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(56) Documents Cited:
GB 0464330 A **WO 2022/069715 A1**
WO 2020/201698 A1 **US 4475941 A**

(58) Field of Search:
INT CL **A01N, A01P**
Other: **WPI, EPODOC, Patent Fulltext, CAS ONLINE**

(54) Title of the Invention: **Fungicides and uses thereof**
Abstract Title: **Organosulfur and organonitrogen ions as fungicides**

(57) Use of at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$, and $R\text{-}N(H)C(NH2)_2^{+}$, and further comprising an agriculturally acceptable counterion (such as chloride, iodide, sulfate); wherein R is a C_8-C_{32} straight chain or branched alkyl (e.g C_{12}-C_{18} straight chain); and, where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl (most preferably, methyl); to kill, suppress or destroy at least one of fungal spores, hyphae and hyphal fragments in soil. The spores may be *Fusariumoxysporum* f.sp *cubense* Tropical Race 4 spores. Most preferred are *n*-octadecyl dimethylsulfonium, *n*-hexadecyl trimethylammonium and *n*-dodecyl guanidinium. Also claimed are methods of destroying, controlling or suppressing *Fusariumoxysporum* f.sp *cubense* Tropical Race 4 spores and strains, variants and pathotypes thereof, the method comprising the step of contacting the fungus with at least one compound as described herein.

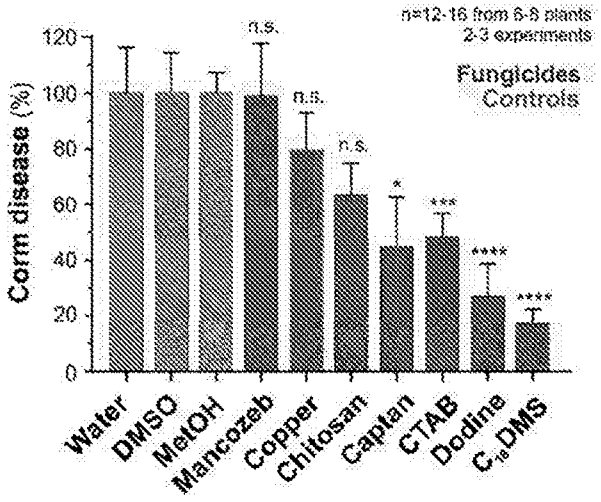

Fig. 11

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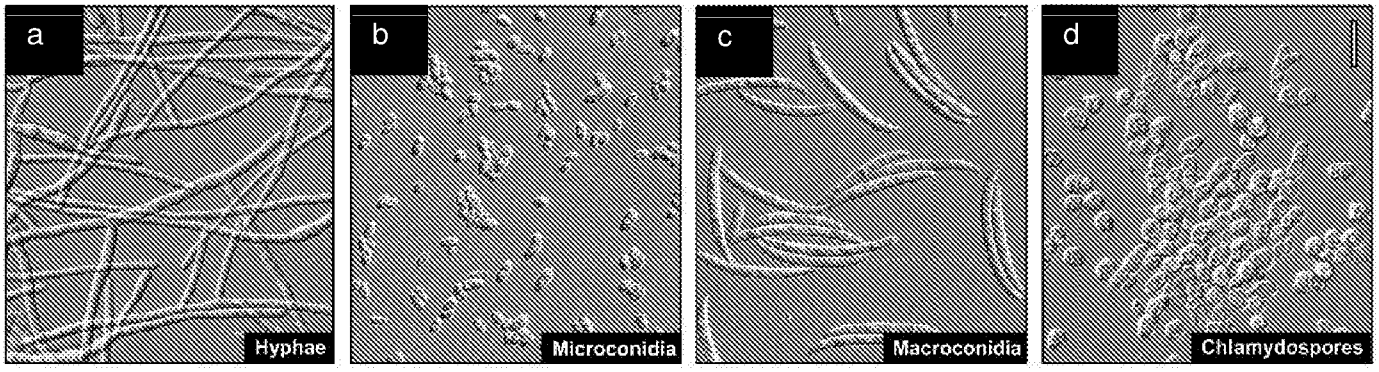


Fig. 1

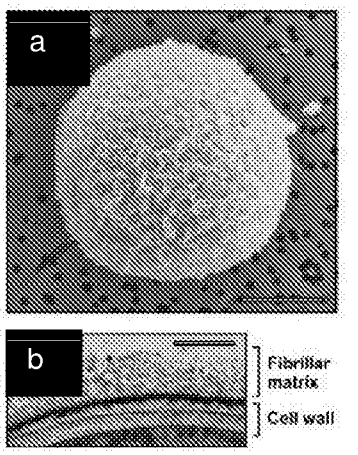


Fig. 2

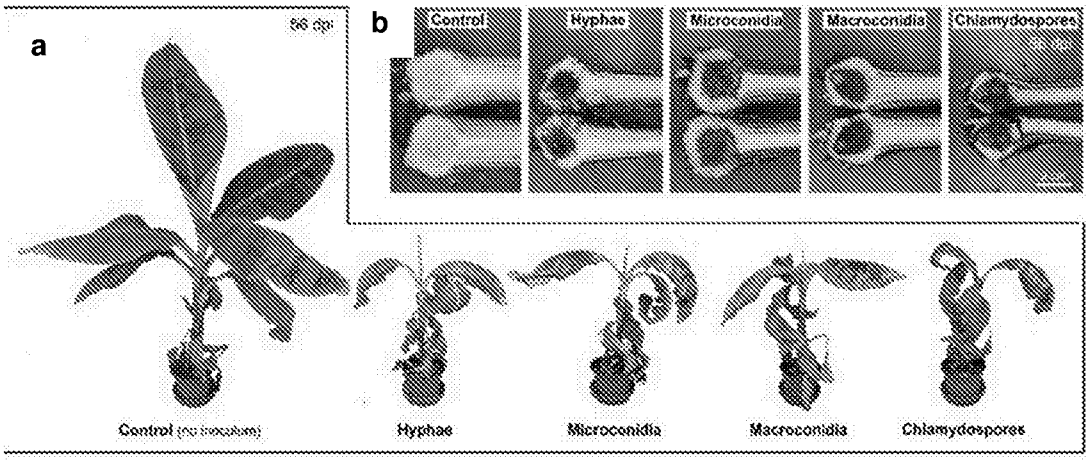


Fig. 3

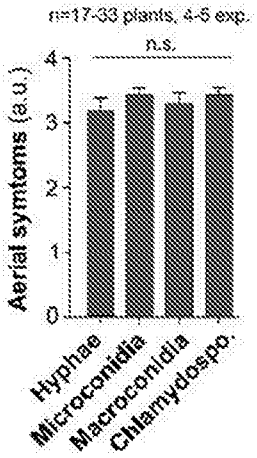


Fig. 4

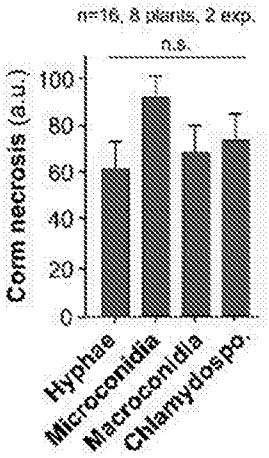


Fig. 5

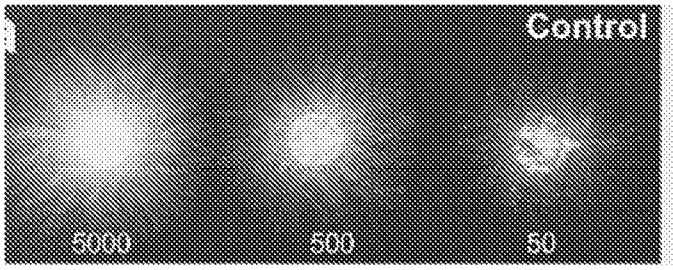


Fig. 6

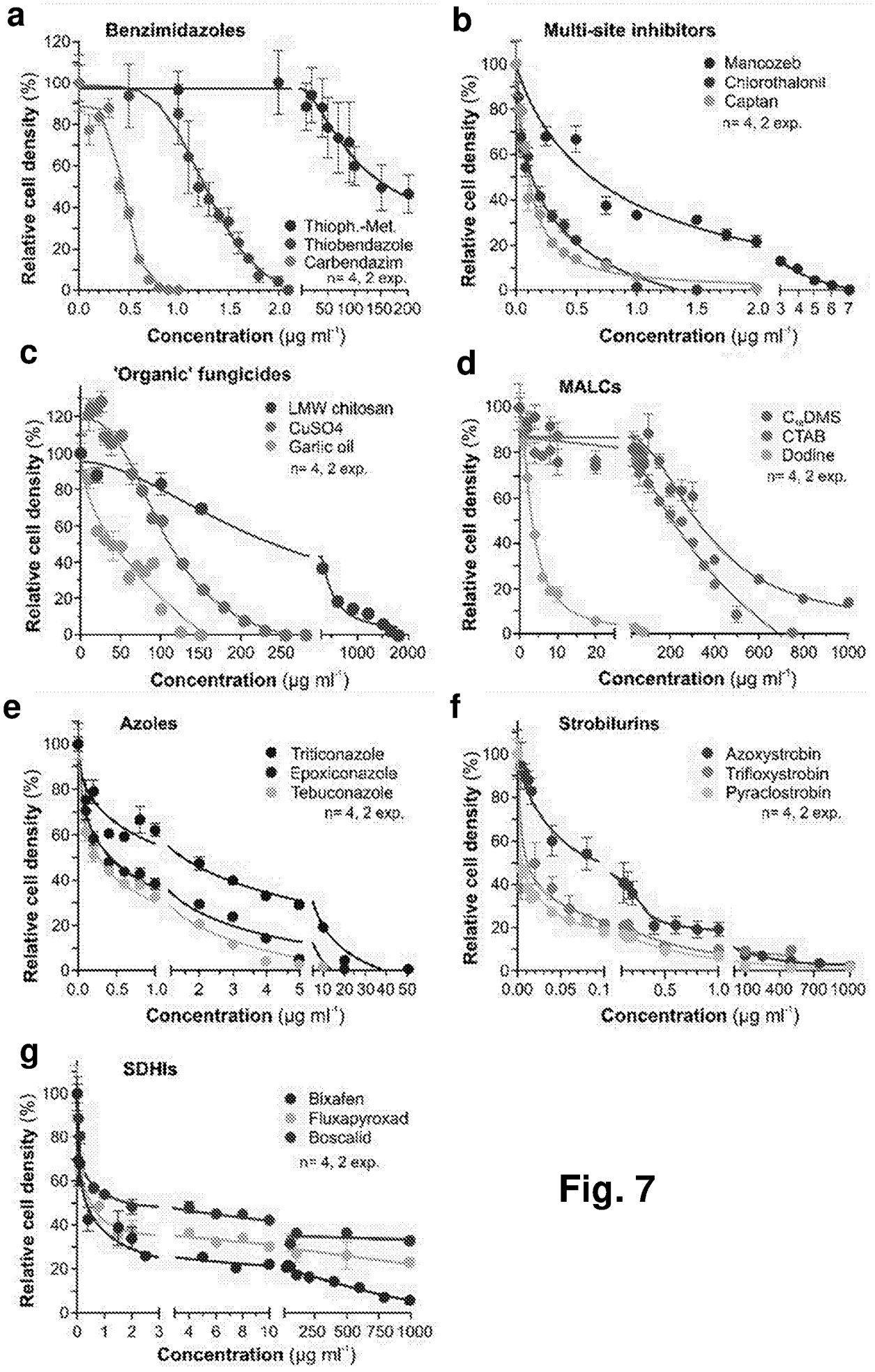

a
Benzimidazoles
Relative cell density (%)
Concentration (µg ml⁻¹)
Thioph.-Met.
Thiobendazole
Carbendazim
n= 4, 2 exp.
b
Multi-site inhibitors
Mancozeb
Chlorothalonil
Captan
n= 4, 2 exp.
c
'Organic' fungicides
LMW chitosan
CuSO4
Garlic oil
n= 4, 2 exp.
d
MALCs
C16DMS
CTAB
Dodine
n= 4, 2 exp.
e
Azoles
Triticonazole
Epoxiconazole
Tebuconazole
n= 4, 2 exp.
f
Strobilurins
Azoxystrobin
Trifloxystrobin
Pyraclostrobin
n= 4, 2 exp.
g
SDHIs
Bixafen
Fluxapyroxad
Boscalid
n= 4, 2 exp.

Fig. 7

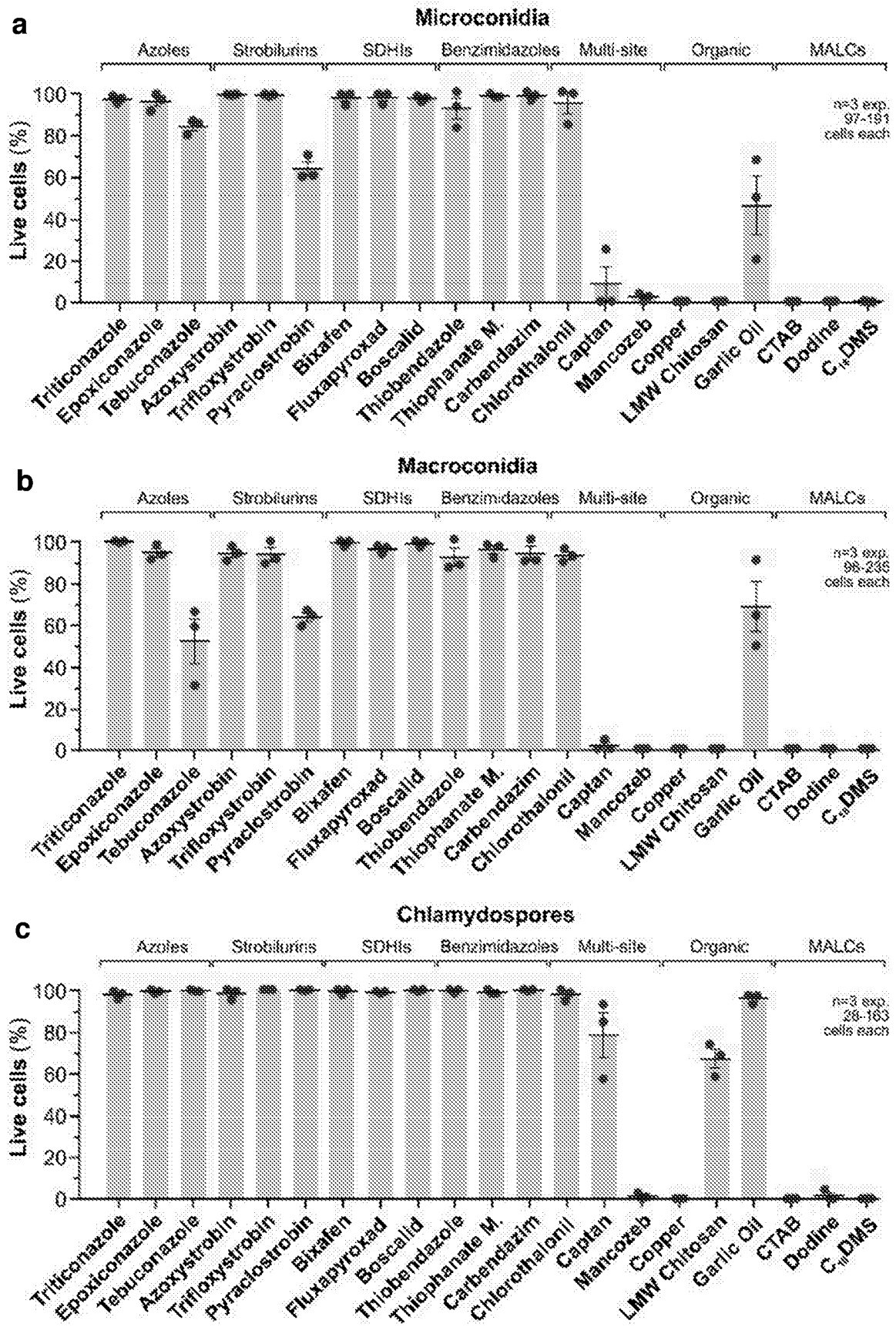

a
Microconidia
Azoles
Strobilurins
SDHIs
Benzimidazoles
Multi-site
Organic
MALCs
Live cells (%)
n=3 exp.
97-191
cells each
Triticonazole
Epoxiconazole
Tebuconazole
Azoxystrobin
Trifloxystrobin
Pyraclostrobin
Bixafen
Fluxapyroxad
Boscalid
Thiobendazole
Thiophanate M.
Carbendazim
Chlorothalonil
Captan
Mancozeb
Copper
LMW Chitosan
Garlic Oil
CTAB
Dodine
C18DMS
b
Macroconidia
n=3 exp.
98-236
cells each
c
Chlamydospores
n=3 exp.
28-163
cells each

Fig. 8

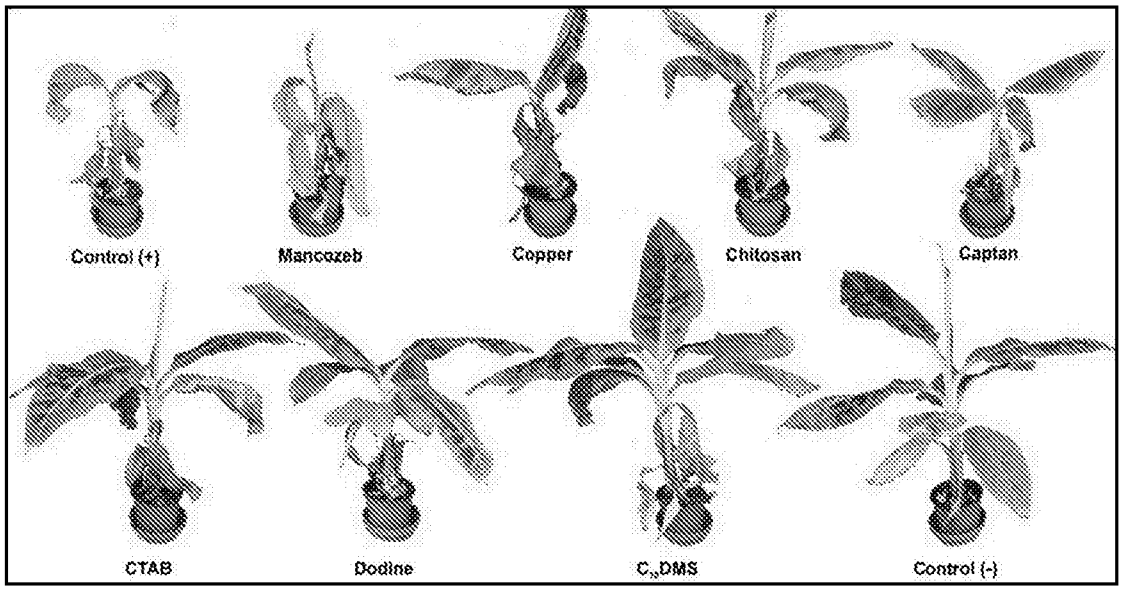


Fig. 9

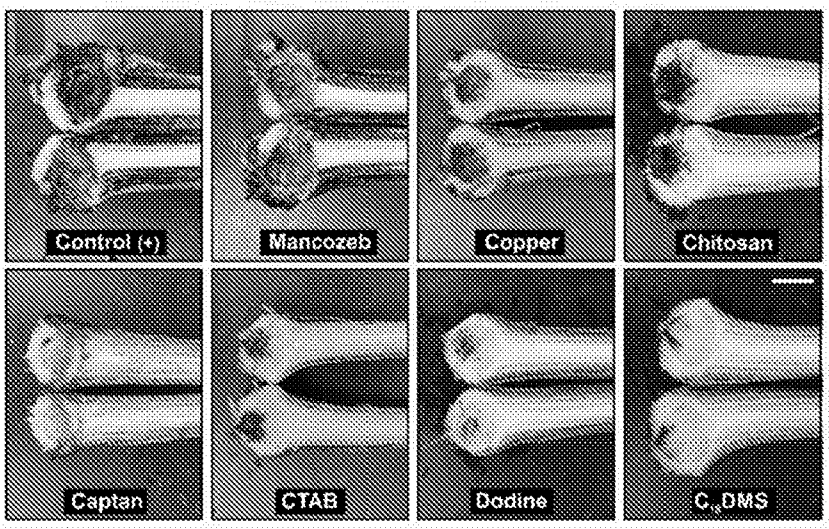


Fig. 10

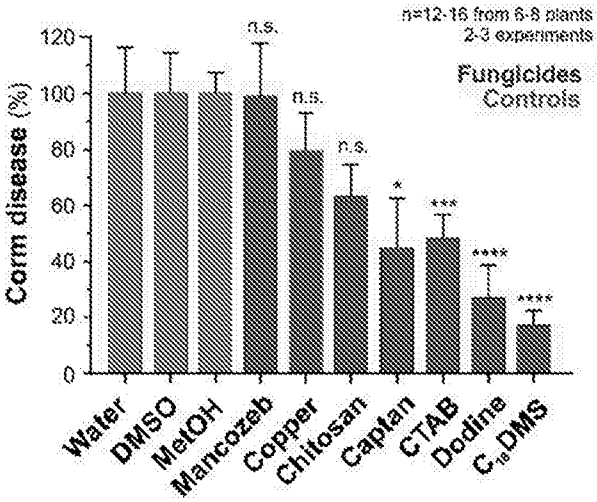


Fig. 11

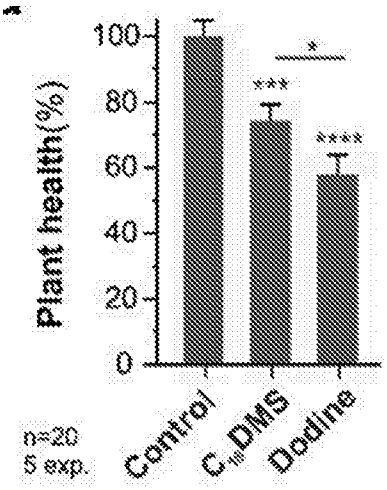


Fig. 12

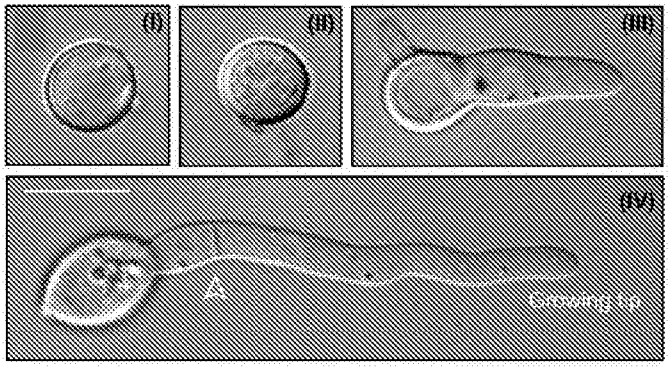


Fig. 13

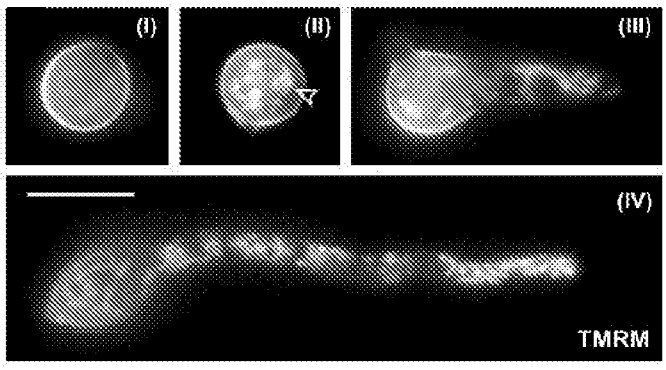


Fig. 14

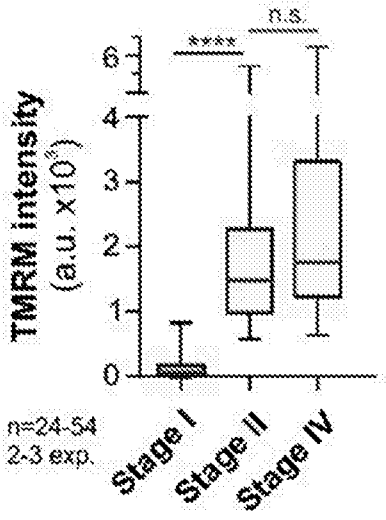


Fig. 15

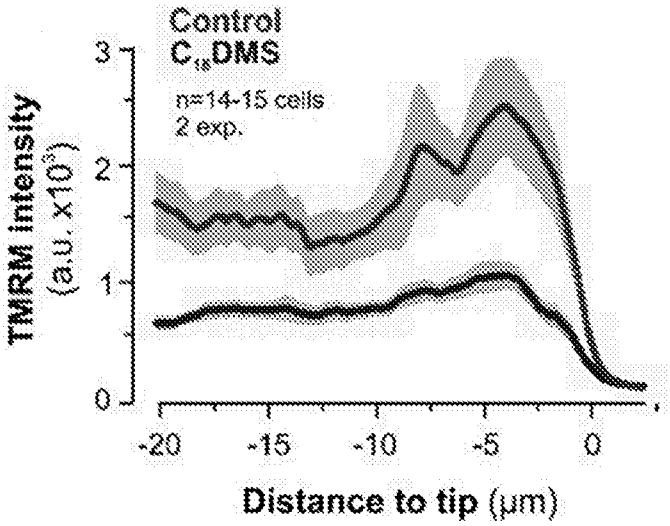


Fig. 16

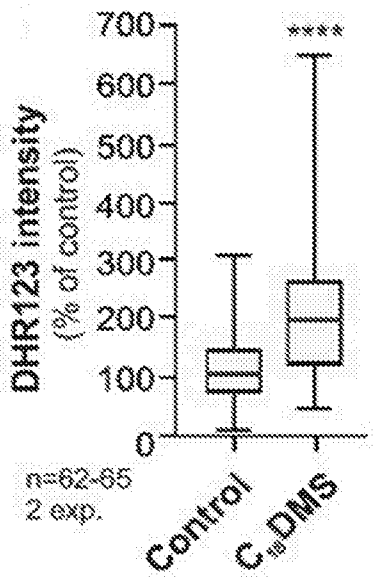


Fig. 17

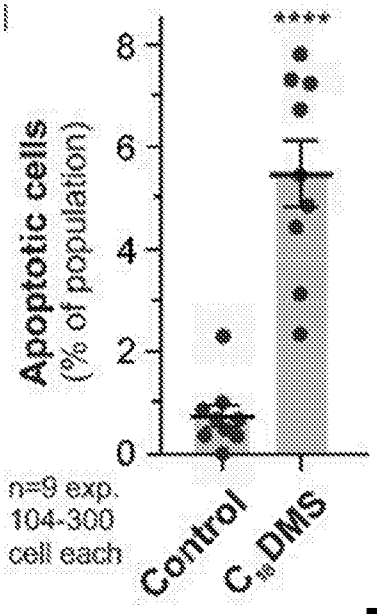


Fig. 18

The following terms are registered trade marks and should be read as such wherever they occur in this document:

Soniprep
Certomat
Silwet
Kristalon
Excel
Prism
Photoshop

Fungicides and Uses Thereof

Technical Field of the Invention

The present invention relates to the use of single alkyl chain cationic compounds to kill fungal spores in soil. The invention further relates to single alkyl chain cationic compounds for use against *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4, and other diseases caused by fungi and oomycetes.

Background to the Invention

Bananas are amongst the most popular fruits eaten world-wide. Indeed, they are the 8th most important global food crop, produced in over 150 countries at >115 million metric tons *per annum* (2019; http://www.fao.org). Banana production is of particular importance for the world's developing countries. As an export crop, bananas support local economies, but also are a staple food for almost half a billion people in the producing countries. As such, bananas are of high importance, both as an essential calorie crop and a revenue-generating trade commodity.

The world's banana supply has a history of being challenged by the fungus *Fusarium oxysporum*. Leading up to 1960s *F. oxysporum* f.sp. *cubense* Race 1 was responsible for the decimation of the then commercially dominant Gros Michel banana variety. With the introduction of Cavendish bananas, this threat was overcome, as this variety proved to be resistant against Race 1. However, with the appearance of *F. oxysporum* f.sp. *cubense* Tropical Race 4 (FocTR4), the world's banana supply faces a new and serious challenge. First identified in Taiwan in 1967, this aggressive FocTR4 spread from Asia to Australasia, Middle East, Indian sub-continent and Africa, finally reaching South America in 2019. As FocTR4 causes Panama disease in Cavendish bananas, this threat is

of high significance, as this variety account for ~40% of world banana production and all exports.

F. oxysporum f. sp. *cubense* Tropical Race 4 produces infectious hyphae and 3 morphologically distinct asexual spore forms, that is, macroconidia, microconidia and chlamydospores. The thick-walled and melanised chlamydospores, in particular, can cope with adverse environmental conditions and can persist in soils or decaying plant debris for decades. Moreover, chlamydospores can be silent passengers on non-host species or carried on contaminated soil on footwear, farm equipment or tools, so spreading the disease. The conidial forms play important roles in host colonisation. Upon favourable conditions, such as the presence of banana roots, chlamydospores germinate in the soil. These germlings penetrate the root tissue, invade the root cortex by hyphal growth, enter the endodermis and colonise the xylem vessels. This results in blockage of the xylem, with yellowed and wilting leaves, thence browning of the vasculature and corm necrosis, splitting of the pseudostems and eventually to plant death.

Different strategies have been adopted in attempts to control FocTR4, including (i) crop husbandry techniques, such as removing infected tissues (Pegg et al, 2019), (ii) use of biological control agents (Bubici et al, 2019); (iii) efforts to generate resistant varieties (Dale et al, 2017; Naim et al, 2018) and (iv) robust biosecurity protocols to reduce the spread of contaminated plants and soil (Bubici et al, 2019). However, none of these approaches have been particularly effective.

Typically, our best defence against fungal plant pathogens has been the use of chemical fungicides. However, studies on the effect of fungicides on FocTR4 *in vitro* and *in vivo* have either been unsuccessful or resulted in contradictory results. Consequently, it is

thought that "chemical measures are of limited or questionable efficacy"(Pegg et al, 2019; Ploetz, 2015b). Moreover, the fungus is thought to be innately resistant to fungicides (Al-Hatmi et al, 2016).

Furthermore, obtaining a clear view of fungicide efficacy on treating Panama disease and other fungal diseases can be challenging. For instance, *in vitro* agar plate-based assays do not reveal a fungicide's actual killing potential in the fungus *per se*. Moreover, bare root assays do not represent the situation in a field. This often means that fungicides which display efficacy in such tests cannot replicate the same performance in the field. Soil, in particular, may attenuate fungicide efficacy by limiting exposure of or sequestering the applied fungicides and hindering them from reaching the pathogens (Nelson, 1996).

This brings a need for compounds and compositions that can be used to safely and effectively kill or suppress fungal spores, and in particular FocTR4 fungal spores, *in situ* in soil.

There also exists a need for compounds and compositions that can be used effectively against FocTR4 and in the treatment of FocTR4-mediated fungal diseases, especially FocTR4-mediated banana wilt (Panama disease).

It is an aim of embodiments of the present invention to address these requirements by providing compounds which provide one or more of the following advantages:

- Ability to be used to effectively kill or suppress fungal spores *in situ* in soil, preferably FocTR4 fungal spores, without loss of efficacy.
- Ability to effectively kill or suppress fungal spores in soil that is on or coated on a plant or a part thereof with minimal phytotoxicity.

- Ability to effectively kill or suppress fungal spores in soil in which fruit-bearing plants, preferably banana plants, are planted, whilst displaying minimal phytotoxicity.
- Ability to kill or suppress fungal spores effectively in soil at low compound loading concentrations without suffering from soil inactivation.
- Ability to be used effectively against FocTR4, preferably in the treatment of FocTR4-mediated fungal diseases, most preferably FocTR4-mediated banana wilt.
- Limited or no toxicity.

It is also an aim of embodiments of the invention to overcome or mitigate at least one problem of the prior art, whether expressly described herein or not.

Summary of the Invention

According to a first aspect of the invention, there is provided the use of at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$ and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl;

to kill or suppress or destroy at least one of the group comprising spores, hyphae and hyphae fragments in soil.

The spores may be fungal spores or oomycete spores. In some embodiments the spores are fungal spores.

All further reference to "hyphae" will includes "hyphae fragments" as an alternative.

Such compounds are able to kill fungal spores and/or their hyphae *in situ* in soil effectively without suffering from soil-derived inactivation. Furthermore, the compounds are effective even at low loadings and show minimal phytotoxicity towards plants or parts of plants in contact with the soil, including fruit-bearing plants growing in the soil.

In some embodiments, the fungal spores comprise plant pathogenic fungal spores and/or hyphae. The fungal spores may comprise spores of at least one fungal division independently selected from the group comprising: *Basidiomycetes, Ascomycetes, Deuteromycetes*, and combinations thereof. Amongst these, but not exclusively, are *Puccinia spp., Ustilago spp , Tilletia spp., Uromyces spp., Phakopsora spp., Rhizoctonia spp., Erysiphe spp., Sphaerotheca spp., Podosphaera spp., Uncinula spp., Helminthosporium spp., Rhynchosporium spp., Pyrenophora spp., Monilinia spp., Sclerotinia spp., Septoria spp. (Mycosphaerella spp., Zymoseptoria spp.), Venturia spp., Botrytis spp., Alternaria spp., Cercospora spp., Cercosporella herpotrichoides, Colletotrichum spp.,* and *Pyricularia oryzae*.

In preferred embodiments, the fungal spores comprise *Fusarium* spores and/or hyphae. The *Fusarium* spores and/or hyphae may comprise at least one plant pathogenic *Fusarium* spores and/or hyphae independently selected from the group comprising: spores of any one of the *Fusarium oxysporum* species, *Fusarium affine* species, *Fusarium arthrosporioides*, *Fusarium fujikuroi*, *Fusarium avenaceum*, *Fusarium circinatum*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium*

incarnatum, *Fusarium langsethiae*, *Fusarium mangiferae*, *Fusarium merismoides*, *Fusarium pallidoroseum*, *Fusarium proliferatum*, *Fusarium pseudograminearum*, *Fusarium redolens*, *Fusarium sacchari*, *Fusarium solani*, *Fusarium sporotrichioides*, *Fusarium sterilihyphosum*, *Fusarium subglutinans*, *Fusarium sulphureum*, *Fusarium tricinctum*, *Fusarium verticillioides*, spores of the *Fusarium virguliforme* species, and combinations thereof.

In preferred embodiments, the fungal spores comprise *Fusarium* spores of the *Fusarium oxysporum* species. The *Fusarium oxysporum* spores may comprise plant pathogenic *Fusarium oxysporum* spores independently selected from the group comprising: spores of the *Fusarium oxysporum* f.sp *cubense* subspecies and morphotypes, f.sp. *asparagi* subspecies, f.sp. *batatas*, f.sp. *betae*, f.sp. *cattleyae*, f.sp. *cannabis*, f.sp. *cepae*, f.sp. *ciceris*, f.sp. *citri*, f.sp. *coffea*, f.sp. *albedinis*, f.sp. *cyclaminis*, f.sp. *herbemontis*, f.sp. *dianthi*, f. sp. *fragariae*, f.sp. *gladioli*, f.sp. *koae*, f.sp. *lactucae*, f.sp. *lentis*, f.sp. *lilli*, f.sp. *lini*, f.sp. *lycopersici*, f.sp. *medicaginis*, f.sp. *melonis*, f.sp. *momordicae*, f.sp. *narcissi*, f.sp. *nicotianae*, f.sp. *niveum*, f.sp. *palmarum*, f.sp. *passiflorae*, f.sp. *perniciosum*, f.sp. *phaseoli*, f.sp. *pisi*, f.sp. *radicis-lycopersici*, f.sp. *ricini*, f.sp. *strigae*, f.sp. *tuberosi*, f.sp. *tulipae*, spores of the f.sp. *vasinfectum* subspecies, and combinations thereof.

In preferred embodiments, the fungal spores comprise spores of the *Fusarium oxysporum* f.sp *cubense* subspecies. The fungal spores may comprise spores of at least one of the group comprising *Fusarium oxysporum* f.sp *cubense* Race 1, Race 2, Race 3 and Race 4 (or any new morphotype which may arise). In preferred embodiments, the fungal spores comprise spores of a *Fusarium oxysporum* f.sp *cubense* Tropical Race or a variant, strain or pathotype thereof.

In a particularly preferred embodiment, the fungal spores comprise spores of *Fusarium oxysporum* f.sp *cubense* Tropical Race 4 or a variant, strain or pathotype thereof. The compounds of the invention display excellent performance at killing and suppressing such fungal spores in soil, despite this being the most aggressive strain of the fungus to date.

The spores may comprise sexual and/or asexual spores, preferably asexual spores.

In some embodiments, the spores comprise one or more spore types independently selected from the group comprising: conidia, sporangiospores, zygospores, ascospores, basidiospores, aeciospores, uredospores, teliospores, oospores, and combinations thereof.

In preferred embodiments, the spores comprise one or more spore types independently selected from the group comprising: microspores (microconidia), macrospores (preferably macroconidia), and chlamydospores.

The compounds of the invention show particularly high efficacy at killing and suppressing these persistent spore-forms and/or their hyphae. Particularly surprisingly, the compounds are highly effective against the thick-walled and melanised chlamydospores, which are otherwise able to cope with adverse environmental conditions and can persist in soils or decaying plant debris for decades.

In some embodiments, the soil comprises a single type of spore and/or hyphae. The spore type may be independently selected from the group comprising: microspores (preferably microconidia), macrospores (preferably macroconidia), and chlamydospores. In a particularly preferred embodiment, the single type of spore is a chlamydospore. Alternatively, the soil may comprise at least 2, or at least 3 different spore types. The spore types may preferably be independently selected from the above group. The soil

may comprise two spore types independently selected from the group comprising: microspores (preferably microconidia) and macrospores (preferably macroconidia); microspores (preferably microconidia) and chlamydospores; and macrospores (preferably macroconidia) and chlamydospores. Preferably, the soil may comprise all three of microspores (preferably microconidia), macrospores (preferably macroconidia), and chlamydospores.

In some embodiments, the spores and/or their hyphae may be present on or at an exposed surface of the soil, in the internal body of or within the soil, or both on or at an exposed surface and in the body of the soil. The spores and/or their hyphae may be present on or within at least 0.0001. 0.001. 0.01. 0.1. 1. 2. 3. 4 or at least 5% of the exposed surface of the soil, or at least 10, 20, 30, 40, or at least 50% of the exposed surface of the soil. The spores and/or their hyphae may be present at or on no greater than 95% of the exposed surface of the soil, or no greater than 90, 80, 70, 60, or no greater than 50% of the exposed surface of the soil. The spores and/or their hyphae may be present in at least 5% of the internal body of the soil, or at least 10, 20, 30, 40, or at least 50% of the internal body of the soil. The spores and/or their hyphae may be present in no greater than 95% of the internal body of the soil, or no greater than 90, 80, 70, 60, or no greater than 50% of the internal body of the soil. In some embodiments, the spores and/or their hyphae are present throughout at least 5% of the soil, or at least 10, 20, 30, 40, or at least 50% of the soil. The spores may be present throughout no greater than 95% of the soil, or no greater than 90, 80, 70, 60, or no greater than 50% of the soil.

In some embodiments, the spores and/or their hyphae are equally distributed throughout the soil. In other embodiments, the concentration of spores and/or their hyphae in the internal body of the soil may be greater than the concentration at or on the exposed

surface of the soil. Alternatively, the concentration of spores and/or their hyphae at or on the exposed surface of the soil may be greater than the concentration in the internal body of the soil.

In some embodiments, the spores and/or their hyphae are present in the soil in a total amount of at least 1, 2, 3, 4, 5, 10, 25, 50 100, 500, 1000, or 5000 spores and/or their hyphae/gram of soil, or at least 10000, 15000, 20000, 25000, 30000, 35000, 40000, 45000, 50000, 55000, 60000, 65000, 70000, 75000, 80000, 85000, 90000, 95000, or at least 100000 spores and/or their hyphae/gram of soil. The spores and/or their hyphae may be present in a total amount of no greater than 1000000 spores and/or their hyphae/gram of soil, or no greater than 950000, 900000, 850000, 800000, 750000, 700000, 650000, 600000, 550000, 500000, 450000, 400000, 350000, 300000, 250000, 200000, or no greater than 150000 spores/gram of soil. The spores and/or their hyphae may be present in a total amount of between 10000-1000000 spores and/or their hyphae/gram of soil, or of between 15000-900000, 20000-800000, 25000-700000, 30000-500000, 35000-400000, 40000-300000, 45000-250000, or preferably 50000-200000, 55000-150000, 60000-140000, 65000-130000, 70000-120000, 80000-115000, or preferably of between 90000-110000 spores and/or their hyphae/gram of soil.

The compounds of the invention are highly effective against even such high concentrations of spores and/or their hyphae in the soil.

In some embodiments, the soil is on, surrounding or coated on a plant or part thereof. The compounds of the invention are able to kill and suppress spores and/or their hyphae in such soil with minimal phytotoxicity towards the plant or part thereof. The plant may be a host species for the fungal spores and/or their hyphae and may be susceptible to attack

or parasitisation by the fungus. Alternatively, the plant may be a non-host species for the fungal spores and/or their hyphae and may not be susceptible to attack or parasitisation by the fungus.

In some embodiments, the soil is on, surrounding or coated on a plant or part of a plant that is planted in the soil. The plant may be independently selected from the group comprising: monocots, dicots and tree species. The plant may be independently selected from the group comprising a cereal (such as wheat, barley, rye, oats, rice, maize, sorghum, etc.), a fruit tree or plant (such as of apples, pears, plums, peaches, cherries, bananas, grapes, strawberries, raspberries, blackberries, etc.), a citrus tree (such as of oranges, lemons, mandarins, grapefruit, etc.), a legume (such as beans, peas, lentils, soybean, etc.), a vegetable (spinach, lettuce, asparagus, cabbage, carrots, onions, tomatoes, potatoes, eggplants, peppers, etc.), Cucurbitaceae (pumpkins, zucchini, cucumbers, melons, watermelons, etc.), oleaginous plants (sunflower, rape, peanut, castor, coconut, etc.), tobacco, coffee, tea, cocoa, sugar beet, sugar cane, cotton, horticultural plants, and combinations thereof.

In some embodiments, the soil is on, surrounding or coated on a part of the plant. The plant part may be an edible foodstuff or an inedible plant part. The plant part may be independently selected from the group comprising: fruit, seeds, nuts, flowers, roots, stems/corms, leaves, stalks, bracts, and combinations thereof.

In preferred embodiments, the soil is on, surrounding or coated on a herbaceous flowering plant or a part thereof. In a particularly preferred embodiment, the plant is a banana plant. The banana plant may preferably be planted in the soil. The banana plant may be of the *Musa acuminata*, *Musa balbisiana*, or *Musa* x *paradisiaca* species.

Preferably, the banana plant may be of the *Musa acuminata* species and may be of a genome group independently selected from the group comprising: diploid *Musa acuminata* (AA group), triploid *Musa acuminata* (AAA group), and tetraploid *Musa acuminata* (AAAA group). The banana plant may preferably be of the AAA group and may more preferably be of the Cavendish subgroup. The banana plant may be a member of the Cavendish subgroup independently selected from the group comprising: Dwarf Cavendish, Giant Cavendish, Grand Nain, Masak Hijau, and Robusta.

Such plants are adversely affected by *Fusarium oxysporum* f.sp. *cubense* fungi, which cause banana wilt disease. In particular, *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (Foc-TR4) is resistant to most fungicides and poses major problems for banana production. The compounds of the invention show minimal phytotoxicity towards such plants. Further, the compounds are able to kill/suppress fungal spores, including highly persistent Foc-TR4 spores, in soil. This allows for protection of the plants from disease without causing harm to them in the process.

In some embodiments, the soil is on, surrounding or coated on a material independently selected from the group comprising: plastic, metal, wood, paper, textiles (natural and synthetic), leather, fibres, glass, composite materials, minerals, stone, concrete, plaster, ceramic, rubber, and combinations thereof. In some embodiments, the material comprises an artefact or a part thereof, which may be independently selected from the group comprising: footwear, farm equipment, and tools. Fungal spores, particularly chlamydospores, can be silent passengers on contaminated soil on artefacts, such as footwear, farm equipment or tools, which can lead to spreading of the spores and thus spreading of fungal disease.

The soil may be any soil type.

In some embodiments, the R group of at least one compound is a C8-C30 straight chain or branched alkyl, or a C8-C28, C8-C26, C8-C24, C8-C22, C8-C20, C8-C18, C8-C16, C8-C14, C8-C12, or a C8-C10 straight chain or branched alkyl.

In some embodiments, the R group of at least one compound is a C10-C32 straight chain or branched alkyl, or a C12-C32, C14-C32, C16-C32, C18-C32, C20-C32, C22-C32, C24-C32, C26-C32, C28-C32, or a C30-C32 straight chain or branched alkyl.

In some embodiments, the R group of at least one compound is a C10-C30 straight chain or branched alkyl, or a C12-C30, C14-C30, C16-C30, C18-C30, C20-C30, C22-C30, C24-C30, C26-C30, C28-C30, C10-C28, C12-C28, C14-C28, C16-C28, C18-C28, C20-C28, C22-C28, C24-C28, C26-C28, C10-C26, C12-C26, C14-C26, C16-C26, C18-C26, C20-C26, C22-C26, C24-C26, C10-C24, C12-C24, C14-C24, C16-C24, C18-C24, C20-C24, C22-C24, C10-C22, C12-C22, C14-C22, C16-C22, C18-C22, C20-C22, C10-C20, C12-C20, C14-C20, C16-C20, C18-C20, C10-C18, C12-C18, C14-C18, C16-C18, C10-C16, C12-C16, C14-C16, C10-C14, C12-C14, or a C10-C12 straight chain or branched alkyl.

In preferred embodiments, R is a straight chain alkyl. The R group may be substituted or unsubstituted, preferably unsubstituted. The R group may be a C8-C30 straight chain alkyl, or a C8-C25, C8-C20, or preferably a C10-C20, more preferably a C10-C18, or most preferably a C12-C18 straight chain alkyl.

In some preferred embodiments, R is a straight chain or branched C8-C15 alkyl, or a C8-C14, C10-C14, or a C10-C12 alkyl. In some preferred embodiments, R is a straight chain or branched, and preferably a straight chain C12 alkyl. In such embodiments, the

compound may preferably be an alkyl guanidinium of the formula: R-N(H)C(NH_2)$_2^+$. In a particularly preferred embodiment, at least one compound is *n*-dodecyl guanidinium.

In other preferred embodiments, R is a straight chain or branched C14-C32 alkyl, or a C14-C28, C14-C24, C14-C22, C14-C20, C16-C20, or a C16-C18 alkyl. In some preferred embodiments, R is a straight chain or branched, and preferably a straight chain C16 alkyl. In other preferred embodiments, R is a straight chain or branched, and preferably a straight chain C18 alkyl. In such embodiments, the compound may preferably be an alkyl ammonium of formula: R-N^+(R')$_3$, or an alkyl sulfonium of formula: R-S^+(R')$_2$. Preferably, at least one compound may be an alkyl ammonium of formula C16-N^+(R')$_3$ and/or an alkyl sulfonium of formula C18-S^+(R')$_2$.

In some embodiments, when present, one R' group of at least one compound may be different to the other R' group(s). In some embodiments, all R' groups of the compound may be different to each other. In preferred embodiments, all R' groups of the compound are the same.

In some embodiments, when present, at least one R' of at least one compound is methyl, ethyl, and/or propyl. At least one R' may preferably be methyl and/or ethyl. In some embodiments, at least one R' is methyl and at least one R' is ethyl. In particularly preferred embodiments, when present, each R' of the compound is methyl.

In especially preferred embodiments, at least one compound is independently selected from the group comprising: *n*-octadecyl dimethylsulfonium (C18-DMS), *n*-hexadecyl trimethylammonium (cetrimonium), and *n*-dodecyl guanidinium (dodine). Such compounds have been found to be particularly effective against fungal spores in soil, and particularly FocTR4 fungal spores.

The compounds of the invention may comprise any suitable counter anion. In some embodiments, at least one compound comprises a counterion independently selected from the group comprising: a halide, hydroxide, sulfate, phosphate, hydrogen sulfate, and acetate. The halide may preferably be independently selected from the group comprising: chloride, bromide, and iodide. At least one compound may preferably comprise a halide and/or acetate counterion. In some embodiments, at least one compound is an alkyl sulfonium and/or alkyl ammonium, preferably as described above, with a halide counterion, which may preferably be independently selected from the group comprising: chloride, bromide, and iodide. At least one compound may preferably be an alkyl sulfonium, preferably as described above, with an iodide counterion. At least one compound may preferably be an alkyl ammonium, preferably as described above, with a chloride or bromide counterion, preferably with a bromide counterion. In some preferred embodiments, at least one compound is an alkyl guanidium, preferably as described above, with an acetate counterion. At least one compound may be an *n*-hexadecyl trimethylammonium halide, which may be independently selected from the group comprising: *n*-hexadecyl trimethylammonium chloride, *n*-hexadecyl trimethylammonium bromide, *n*-hexadecyl trimethylammonium iodide, and combinations thereof. At least one compound may be an *n*-octadecyl dimethylsulfonium halide, which may be independently selected from the group comprising: *n*-octadecyl dimethylsulfonium chloride, *n*-octadecyl dimethylsulfonium bromide, *n*-octadecyl dimethylsulfonium iodide, and combinations thereof. At least one compound may be an *n*-dodecyl guanidinium halide, which may be independently selected from the group comprising: *n*-dodecyl guanidinium chloride, *n*-dodecyl guanidinium bromide, *n*-dodecyl guanidinium iodide, and combinations thereof.

In some embodiments, the or each compound is present as or in a composition.

In some embodiments, the composition comprises a single compound of the invention. In other embodiments, the composition comprises multiple different compounds of the invention. The composition may comprise at least 2, 3, 4, 5, 6, 7, 8, 9, at least 10, or more different inventive compounds. In some embodiments, the compounds in the composition may all be of the same chemical type. In such embodiments, all of the compounds in the composition may be of one chemical type independently selected from the group comprising: an alkyl sulfonium of formula: $R\text{-}S^{+}(R')_2$; an alkyl ammonium of formula: $R\text{-}N^{+}(R')_3$; and an alkyl guanidinium of formula: $R\text{-}N(H)C(NH_2)_2^{+}$.

In other embodiments, two or at least two of the inventive compounds in the composition may be of different chemical types. In some embodiments, at least one compound in the composition is an alkyl sulfonium and at least one compound in the composition is an alkyl ammonium. At least one compound in the composition may be an alkyl sulfonium and at least one compound may be an alkyl guanidinium. At least one compound in the composition may be an alkyl ammonium and at least one compound in the composition may be an alkyl guanidinium.

In some embodiments, three inventive compounds in the composition may be of different chemical types. In preferred embodiments, at least one compound in the composition is an alkyl sulfonium, at least one compound is an alkyl ammonium, and at least one compound is an alkyl guanidinium.

The antifungal composition may comprise one or more further antifungal agents, in addition to the compounds of the invention. Further antifungal agents may be independently selected from the group comprising: azoles; amino-derivatives;

strobilurins; specific anti-oidium compounds; aniline-pyrimidines; benzimidazoles and analogues; dicarboximides; polyhalogenated fungicides; systemic acquired resistance (SAR) inducers; phenylpyrroles; acylalanines; anti-peronosporic compounds; dithiocarbamates; arylamidines; phosphorous acid and its derivatives; fungicidal copper compounds; plant-based oils (botanicals); chitosan; sulfur-based fungicides; fungicidal amides; nitrogen heterocycles; and combinations thereof.

The composition may comprise a solution, suspension, emulsion, powder, paste, granules, gel, mousse or dust, for example. In preferred embodiments, the composition comprises a solution, and preferably an aqueous solution.

The composition may include a carrier. The carrier may comprise a solvent, which may be water or an aqueous solvent. Thus, the composition may be an aqueous composition of at least one compound of the invention in water, or an aqueous solvent. The aqueous solvent may comprise water and a co-solvent, which may be selected from methanol and ethanol, for example. Preferably, the composition may be an aqueous composition of the compound in water, preferably distilled water, and most preferably double-distilled water.

In some embodiments, the composition contains the compounds of the invention, preferably as part of an aqueous solution, at a total concentration of at least 0.02 μg/mL, or at least 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2, 2.5, 5, 7.5, 10, 15, 20, 30, 40, 50, 60, 70, 75, 100, 150, or at least 200 μg/mL. The composition may contain the compounds of the invention at a total concentration of no greater than 1200 μg/mL, or no greater than 1100, 1000, 900, 800, 700, 600, 500, 400, 300, or no greater than 250 μg/mL.

In preferred embodiments, the composition contains the compounds of the invention at a total concentration of between 0.1-500 μg/mL.

The composition may contain the compounds of the invention at a total concentration of between 0.5 – 1500, 0.5 – 1000, 0.5 – 750, 0.5-500, 1-500, 5-500, 10-500, 25-500, 50-500, 75-500, 100-500, 150-500, or between 200-500 μg/mL. The composition may contain the compounds of the invention at a total concentration of between 0.1-450, 0.1-400, 0.1-350, 0.1-300, 0.1-250, 0.1-200, 0.1-150, 0.1-100, 0.1-50, 0.1-25, or between 0.1-10 μg/mL. The composition may preferably contain the compounds of the invention at a total concentration of between 0.5-400, 1-350, 5-300, 10-250, 25-250, 50-250, 100-250, or of between 150-250 μg/mL.

Surprisingly, the compounds of the invention are effective at killing and suppressing fungal spores in soil at even such low concentrations.

In some embodiments the composition may comprise an adjuvant or penetrant. The adjuvant may enhance the efficacy of the compounds of the invention. The mechanisms of action of suitable adjuvants include buffering water to a pH at which a specific compound is most active, water conditioning (for example when using hard water to solvate or dilute the compound, they may reduce the potential inhibitory effects of minerals such as calcium and magnesium), reducing the surface tension of water ("wetting agents") so that the composition has greater coverage of surface to which it is applied. Adjuvants may contain solvents, additives, surfactants and oils which make soil more penetrable to the compound; or they may contain nutrients which may assist the compound when applied to soil housing crops or other plants, such as to encourage plant growth. Other suitable adjuvants are defoaming agents, adjuvants which reduce drift

during spraying of the compound, and adjuvants which may reduce evaporation and compound volatility.

The adjuvant may be a compound independently selected from the group comprising: a pH buffering agent; a water conditioning agent; a wetting agent; a plant growth enhancer; a defoaming agent; a spray drift reducing agent; an evaporation reducing agent; and combinations thereof.

The adjuvant may be in the form of a crop protectant spray additive and/or a surfactant. The adjuvant may preferably be a non-ionic spreading and a penetration aid; and/or act to reduce surface tension of the composition.

The adjuvant may be an additive for crop protectant sprays, such as a surfactant; a non-ionic spreading and a penetration aid; and/or act to reduce surface tension of the composition, for example.

The adjuvant may comprise an activator adjuvant or a utility adjuvant.

Activator adjuvants are compounds that when added to the composition, enhance its antifungal activity. Activator adjuvants include surfactants, oil carriers such as phytobland (not harmful to plants) oils, crop oils, crop oil concentrates (COCs), vegetable oils, methylated seed oils (MSOs), petroleum oils, and silicone derivatives, as well as nitrogen fertilizers, for example.

Utility adjuvants, which are sometimes called spray modifiers, alter the physical or chemical characteristics of the composition making it easier to apply.

One or more oils may be used as an adjuvant carrier or diluent for the compound.

Salts may also be used as activator adjuvants, such as, for example, to increase the uptake and effect of the compound in a target surface and/or material (soil).

One or more surfactant adjuvants may be present in the composition to facilitate or enhance the emulsifying, dispersing, spreading, sticking or wetting properties of the composition.

Surfactants reduce surface tension in the spray droplets of the composition, when the composition is applied to a material, which aids the composition to spread out and cover the target material with a thin film, leading to more effective or quicker absorption of the composition into the material.

The surfactant may be chosen to enhance the antifungal properties of the composition, through any one or more of: a) making the composition spread more uniformly on a material to which the composition is applied; b) increasing retention (or 'sticking') of the composition on the material; c) preventing crystallization of the composition; and/or d) slowing the drying of the composition.

Each surfactant may be independently selected from the group comprising: a non-ionic surfactant, an ionic surfactant, an amphoteric surfactant, a zwitterionic surfactant, and combinations thereof.

Non-ionic surfactants are generally biodegradable and are compatible with many fertilizers and so may be preferable in compositions of the invention when used in crop or plant protection applications. Some non-ionic surfactants are waxy solids and require the addition of a co-solvent (such as alcohol or glycol) to solubilize into liquids. Glycol co-solvents are generally preferred over alcohols, as the latter are flammable, evaporate

quickly, and may increase the number of fine spray droplets (making the formulation likely to drift when sprayed).

The non-ionic surfactant may preferably comprise an organosilicone or silicone surfactant (including siloxanes and organosiloxanes). Organosilicone surfactants significantly reduce surface tension of the composition, enabling the composition, in use, to form a thin layer on a surface to which it is applied. Silicone surfactants also provide a protective effect to the compositions of the invention by making the compositions very difficult to wash off after they are applied. Silicone surfactants can also influence the amount/rate of compound absorption through the soil.

In other embodiments, the non-ionic surfactant may comprise a carbamide surfactant (also known as a urea surfactant). The carbamide surfactant may comprise monocarbamide dihydrogen sulfate, for example.

Suitable ionic surfactants include cationic surfactants and anionic surfactants. Suitable cationic surfactants include tallow amine ethoxylates. Suitable anionic surfactants include sulfates, carboxylates, and phosphates attached to lipophilic hydrocarbons, including linear alkylbenzene sulfonates, for example.

Amphoteric surfactants contain both a positive and negative charge and typically function similarly to non-ionic surfactants. Suitable amphoteric surfactants include lecithin (phosphatidylcholine) and amidopropylamines, for example.

Utility adjuvants, which are sometimes called spray modifiers, alter the physical or chemical characteristics of the compositions of the invention making the composition easier to apply, which may increase the persistence of the composition in the environment or treatment area in which the composition is present.

Examples of different functional categories of utility adjuvants suitable for use in the compositions and uses of the invention include wetting agents, spreading agents, drift control agents, foaming agents, dyes, thickening agents, deposition agents (stickers), water conditioning agents, humectants, pH buffers, de-foaming agents, anti-foaming agents and UV absorbents. Some utility adjuvants may function in more than one of the aforesaid functional categories. Some activator adjuvants are also utility adjuvants.

Wetting agents or spreading agents lower surface tension in the compositions. Since these agents are typically non-ionic surfactants diluted with water, alcohol, or glycols they may also function as activator adjuvants (surfactants). However, some wetting or spreading agents affect only the physical properties of the composition, and do not affect the behaviour of the composition once it is in contact with soil.

Drift control agents may be used to reduce spray drift of the composition, which most often results when fine (< 150 μm diameter) spray droplets are carried away from the target area by air currents. Drift control agents alter the viscoelastic properties of the spray solution, yielding a coarser spray with greater mean droplet sizes and weights, and minimizing the number of small, easily-airborne droplets. Suitable drift control agents may comprise large polymers such as polyacrylamides, polysaccharides and certain types of gums.

Suitable deposition agents (stickers) include film-forming vegetable gels, emulsifiable resins, emulsifiable mineral oils, vegetable oils, waxes, and water-soluble polymers, for example. Deposition agents may be used to reduce losses of composition from the target plant, due to the evaporation of the composition from the target surface, or beading-up and falling off of the composition. Deposition agents are particularly suitable for

compositions of the invention in the form of dry (wettable) powder and granule formulations.

De-foaming and antifoam agents reduce, supress or destroy the formation of foam in containers in which the compositions of the invention may be contained. Suitable de-foaming agents include oils, polydimethylsiloxanes and other silicones, alcohols, stearates and glycols, for example.

The adjuvant or adjuvants may comprise BREAK-THRU® S 240, BREAK-THRU® SP 131, BREAK-THRU® SP 133, BREAK-THRU® S 233, BREAK-THRU® OE 446, Aduro (RTM) and/or Transport Ultra (RTM). BREAK-THRU® S 240 is a polyether trisiloxane that imparts super spreading and dramatically reduces surface tension. BREAK- THRU SP131 is composed of polyglycerol fatty esters and polyglycols, and it improves the performance of antifungal compounds.

BREAK-THRU® SP 133 is based on polyglycerol esters and fatty acid esters. BREAK-THRU® S 233 is a non-ionic trisiloxane surfactant, which increases the deposition of agrichemical sprays and improves the penetration of pesticide actives into plant tissue. BREAK-THRU® OE 446 is a polyether polysiloxane.

Transport Ultra (RTM) comprises a blend of non-ionic surfactants, ammoniated ions, water conditioning agents and an antifoam agent.

Aduro (RTM) comprises a monocarbamide dihydrogen sulfate and alkylamine ethoxylates.

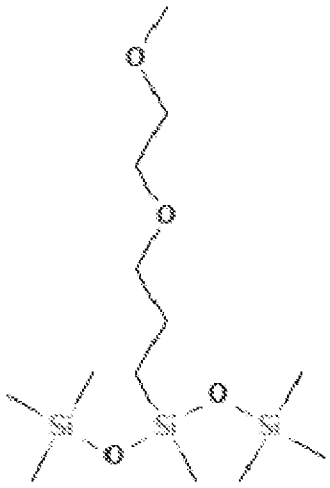
In some preferred embodiments, at least one adjuvant is selected from a silicone, a siloxane, an alkylamine ethoxylate or a carbamide. Said adjuvants are particularly useful

at enhancing the effect of the compound, or otherwise increasing or speeding up the take-up of the compound.

In some embodiments, the adjuvant may comprise: a non-ionic surfactant; and/or antifoam agent; and/or ammonium ions; and/or water-conditioning agent; and/or polyether-polymethylsiloxane-copolymer; and/or polyether polysiloxane; and/or polyglycerol fatty esters and polyglycols; and/or polyglycerol esters and fatty acid esters; and/or non-ionic trisiloxane.

In preferred embodiments, the composition comprises at least one surfactant, which may be a non-ionic surfactant. In some embodiments, the composition comprises at least one silicone or siloxane, which silicone or siloxane may act as a surfactant and/or an anti-foam agent and/or a wetting agent.

Most preferably, the composition may comprise at least one organosilicon non-ionic surfactant adjuvant. The organosilicon non-ionic surfactant may comprise a polyalkyleneoxide modified siloxane, preferably a polyalkyleneoxide modified trisiloxane, and most preferably a polyalkyleneoxide modified heptamethyl trisiloxane. In preferred embodiments, the organosilicon non-ionic surfactant comprises a polyethyleneoxide modified heptamethyl trisiloxane. In particularly preferred embodiments, the organosilicon non-ionic surfactant comprises a 2-[methoxy(polyethyleneoxy)propyl]heptamethyltrisiloxane, preferably 3-(2-methoxyethoxy)propyl-methyl-bis(trimethylsilyloxy)silane (Silwet (RTM) L-77), which has the formula:



Such organosilicon non-ionic surfactant adjuvants ensure even penetration of the inventive compounds through the soil and ensure an even distribution of chemistries in the soil. This allows for the composition to simply be applied to an exposed surface of the soil and rapidly reach and kill spores that are even deep within the internal body of the soil.

In some embodiments, the composition may comprise 2, 3, 4, or at least 5 different adjuvants. In preferred embodiments, the composition comprises a single adjuvant, preferably an organosilicon non-ionic surfactant adjuvant as described above.

In some embodiments, the composition comprises an adjuvant, preferably a non-ionic surfactant adjuvant as described above, in a total amount of at least 0.002 vol%, or at least 0.004, 0.006, 0.008, 0.01, 0.015, or at least 0.02 vol%. The composition may comprise an adjuvant, preferably a non-ionic surfactant adjuvant in a total amount of no greater than 5 vol%, or of no greater than 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or of no greater than 0.1 vol%. The composition may comprise an adjuvant, preferably a

non-ionic surfactant adjuvant in a total amount of between 0.01-0.5 vol%, or between 0.05-0.4, 0.05-0.3, 0.05-0.2, or of between 0.05-0.15 vol%.

The use may comprise contacting the spore-containing soil with the inventive compound or composition to kill or suppress the fungal spores. The spore-containing soil may preferably be contacted with the compound or composition by applying the compound or composition to an exposed surface of the soil.

The use may comprise spraying or pouring the compound or composition composition onto an exposed surface of the soil. In other embodiments, the use may comprise spreading the composition over the exposed surface of the soil.

Alternatively, the compound or composition may be applied directly into the body of the soil, such as by injection or irrigation.

In some embodiments, the inventive compound or composition is incubated with the spore and/or hyphae-containing soil for at least 1 day in total, or at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or at least 60 days in total. The inventive compound or composition may be incubated with the spore and/or hyphae-containing soil for no greater than 150 days in total, or for no greater than 125, 100, 95, 90, 85, 80, 75, 70, or no greater than 65 days in total. The compound or composition may be incubated with the spore-containing soil for between 1-150 days in total, or between 5-140, or between 10-130, or between 20-120, or between 30-110, or between 40-100, or between 45-80, or between 50-70, or between 55-65 days in total.

In some embodiments, the use may comprise applying the inventive compound or composition to the spore and/or hyphae-containing soil, followed by incubating the soil with the compound or composition. The use may further comprise re-applying the

inventive compound or composition to the spore and/or hyphae-containing soil. The use may comprise re-applying the compound or composition to the soil in the same amount/concentration as the initial application. Alternatively, the re-applied compound or composition may be of a higher or lower amount/concentration. The use may comprise re-applying the compound or composition to the soil after at least 30 minutes incubation, or at least 1 h, 2, 3, 4, 5, 10, 15, or at least 20 h incubation, or at least 1 day, 2, 3, 4, 5, 6, or at least 7 days incubation. The use may comprise re-applying the compound or composition to the soil after no greater than 60 days incubation, or after no greater than 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, or no greater 7 days incubation. The use may comprise re-applying the compound or composition to the soil after between 1-30 days incubation, or between 1-20, 1-15, 1-10, 2-9, 3-9, 4-9, 5-9, 6-9, or most preferably between 6-8 days incubation.

In some embodiments, the spore and/or hyphae-containing soil is contacted/incubated with the compound or composition comprising the inventive compound at a total concentration of at least 0.01 ng of the compound per spore, or at least 0.02, 0.03, 0.04, 0.05, 0.075, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2, 0.22, or at least 0.24 ng of the compound per spore. The spore and/or hyphae-containing soil may be contacted/incubated with the compound or composition comprising the inventive compound at a total concentration of no greater than 1.5 ng of the compound per spore, or no greater than 1.25, 1, 0.75, 0.5, 0.4, or of no greater than 0.3 ng of the compound per spore. The spore and/or hyphae-containing soil may be contacted/incubated with the compound or composition comprising the inventive compound at a total concentration of between 0.02-1 ng of the compound per spore, or preferably of between 0.05-0.5, 0.05-0.45, 0.05-0.4, 0.1-0.4, 0.1-

0.35, 0.15-0.35, 0.15-0.3, or preferably of between 0.2-0.3, or of between 0.22-0.28 ng of the compound per spore and/or its hyphae.

In some embodiments, the spore and/or hyphae-containing soil is contacted/incubated with the compound or composition comprising the inventive compound at a total concentration of at least 0.5 µg of the compound per gram of soil, or at least 1, 2, 3, 4, 5, 10, 15, 20, or at least 25 µg of the compound per gram of soil. The spore and/or hyphae-containing soil may be contacted/incubated with the compound or composition comprising the inventive compound at a total concentration of no greater than 150 µg of the compound per gram of soil, or of no greater than 125, 100, 75, 50, 40, or of no greater than 30 µg of the compound per gram of soil. The spore and/or hyphae-containing soil may be contacted/incubated with the compound or composition comprising the inventive compound at a total concentration of between 2-100 µg of the compound per gram of soil, or preferably of between 5-50 µg of the compound per gram of soil

5-45, 5-40, 10-40, 10-35, 15-35, 15-30, or preferably of between 20-30, or of between 22-28 µg of the compound per gram of soil.

The compounds of the invention can be used in such low amounts, whilst still successfully killing/suppressing fungal spores and/or their hyphae in soil without suffering from soil-derived inactivation.

In other embodiments, non-spore and/or hyphae-containing soil may be contacted with the inventive compound or composition. The soil may be susceptible to fungal spore infection and the inventive compounds may act to prevent the growth/introduction of fungal spores and/or their hyphae in the non-spore-containing soil.

According to a second aspect of the invention, there is provided a composition comprising at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$, and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl;

for use in the treatment of at least one fungal disease selected from *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4-mediated fungal disease and any strains, variants and pathotypes thereof.

The composition of the second aspect of the invention is preferably the composition of the first aspect of the invention. Statements of invention above relating to the composition of the first aspect of the invention or to any of its components may equally be applied to the second aspect of the invention. Other statements of invention above relating to the first aspect of the invention may also be applied *mutatis mutandis* to the second aspect of the invention.

In some embodiments, the fungal disease is a *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4-mediated plant disease. In a particularly preferred embodiment, the FocTR4-mediated fungal disease is a disease of a herbaceous flowering plant. The plant may be a plant of the order Zingiberales. In some embodiments the plant may be of the family *Musa*ceae or *Zingiberaeceae*. In some embodiments the plant may be a plant from the genus *Musa* or *Zingiber*. In some embodiment the plant may be selected from the group comprising banana, ginger or plantain. In preferred embodiments the plant is a

banana plant, which may be selected from the group comprising *Musa acuminata*, *Musa balbisiana*, *Musa* × *paradisiaca* and any hybrid thereof, and may be preferably as described in the first aspect of the invention. Most preferably, the FocTR4-mediated fungal disease is FocTR4-mediated banana wilt. Little success has been found previously when attempting to treat FocTR4-mediated banana wilt with fungicides, including fungicides of the major classes. FocTR4 is able to form fungicide-tolerant persister cells, allowing the fungus to survive high fungicide loads. Surprisingly, the compounds of the invention display outstanding performance at treating FocTR4-mediated banana wilt *in vivo*. The compounds can be administered at low concentrations (preferably as described for the first aspect of the invention) and display no phytotoxicity.

In some embodiments, the inventive composition may treat the FocTR4-mediated fungal disease by killing or suppressing FocTR4 fungus in soil. Preferably, the composition may treat the disease by killing or suppressing FocTR4 fungal spores in soil. The soil may be on, surrounding or coated on a plant or part of a plant, as described for the first aspect of the invention above. Statements of invention for the first aspect of the invention above relating to killing or suppressing fungal spores in soil may equally be applied to the second aspect of the invention.

According to a third aspect of the invention, there is provided the use of at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$, and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl;

as an antifungal agent against at least one fungal disease selected from *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4-mediated fungal disease and any strains, variants and pathotypes thereof.

The at least one compound is preferably the at least one compound of the first aspect of the invention. Statements of invention above relating to the at least one compound of the first aspect of the invention may equally be applied to the third aspect of the invention.

The at least one compound may be present as part of a composition, preferably the composition of the first aspect of the invention. Statements of invention above relating to the composition of the first aspect of the invention or to any of its components may equally be applied to the third aspect of the invention.

The *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4-mediated fungal disease is preferably the FocTR4-mediated fungal disease of the second aspect of the invention, or a variant, strain or pathotype thereof. Statements of invention above relating to the FocTR4-mediated fungal disease of the second aspect of the invention may equally be applied to the third aspect of the invention.

Other statements of invention above relating to the first and second aspects of the invention may also be applied *mutatis mutandis* to the third aspect of the invention.

In preferred embodiments, the inventive compounds are used as antifungals against a FocTR4-mediated plant fungal disease, preferably as described for the second aspect of the invention above.

The use may comprise contacting FocTR4-infected soil or soil that is susceptible to becoming infected by FocTR4 with the inventive compounds. The soil may be on, surrounding or coated on a plant or part of a plant, as described for the first aspect of the invention above. The soil may comprise FocTR4 fungal spores and/or their hyphae, as described for the first aspect of the invention. The inventive compounds may be applied before or after infection of the soil with the fungus.

In other embodiments, the use comprises contacting a plant that is infected with or susceptible to becoming infected with a FocTR4-mediated fungal disease, or a part of the plant, with the inventive compounds. The inventive compounds may be applied to the plant or part thereof by any suitable method, such as by spraying, spreading, or dusting the plant or part thereof with the inventive compounds. The use may comprise treating seeds of the plant with the inventive compounds. The inventive compounds may be applied before or after infection of the plants or seeds by the fungal disease.

According to a fourth aspect of the invention, there is provided a method of destroying, controlling or suppressing a *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 fungus or a variant, strain or pathotype thereof, the method comprising the step of contacting the fungus with at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$, and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl.

The at least one compound is preferably the at least one compound of the first aspect of the invention. Statements of invention above relating to the at least one compound of the first aspect of the invention may equally be applied to the fourth aspect of the invention.

The at least one compound may be present as part of a composition, preferably the composition of the first aspect of the invention. Statements of invention above relating to the composition of the first aspect of the invention or to any of its components may equally be applied to the fourth aspect of the invention.

Other statements of invention above relating to the first, second, and third aspects of the invention may also be applied *mutatis mutandis* to the fourth aspect of the invention.

Controlling or suppressing the fungus may comprise controlling the growth and/or lifespan of the fungus.

The method may comprise destroying, controlling, or suppressing fungus infecting plants (including cut or growing plants or plant parts), seeds, soil or an ecosystem.

The method may comprise contacting FocTR4-infected soil with the inventive compounds, as described for the third aspect of the invention above.

In some embodiments, the method comprises contacting a plant that is infected with a FocTR4-mediated fungal disease, or a part of the plant, with the inventive compounds, as described for the third aspect of the invention above.

According to a fifth aspect of the invention, there is provided a method of preventing *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 fungal growth or growth of any strains, variants and pathotypes thereof, on or in a material, the method comprising the step of applying to the material at least one compound with a formula independently

selected from the group comprising: R-$S^+(R')_2$, R-$N^+(R')_3$, and R-$N(H)C(NH_2)_2^+$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl.

The at least one compound is preferably the at least one compound of the first aspect of the invention. Statements of invention above relating to the at least one compound of the first aspect of the invention may equally be applied to the fifth aspect of the invention.

The at least one compound may be present as part of a composition, preferably the composition of the first aspect of the invention. Statements of invention above relating to the composition of the first aspect of the invention or to any of its components may equally be applied to the fifth aspect of the invention.

Other statements of invention above relating to the first, second, third, and fourth aspects of the invention may also be applied *mutatis mutandis* to the fifth aspect of the invention.

The material may comprise plant matter, seeds, or soil. The material alternatively be independently selected from the group comprising: plastic, metal, wood, paper, textiles (natural and synthetic), leather, fibres, glass, composite materials, minerals, stone, concrete, plaster, ceramic, rubber, and combinations thereof. In some embodiments, the material comprises an artefact or a part thereof, which may be independently selected from the group comprising: footwear, farm equipment, and tools.

Detailed Description of the Invention

In order that the invention may be more clearly understood embodiments thereof will now be described, by way of example only, with reference to the accompanying drawings, of which:

Figure 1 morphology of FocTR4 (a) hyphae; (b) microconidia; (c) macroconidia; and (d) chlamydospores. Scale bar = 20 μm.

Figure 2 (a) scanning electron micrograph of a chlamydospore. Scale bar = 2 μm; and (b) ultrastructure of the cell wall of a chlamydospore. The inner wall is layered, while the outer wall is a fibrillar matrix. Scale bar = 0.5 μm.

Figure 3 (a) banana plants and (b) banana corms at 56 days after infection (dpi) with FocTR4 hyphae, microconidia, macroconidia or chlamydospores. All morphotypes cause leaf wilting and corm necrosis.

Figure 4 quantitative assessment of disease symptoms on banana plant leaves after infection with FocTR4 morphotypes (hyphae, microconidia, macroconidia or chlamydospores). Plants range from healthy to dead (0-4). Average scores and standard error of the mean (SEM) from 17-33 plants in 4-5 independent experiments are given. Bars represent mean±SEM; sample sizes are indicated; statistical analysis used one-way ANOVA; n.s.= non-significant difference amongst data sets to control at two-tailed P= 0.7174.

Figure 5 quantitative assessment of necrosis in banana plant corm tissue after infection with FocTR4 morphotypes (hyphae, microconidia, macroconidia or chlamydospores). Bars represent mean±SEM; sample

sizes are indicated; statistical analysis used one-way ANOVA; n.s.= non-significant difference amongst data sets to control at two-tailed P=2590.

Figure 6 FocTR4 colonies on potato dextrose agar (PDA) plates after 2d growth at 25°C. Colonies contain of central region of densely-grown cells and a "corona" of more diffuse spreading hyphae. The number of inoculated microconidia per colony are indicated.

Figure 7 growth curves of FocTR4 on PDA plates, supplemented with fungicides, after growth for 2d at 25 °C. 7 fungicide classes were tested, each represented by 3 compounds; (a) benzimidazoles; (b) multi-site inhibitors; (c) 'organic' fungicides; (d) MALCs = mono-alkyl lipophilic cations (compounds of the invention); (e) azoles; (f) strobilurins; (g) SDHIs = succinate dehydrogenase inhibitors. All data points in are given as mean ± SEM from 4 measurements in 2 independent experiments.

Figure 8 relative number of living (a) microconidia, (b) macroconidia, and (c) chlamydospores (LIVE/DEAD (RTM) staining negative) after 10d treatment with fungicides. Fungicide classes are indicated above the bars. Bars represent the mean proportion (±SEM) of cells that did not take up LIVE/DEAD (RTM) staining, thus were considered alive; dots represent averages of 3 independent experiments. All spore types in were incubated for 10d in ddH_2O at 25°C and under rotation; concentrations used were in category I (=inhibition on agar plates at <10 µg ml^{-1}): thiobendazole, 10 µg ml^{-1}; carbendazim, 4.5 µg ml^{-1}; chlorothalonil, 5 µg ml^{-1}; captan, 10 µg ml^{-1}; mancozeb, 35 µg ml^{-1}; in category II (=inhibition on agar plates

at >10 μg ml^{-1}): copper, 100 μg ml^{-1} (applied as Copper(II) sulfate pentahydrate); LMW chitosan, 100 μg ml^{-1} (applied as a lactate salt); garlic oil, 100 μg ml^{-1}; CTAB (Cetrimonium bromide), 100 μg ml^{-1}; dodine, 100 μg ml^{-1}; C_{18}DMS; 100 μg ml^{-1}; triticonazole, 100 μg ml^{-1}; epoxiconazole, 100 μg ml^{-1}: tebuconazole, 100 μg ml^{-1}; azoxystrobin, 100 μg ml^{-1}; trifloxistrobin, 100 μg ml^{-1}; pyraclostrobin, 100 μg ml^{-1}; bixafen, 100 μg ml^{-1}; fluxapyroxad, 100 μg ml^{-1}; boscalid, 100 μg ml^{-1}; thiophanate methyl, 100 μg ml^{-1}.

Figure 9 whole-plant symptoms of Panama disease at 56d after plant root inoculation with FocTR4 chlamydospores, followed by 2 applications of fungicides (labelled in figure) or the solvents alone (0.14% DMSO or 0.16% methanol in water (Control (+))). The negative control plant (Control (-)) was only treated with the solvent and not inoculated with spores.

Figure 10 corm necrosis in banana plants at 56 d after plant root inoculation with chlamydospores, followed by 2 treatments with fungicides (labelled in figure). Control(+) indicates inoculation with spores, followed by treatment with 0.16% methanol (positive control).

Figure 11 quantitative assessment of darkening of corm tissue after infection with chlamydospores followed by 2 treatments with fungicides. Banana corm necrosis was analysed 56 d after the first treatment. First 3 bars: Controls; remaining bars: fungicide treatments. Bars show mean±SEM from 12-16 measurements of 6-8 plants from 2 experiments; statistical comparison

used Student's t-testing with Welch correction; n.s.= non-significant difference to respective control at two-tailed error probability of P=0.965 (Mancozeb), P=0.3425 (Copper) and P=0.0753 (Chitosan); *= significant difference to control at two-tailed P=0.0261; ***= significant difference to control at two-tailed P=0.0001; ****= significant difference to control at two-tailed P<0.0001. Plant infection assays used Chitosan, 200 $\mu g\ ml^{-1}$ (applied as 333 $\mu g\ ml^{-1}$ lactate salt); Copper, 200 $\mu g\ ml^{-1}$ (applied as 786 $\mu g\ ml^{-1}$ copper(II) sulfate pentahydrate); Mancozeb, 70 $\mu g\ ml^{-1}$; Captan, 20 $\mu g\ ml^{-1}$; CTAB, 200 $\mu g\ ml^{-1}$; Dodine, 200 $\mu g\ ml^{-1}$; C_{18}DMS, 200 $\mu g\ ml^{-1}$.

Figure 12 Bar chart showing plant health, assessed by measuring all aerial tissues in photographic images of 56d-old plants, infected with chlamydospores and treated with C_{18}DMS or dodine. C_{18}DMS is significantly more protective than dodine. Control indicates healthy plants that were not infected but were treated with the solvent methanol.

Figure 13 developmental stages of chlamydospore germination in potato dextrose medium. Dormant spores appear smooth (stage I), but before germination appear granular (stage II). Single germ tubes are formed that form septa (arrowhead), extend at their growing tip and develop into hyphae (stage IV). Scale bar= 10 μm.

Figure 14 mitochondrial membrane potential, visualised by the cell-penetrating dye TMRM, in chlamydospores at different stages of germination. Almost no TMRM staining is seen in un-germinated spores (stage I), but

mitochondria-associated fluorescence appears shortly before the germ tube emerges (stage II; arrowhead). Scale bar= 10 µm.

Figure 15 mitochondrial membrane potential, indicated by TMRM fluorescence, in chlamydospores at stage (I) and (II) and in the germ tube (IV). Data are not normally distributed (Shapiro-Wilk test, $P<0.05$) and are shown as Whiskers' plots with 25/75 percentiles and median. Statistical comparison with control non-parametric Mann-Whitney testing. Treatments were 10 µg ml^{-1} C_{18}DMS for 30 minutes.

Figure 16 intensity profile of TMRM fluorescence, indicative of mitochondrial membrane potential, along germ tubes of 7h-old germlings of FocTR4, treated with the solvent methanol (top curve) and 10 µg ml^{-1} C_{18}DMS (bottom curve) for 30 min. Values are shown as mean ± SEM of 14-15 germlings. C_{18}DMS depolarises mitochondria. n.s.= non-significant difference at two-tailed $P=0.093$; ****= significant difference at two-tailed $P<0.0001$.

Figure 17 abundance of reactive oxygen species (ROS), labelled with DHR123, in control and C18DMS-treated FocTR4 cells. Treatments were 10 µg ml^{-1} C_{18}DMS for 30 min. Control indicates the use of an equivalent volume of the solvent methanol. Data are not normally distributed (Shapiro-Wilk test, $P<0.05$) and are shown as Whiskers' plots with 25/75 percentiles and median. Statistical comparison with control non-parametric Mann-Whitney testing. n.s.= non-significant difference at two-tailed $P=0.093$; ****= significant difference at two-tailed $P<0.0001$.

Figure 18 induction of cellular apoptosis, visualised by FITC-VAD(OMe)-FMK staining, in control and C_{18}DMS-treated FocTR4 cells. Treatments were 10 µg ml-1 C18DMS for 24h. Control indicates the use of an equivalent volume of the solvent methanol. Data are given as mean±SEM; dots represent independent experiments. Statistical comparison with control Student's t-testing with Welch correction. n.s.= non-significant difference at two-tailed P=0.093; ****= significant difference at two-tailed P<0.0001.

Examples

Ability of FocTR4 morphotypes to cause Panama disease in bananas

FocTR4 macroconidia, microconidia, chlamydospores and hyphae were prepared at >95% purity (Figure 1a-d), as follows:

Media used for production of chlamydospores, macrospores, microspores and hyphae were supplemented with PenStrep at a final concentration of 50 mg/l (Sigma–Aldrich, Gillingham, UK).

Microconidia: 100 ml potato dextrose broth (PDB, Sigma Aldrich, Gillingham, UK) were inoculated from -80°C glycerol stocks and grown at 26°C, 150 rpm, for 7 days, thence filtered through two layers of Miracloth (Merck Millipore, Hertfordshire, UK), pelletted by centrifugation and washed 3-times with sterile distilled water (SDW). The purity was 100% (100±0.0 %, n=3 preparations, >100 cells each).

Hyphae: Microconidia were incubated in yeast peptone dextrose broth (YPD) at 26°C, 150 rpm, for 4d. Hyphae were collected by filtering through Miracloth, followed by re-submersion into SDW. Hyphal preparations were 97.3±0.9% pure (n=3).

Chlamydospores: ~10^8 microconidia per litre were inoculated into soil broth (250 g of John Innes No. 2 soil to 1 litre of SDW supplemented with 0.5% (w/v) glucose) and grown at 26°C, 120 rpm, for 14-28 days. Chlamydospores, attached to hyphae, were harvested by filtering through Miracloth, and suspended in 40 ml SDW. Chlamydospores were released from hyphae using a Soniprep 150 sonicator (Sanyo, Osaka, Japan) for 5 minutes, using a 19 mm titanium probe. Residual hyphal fragments were removed by Miracloth filtration. Finally, chlamydospores were pelletted by centrifugation and washed 3-times with sterile distilled water (SDW). The purity of chlamydospore preparations was 95.5±1.4% (n=3).

Macroconidia: Microconidia were plated on yeast peptone dextrose agar (bacteriological peptone, 20 g/l; glucose, 20 g/l; agar, 15 g/l) for 10d at 26°C under constant illumination (Certomat BS-1 growth cabinet, Satorius, Göttingen, Germany). Cells were harvested into 5 ml SDW, filtered through Miracloth, pelletted by centrifugation and washed 3-times with sterile distilled water (SDW). The purity of macrospore preparations was 94.2±1.9% (n=3).

Chlamydospores are thought to be the most durable and infectious spore form. These spherical cells are enveloped in a thick cell wall covered by a fibrillar matrix (Figure 2a, b). Upon germination in potato dextrose broth (PDB), these spores formed short hyphae, which contained a cluster of mitochondria near the growing apex.

A robust and quantitative banana infection assay was designed to investigate if the FocTR4 spores cause Panama disease in bananas. Procedures were standardised (i) using whole plant-in-soil assays and (ii) with pre-weighed soil in pots; (iii) top-drenching 8-12 week-old bananas with 50 ml water, containing a defined amount of fresh inoculum, to

avoid run-off from the pots of inoculum, (iv) adding the adjuvant 3-(2-methoxyethoxy)propyl-methyl-bis(trimethylsilyloxy)silane (Silwet L-77), to ensure even distribution within the soil; finally (v) watering plants, placed on saucers, from the bottom.

8 to 12 week-old banana plants (Cavendish, Great Nairn), which carried 4-5 fully formed leaves, were kept in 12 cm, 500 mL plastic pots containing 400 g John Innes No. 2 soil (J Arthur Bowers, Dungannon, UK). The root system of the plants was infected with purified hyphae, microconidia, macroconidia and chlamydospores, as follows:

The root system was wounded by cutting once through the entire soil area, at a 45-degree angle, with a garden trowel (3/8 gauging trowel, Marshalltown Tools, Braintree, Essex, UK). 50 ml of cell suspensions, containing microconidia, macroconidia, chlamydospores or hyphae were added to the soil, resulting in $1x10^5$ propagules per gram soil. The inoculated plants were grown for 56 days in a greenhouse on a long-day light cycle (16h light: 8h dark), using artificial light (SON-T 230V 600W bulbs; Philips, Amsterdam, The Netherlands) at 26°C during the day and 24°C at night. The plants were watered daily and fertilised weekly with Yara Tera™ Kristalon™ Label fertiliser solution (Yara UK Ltd, Grimsby, UK) at a rate of 0.5 g/l. (i) External disease symptoms on whole plants were assessed and (ii) corm necrosis was determined as an internal marker of disease severity. Plant health was scored using a 0–4 scale (0= healthy; 1= slight chlorosis and wilting with no petiole buckling; 2= moderate chlorosis and wilting with some petiole buckling and/or splitting of leaf bases; 3= severe chlorosis, wilting, petiole buckling and dwarfing of the newly emerged leaf; and 4= dead. Necrosis in corms was measured in digital images of split corms, taken at standardised conditions. Images were converted to grey-scale and contrast-inverted, using MetaMorph, which converted dark necrosis areas

into bright signals. In each image, the average pixel signal intensity in both halves of the corm and in a defined background area was measured. Image variations were normalised by adjusting all background signal intensities, providing corrected values for signal intensities in corms. The values of non-infected plants (negative control) were subtracted from all measurements. The values for infected plants that were treated with water, DMSO or methanol (infected "solvent" only controls) were set to 100% (=maximal symptoms) and measurements related to their respective treatments (copper and chitosan to water control; mancozeb to DMSO control; all other compounds to methanol control). Data analysis and presentation used Excel and Prism6.

All morphotypes caused wilting, with leaves being necrotic and/or chlorotic (Figure 3a). These symptoms are due to the infection of the vascular system by the fungus, which can be directly monitored by tissue necrosis in the corm of the plant. Consistently, it was found that all morphotypes induce necrosis of the plant corm (Figure 3b). These external and internal disease symptoms were quantitatively assessed as described above. Averaging these symptom values, derived from 17-33 plants in 4-5 experiments, it was found that all morphotypes were equally virulent (Figure 4, average ~3.4; one-way ANOVA test, not different at two-tailed P=0.7174). In addition, corm necrosis was measured using contrast-inverted images of infected corms. Again, no significant difference between all morphotypes was found (Figure 5, one-way ANOVA test, not different at two-tailed P=0.2590). Thus, it was concluded that all morphotypes can cause Panama disease.

FocTR4 forms colonies in the presence of major fungicide classes

Experimental: YPD agar plates (for growth of *Z. tritici* IPO323) and potato dextrose agar plates (for growth of FocTR4) were supplemented with fungicides or their respective solvents (100% control). FocTR4 microconidia suspensions were diluted (concentrations were 10^6 cells ml^{-1}, 10^5 ml^{-1}, 10^4 ml^{-1}, 10^3 ml^{-1}; here and in the following cells were counted using a Cellometer Auto 1000 cell counter (Nexcelom Biosciences, Lawrence, USA)). Drops of 5 µl cell suspension were placed on the agar plates, followed by incubation for 2d at 25°C. For *Z. tritici* assays, diluted cell suspensions were prepared (concentrations were 10^6 cells ml^{-1}, 0.5 x 10^6 ml^{-1}, 0.25 x 10^6 ml^{-1}, 1.25 x 10^5, 6.25 x 10^4). Drops of 5 µl were placed on the agar plates, followed by incubation for 5d at 18°C. Digital images of plates were obtained using an Epson Perfection V750 Pro scanner (Epson, Hemel Hempstead, UK). Growth inhibition was assessed by measuring the integrated intensity of the third highest dilution for IPO323 and second dilution for FocTR4. Digital images were converted to grey-scale using Photoshop CS6 (Adobe Inc., San Jose, USA), an area of interest was drawn around the colony and the integrated intensity of all pixels in this area determined. Next, the area of interest was moved in fungal cell-free region and the integrated intensity of this background was measured. After correcting for this background, the values for colony formation on control plates, containing the solvents only, were set to 100% and all other measurements were compared. The resulting values represented the relative cell density (=colony brightness) and were plotted in Prism6 to obtain fungicide dose-response curves.

Results: Growth of microconidia on the fungicide-supplemented potato dextrose agar (PDA) plates at 2d were quantitatively assessed. The fungicide-susceptible pathogen *Z. tritici* was used as a reference. Colony formation was assessed and compared with that of *Z. tritici*, grown under comparable conditions. 21 compounds were chosen, belonging to

7 main classes of fungicides, including succinate dehydrogenase inhibitors (SDHIs: fluxapyroxad, bixafen, boscalid), strobilurins (azoxystrobin, pyraclostrobin, trifloxystrobin), azoles (epoxiconazole, tebuconazole, triticonazole), benzimidazoles (carbendazim, thiobendazole, thiophanate methyl), multi-site fungicides (captan, chlorothalonil, mancozeb), "organic" fungicides (low molecular weight chitosan [= LMW chitosan], copper sulfate, garlic oil) and mono-alkyl lipophilic cations (MALCs) (compounds of the invention) (*n*-hexadecyltrimethylammonium bromide (cetrimonium bromide (CTAB)), *n*-dodecylguanidinium (dodine), and *n*-octadecyldimethylsulfonium (C18-DMS)).

When *Z. tritici* strain IPO323 was grown on control plates, smooth colonies were formed. In contrast, FocTR4 colonies (Figure 6) consisted of a peripheral "corona" of hyphae, and central accumulations of "yeast-like" cells. Fungicides prevented FocTR4 hyphal growth, before growth of the central cells were inhibited. This suggested that measuring the colony diameter, as done in previous studies, is not an accurate method to monitor fungicide effects on FocTR4. A method was developed using the bright appearance of colonies on dark agar plates in digital images as a measure of growth. This provided dose-response curves for the fungi exposed to all fungicides (curves for FocTR4 - Figure 7a-7g). Using these curves, the fungicide concentrations leading to 50% growth inhibition (EC50) and 100% growth inhibition (EC100) were determined. 16 of the 21 fungicides were highly inhibitory against IPO323 (category I, EC100 of 1-10 µg/ml), whereas only 3 had moderate inhibitory effects (category II, EC100 <41 µg/ml), while copper (applied as copper sulfate) and LMW chitosan (applied as a lactate salt) had only low inhibitory effects on colony formation (category III, EC100 ~1,100-1,200 µg/ml). In contrast, FocTR4 colony formation was much less inhibited by the fungicides. Only 5

fungides showed strong inhibitory effects (category I, EC100 of 1-10 µg/ml: carbendazim, thiobendazole, chlorothalonil, captan, mancozeb), whereas 6 compounds had only moderate effects on colony formation (category II, EC100 of 10-263 µg/ml: epoxiconazole, triticonazole, tebuconazole, dodine, garlic oil, copper). In addition, 2 compounds showed low effects, yet still prevented colony growth at high concentrations (category III, EC100 of 690-1790 µg/ml: cetyltrimethylammonium bromide (CTAB) and LMW chitosan). Surprisingly, 8 compounds did not fully inhibit colony formation (category IV, EC100 >1000 µg/ml: azoxystrobin, pyraclostrobin, trifloxystrobin, fluzapyroxad, bixafen, boscalid, thiphanate methyl, *n*-octadecyldimethylsulfonium). A direct comparison of the response of both fungi to each fungicide, given as the ratio of EC50 values (resistance factor RF) and EC100 values (survival factor SF), revealed that FocTR4 showed higher RF values for 15 fungicides (3.1-324.5) and increased SF values for 16 fungicides (SF>4.0; note that RF<3 was not considered). IPO323 shows higher tolerance only against captan (RF= 293.6, 12.1), copper (RF= 4.3, SF= 4.1) and, to a minor extent, to chitosan (RF= 2.7, SF= 1.5). It is worth mentioning that FocTR4 colony formation was highly tolerant to azoles (SF=170.8-1329.9). Moreover, even 1000 µg/ml of strobilurins and SDHIs did not prevent FocTR4 growth (Fig. 7e, 7f). However, colony formation in these fungicides was strongly inhibited at low concentrations, resulting in rapid decline in the dose response curve, but reached a plateau at higher concentrations. Thus, FocTR4 cells, grown on solid medium, are highly tolerant against major fungicide classes.

<u>All three asexual spore forms of FocTR4 survive treatment with most fungicides</u>

The fungitoxicity of the abovementioned 21 fungicides was assessed on chlamydospores, microspores, and macrospores, using quantitative LIVE/DEAD (RTM) staining.

Experimental: FocTR4 macroconidia, microconidia and chlamydospores were prepared as described above and diluted in ddH_20. The cell density was adjusted to 5 x 10^6 ml^{-1} and fungicides were added to a final concentration of 100 µg/ml (azoxystrobin, pyraclostrobin, trifloxystrobin, bixafen, boscalid, fluxapyroxad, triticonazole tebuconazole epoxiconazole, LMW chitosan [applied as 167 µg/ml lactate salt], copper [applied as 393 µg/ml copper(II) sulfate pentahydrate], garlic oil, thiophanate methyl, tebuconazole, CTAB, dodine, *n*-octadecyldimethylsulfonium). Highly efficient category I fungicides were applied at 5-times their EC100 (carbendazim 4.50 µg/ml, captan 10 µg/ml, mancozeb 35 µg/ml, chlorothanonil 5 µg/ml). The suspensions were incubated in the dark on a rotation wheel at 85 rpm at 25°C. Cells were harvested after 10d by centrifugation and stained with the red-fluorescent LIVE/DEAD (RTM) Fixable Red Dead Cell Stain Kit (Molecular Probes (RTM) Thermofisher, UK), used at a final concentration of 1 µl/ml. Cell death was assessed in microscopy, taken with wide-field and red-fluorescence settings at the Olympus inverted microscope. Cells were considered dead if red-fluorescence was found within the borders of the cell. To assess " persister" cell survival of FocTR4 and IPO323 in high concentration of fungicides (3 µg/ml epoxiconazole, 500 µg/ml azoxystrobin and 500 µg/ml fluxapyroxad) pre-cultures were grown for 2d at 25°C or 18°C, 150 rpm and 5 ml used to inoculate fresh medium, supplemented with identical concentrations of fungicides. Samples were taken after 2d, 5d and 9d and mortality assessed using LIVE/DEAD (RTM) staining.

Results: In this assay, dead cells accumulated red fluorescence, whereas living cells excluded the dye. Fungicides in category I (=inhibition on agar plates at <10 µg/ml) were incubated at ~5-times EC100 (carbendazim, 4.5 µg/ml; thiobendazole, 10 µg/ml; chlorothalonil, 5 µg/ml; captan, 10 µg/ml; mancozeb, 35 µg/ml) and fungicides in

category II-IV (=inhibition on agar plates at >10 µg/ml) were incubated at 100 µg/ml on a rotating shaker. After 10d, at 25°C, in water, LIVE/DEAD (RTM) staining was performed and red-fluorescent cells counted. All spore forms survived treatment with 100 µg/ml azoles, strobilurins, and SDHIs, and only tebuconazole and pyraclostrobin caused moderate mortality in macro- and microconidia (Fig. 8a-c), again suggesting that major fungicide classes are not fungitoxic in FocTR4. Surprisingly, several category I compounds, including carbendazim, thiobendazole and chlorothalonil, did not kill all spore forms (Fig. 8a-c), indicating that they inhibit colony formation, but are not fungitoxic to FocTR4 cells. Only mancozeb, copper, and the three inventive mono-alkyl lipophilic cationic compounds effectively killed all micro-, macroconidia and chlamydospores (Fig. 8a-c). The colony formation assays had classified CTAB and *n*-octadecyldimethylsulfonium as category III and category IV, respectively (see above), but these results indicate that they are more effective in liquid culture. Several fungicides showed reduced efficacy in chlamydospores (Fig. 8a-c; e.g. captan and LMW chitosan). Fungal chlamydospores are enclosed by thick cell walls, which makes them durable and allows survival in harsh environments, such as soil. Electron microscopy on FocTR4 chlamydospores revealed that these uni-nuclear cells are surrounded by a fibrillar outer layer and an inner multi-layered wall, which is significantly thicker than that of the other morphotypes. The cell wall can thus act to reduce fungicide efficacy. In summary, 14 of 21 fungicides showed no, or low fungitoxicity in spores. Again, azoles, SDHIs and strobilurins were ineffective against FocTR4.

<u>The inventive compounds protect banana plants against Panama disease</u>

Experiments for fungicide lethality in FocTR4 cultures showed that the multi-site fungicide mancozeb, the "organic fungicide" copper and the three inventive compounds

(CTAB, dodine and *n*-octadecyldimethylsulfonium) were fungitoxic in all spore types. These fungicides were then applied to banana pathogenicity assays. In addition, captan and chitosan were included, which effectively killed micro- and macroconidia, as both are effective in inhibiting growth of FocTR4 on plates. To enhance the effectiveness of these 7 fungicides in soil, the lethal concentrations, determined in liquid culture fungicide mortality assays were doubled, and the fungicide solutions supplemented with the non-ionic surfactant Silwet L-77, to ensure even penetration within the soil. The fungicide solutions used were: LMW chitosan, 200 µg/ml [applied as 333 µg/ml lactate salt]; copper, 200 µg/ml [applied as 786 µg/ml copper(II) sulfate pentahydrate]; mancozeb, 70 µg/ml; captan, 20 µg/ml; CTAB, 200 µg/ml in methanol; dodine, 200 µg/ml; *n*-octadecyldimethylsulfonium, 200 µg/ml; all in double-distilled water and supplemented with 0.1% (v/v) Silwet L-77.

In a first set of experiments, phytotoxicity of the fungicide preparations was tested. Droplets (10 µl) of all fungicide solutions were applied to the newest fully-formed leaves of a 6-8 week-old plant and tissue damage after 24h was monitored. Droplets of 50 mg/ml benzalkonium chloride (BAC), used as positive control, caused leaf necrosis. However, none of the fungicide solutions caused leaf damage. Next, it was tested if root-application of the fungicides affects plant health. 8-week-old plants were watered with all 7 fungicide solutions at the same concentrations and BAC was used as a positive control (50 mg/ml). 50 ml of fungicide solution, or BAC as the positive control, were added to the plant pots twice, on day 0 and on day 7. The appearance of the banana plants was assessed after 21d. Again, BAC treatment caused plant damage and killed the control plants, whereas fungicide-treated plants did not show obvious differences from plants, treated with water only.

As the experiments demonstrated that soil-applied fungicide solutions are not toxic to the plant, all 7 fungicides were tested for their ability to protect banana plants from FocTR4-mediated disease. Chlamydospores were the most resilient against fungicides in liquid culture (see above, Fig. 8a-c) and are most relevant for *Fusarium oxysporum* infection in the field. 8-12 week-old banana plants were infected with this spore form.

8-12-week-old transplant plants, grown in 400 g soil in 500 mL pots were wounded, as described previously. Chlamydospore suspension was poured onto the soil surface at a final concentration of 10^5 spores per gram of soil. After allowing the spores to settle in the soil for 1h, 50 ml fungicide solution, containing Silwet L-77, was added to each pot, thereby wetting the soil evenly without losing any chlamydospores in flow-through liquid. This procedure was repeated after 7d, and plant disease symptoms were assessed after an additional 49 d. Consistent with previous results (see above, Fig. 3a, 3b), chlamydospores caused wilting and death of banana plants (Fig.9, (Control (+)) and induced necrosis of the corm (Fig.10, (Control (+)). Treatment of plants with the various fungicides resulted in banana plants with graduated disease symptoms. Mancozeb afforded no obvious protection against Panama disease (Fig. 9, 10). Indeed, quantitative comparison of corm necrosis against plants, treated with solutions that contained the solvent DMSO alone, showed no significant difference (Fig. 11; Student's-t test, two-tailed P=0.965). Plants treated with copper, chitosan and captan showed attenuated leaf damage in banana plants (Fig. 9) and reduced corm necrosis as compared with the disease control (Fig. 10, 11). However, of the 7 fungicides tested, captan was least reproducible in its protective activity. The best protection was achieved by treatment of banana plants with the compounds of the invention. In particular, plants treated with *n*-octadecyldimethylsulfonium (C_{18}DMS) appeared relatively healthy, with necrosis and

leaf yellowing being restricted to lower and older leaves (Fig. 9; compare "Control (-)" (=non-infected) and C_{18}DMS-treated plants; Fig. 12). Corm necrosis in all inventive compound-treated plants was largely reduced (Fig. 10, 11), with highly significant differences to infected control plants. Again, *n*-octadecyldimethylsulfonium outperformed all other chemistries in preventing corm necrosis, although dodine had a similar corm protective effect (Fig. 11). Thus, it was shown that the compounds of the invention have the potential to protect bananas against FocTR4-induced Panama disease.

The results show that *n*-octadecyldimethylsulfonium has improved protective activity in banana infection assays. Work in *Z. tritici* has shown that *n*-octadecyldimethylsulfonium affects the pathogen cell in multiple ways. As with dodine, this lipophilic cation depolarises the Inner Mitochondrial Membrane (IMM), thereby impairing ATP synthesis. However, *n*-octadecyldimethylsulfonium also induces reactive oxygen species (ROS) production and initiates apoptotic cell death.

It was then investigated how *n*-octadecyldimethylsulfonium kills FocTR4 in the soil. A prerequisite for root infection is the "awakening" of the dormant chlamydospore, followed by germ tube formation and hyphal growth. Chlamydospore germination was triggered in potato dextrose medium and cells stained with the red-fluorescent dye tetramethylrhodamine methyl ester (TMRM), which accumulates in the negatively-charged matrix of healthy and active mitochondria. It was found that TMRM does not stain mitochondria in dormant chlamydospores (Fig. 13, 14; stage I; Fig. 15), suggesting that these cells are inactive. Subsequently, the cytoplasm of chlamydospores appears more granular and TMRM-positive mitochondria appear before a germ tube emerges (Fig. 14, 15; stage II). Mitochondria remain active during germ tube formation and extension (Fig. 13, 14, 15; stage III). A cluster of active mitochondria was found at ~5

µm behind the growing tip of the germ tube (Fig. 13, 14; stage IV; Fig. 16). Next, TMRM fluorescent signal intensity was measured in germlings (stage IV), treated with *n*-octadecyldimethylsulfonium or solvent only (Control). This revealed that the mitochondrial potential was clearly reduced in the presence of the lipophilic cation (Fig. 16). Next, cells were stained with the ROS-detecting dye dihydrorhodamine 123 (DHR123) and the apoptosis marker FITC-VAD(OMe)-FMK. *n*-octadecyldimethylsulfonium induced ROS formation (Fig. 17) and, consequently, triggered apoptosis in FocTR4 germlings (Fig. 18). Thus, it was concluded that *n*-octadecyldimethylsulfonium impacts on FocTR4 by (i) affecting mitochondrial respiration, (ii) inducing aggressive ROS molecules and (iii) initiating apoptotic "cell suicide".

Discussion of results

The results achieved largely support the notion that chemical control of *Fusarium* wilt in banana plants is challenging, particularly in the field. 18 of 21 fungicides from 6 notable fungicide classes were ineffective against FocTR4 *in vitro* and/or *in vivo*. This result was most significant for azoles, SDHIs, strobilurins and benzimidazoles, which had minor effects on colony formation in FocTR4, and/or did not kill FocTR4 microconidia, macroconidia or chlamydospores in liquid culture assays.

Furthermore, most fungicides which inhibited colony formation and/or were fungicidal to FocTR4 spores *in vitro* did not protect banana plants against Panama disease *in vivo*. However, the three tested mono-alkyl lipophilic cationic compounds of the invention displayed significant protection *in planta*. The compounds of the invention were also

found to be effective at even very low concentrations and caused no phytotoxicity in the plants.

Without being bound by theory, the high anti-fungal activity displayed by dodine and *n*-octadecyldimethylsulfonium, in particular, is believed to be related to their primary mechanism of action, which is inhibition of mitochondrial respiration and, consequently, impaired ATP production. Moreover, *n*-octadecyldimethylsulfonium induces the formation of reactive oxygen species at mitochondrial respiration complex I, which, in turn, triggers apoptotic programmed cell death.

The above embodiments are described by way of example only. Many variations are possible without departing from the scope of the invention as defined in the appended claims.

CLAIMS

1. Use of at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$ and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl;

to kill or suppress or destroy at least one of the group comprising fungal spores, hyphae and hyphal fragments in soil.

2. Use of at least one compound as claimed in claim 1, wherein the fungal spores and/or their hyphae comprise *Fusarium* spores, preferably *Fusarium oxysporum* spores and/or their hyphae.

3. Use of at least one compound as claimed in claim 2, wherein the fungal spores comprise *Fusarium oxysporum* f.sp. *cubense* spores, preferably *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 spores or spores of a variant, strain or pathotype thereof.

4. Use of at least one compound as claimed in any preceding claim, wherein the spores comprise asexual spores or sexual spores.

5. Use of at least one compound as claimed in any preceding claim, wherein the spores and/or their hyphae comprise or are derived from one or more spore types independently selected from the group comprising: microspores, macrospores and chlamydospores .

6. Use of at least one compound as claimed in any preceding claim, wherein the spores are present in a total amount of between 50,000-200,000 spores and/or hyphae/gram of soil.

7. Use of at least one compound as claimed in any preceding claim, wherein the soil is on, surrounding or coated on a plant or part thereof.

8. Use of at least one compound as claimed in claim 7, wherein the plant or plant part is an edible foodstuff.

9. Use of at least one compound as claimed in any preceding claim, wherein R is a C10-C20, preferably C12-C18 straight chain alkyl.

10. Use of at least one compound as claimed in any preceding claim, wherein, when present, at least one R', and preferably each R' is methyl.

11. Use of at least one compound as claimed in any preceding claim, wherein the at least one compound is independently selected from the group comprising: *n*-octadecyl dimethylsulfonium, *n*-hexadecyl trimethylammonium, and *n*-dodecyl guanidinium.

12. Use of at least one compound as claimed in any preceding claim, wherein the at least one compound comprises a counterion independently selected from the group comprising: chloride, bromide, iodide, hydroxide, sulfate, phosphate, hydrogen sulfate, and acetate.

13. Use of at least one compound as claimed in any preceding claim, wherein spore-containing soil is incubated with the at least one compound at a

concentration of between 5-50 µg of the at least one compound per gram of soil.

14. Use of at least one compound as claimed in any preceding claim, wherein the at least one compound is applied prophylactically to uninfected or pre-infected soil, before planting of a crop.

15. Use of at least one compound as claimed in any preceding claim, wherein the at least one compound is present as or in a composition.

16. Use of at least one compound as claimed in claim 15, wherein the composition is an aqueous solution containing the at least one compound at a concentration of between 0.1-1000 µg/mL.

17. Use of at least one compound as claimed in claim 15 or 16, wherein the composition further comprises at least one non-ionic or ionic surfactant adjuvant, preferably in an amount of between 0.01-1 vol%.

18. A composition comprising at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$, and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl;

for use in the treatment of at least one fungal disease selected from *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4-mediated fungal disease and any strains, variants and pathotypes thereof.

19. A composition for use as claimed in claim 18, wherein the fungal disease is *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4-mediated banana wilt.

20. A composition for use as claimed in claim 18 or 19, wherein R is a C10-C20, preferably C12-C18 straight chain alkyl.

21. A composition for use as claimed in any one of claims 18 to 20, wherein, when present, at least one R', and preferably each R' is methyl.

22. A composition for use as claimed in any one of claims 18 to 21, wherein the at least one compound is independently selected from the group comprising: *n*-octadecyl dimethylsulfonium, *n*-hexadecyl trimethylammonium, and *n*-dodecyl guanidinium.

23. Use of at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$, and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl;

as an antifungal agent against at least one fungal disease selected from *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4-mediated fungal disease and any strains, variants and pathotypes thereof.

24. A method of destroying, controlling or suppressing a *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 fungus or any strains, variants and pathotypes thereof, the method comprising the step of contacting the fungus with at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$, and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl.

25. A method of preventing *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 fungal growth or growth of any strains, variants and pathotypes thereof, on or in a material, the method comprising the step of applying to the material at least one compound with a formula independently selected from the group comprising: $R\text{-}S^{+}(R')_2$, $R\text{-}N^{+}(R')_3$, and $R\text{-}N(H)C(NH_2)_2^{+}$, and further comprising an agriculturally acceptable counterion;

wherein R is a C8-C32 straight chain or branched alkyl; and

where present, each R' is independently selected from the group comprising: methyl, ethyl, propyl, isopropyl, and butyl.

Intellectual Property Office

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Claims searched:	1-25 (in part)	**Date of search:**	20 May 2022

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-25	WO 2020/201698 A1 (UNIVERSITY OF EXETER), see page 12 lines 7-22, page 23 line 5 to page 25 line 24, examples and claims
X,E	1, 2, 4, 5, 7-12, 14, 15, 18-22	WO 2022/069715 A1 (UNIVERSITY OF EXETER), see examples and [0104]-[0112]
X	1, 4-21	US 4475941 A (AYGLON), see example 6
X	1, 4-21	GB 464330 A (GROVES), see example 1

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC

A01N; A01P

The following online and other databases have been used in the preparation of this search report

WPI, EPODOC, Patent Fulltext, CAS ONLINE

International Classification:

Subclass	Subgroup	Valid From
A01N	0031/02	01/01/2006
A01N	0033/12	01/01/2006
A01N	0047/44	01/01/2006
A01P	0003/00	01/01/2006