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(73) Patenthaver: **UPL Europe Supply Chain GmbH, Suurstoffi 37, 6343 Rotkreuz / Risch, Schweiz**

(72) Opfinder: **TRONSMO, Arne, Haugerudveien 35, NO-1430 Aas, Norge**
AAM, Berit Bjugan, Hoelveien 1, NO-1400 Ski, Norge
EIJSINK, Vincent, Ekornveien 44, NO-1430 Aas, Norge
RAHMAN, Md Hafizur, , 2007 Kjeller, Norge
SÃ¿RLIE, Morten, Nylenda 26, NO-1430 Aas, Norge
HJELJORD, Linda, Høgskoleveien 17, N-1430 Aas, Norge
FILTVEDT, Anne Line, Kappveien 1, NO-1415 Oppegård, Norge

(74) Fuldmægtig i Danmark: **Plougmann Vingtoft A/S, Strandvejen 70, 2900 Hellerup, Danmark**

(54) Benævnelse: **SAMMENSÆTNING OMFATTENDE CHITOSAN OG ET FUNGICID**

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DESCRIPTION

[0001] The present invention relates to a composition for treating, preventing or controlling fungal disease, damage or infection in plants. Specifically the invention relates to a composition comprising chitooligo-saccharides and a fungicide not containing chitopoly- or chitooligo-saccharides, and to methods for treating, preventing or controlling fungal disease, damage or infection in plants using a composition according to claim 1.

[0002] Fungi are eukaryotic organisms that lack chlorophyll and thus do not have the ability to photosynthesize their own food. They obtain nutrients by absorption through tiny thread-like filaments called hyphae that branch in all directions throughout a substrate. A collection of hyphae is referred to as mycelium (pl., mycelia). The hyphae are filled with protoplasm containing nuclei and other organelles. Conidiophores are asexual reproductive structures that develop at the tip of hyphae and produces conidia. Mycelia and conidiophores are the key diagnostic signs associated with diseases caused by fungi and fungal-like organisms (FLOs).

[0003] Fungal diseases of plants can cause severe pre- and post-harvest losses in agricultural crops. Fungi and FLOs including oomycetes cause the great majority of infectious plant diseases and over 8,000 species have been shown to cause disease. Diseases caused by fungi include all white and true rusts, smuts, needle casts, leaf curls, mildew, sooty moulds and anthracnoses; most leaf, fruit and flower spots; cankers; blights; scabs; root, stem, fruit and wood rots; wilts; and leaf, shoot and bud galls. All economically important plants are thought to be susceptible to attack by one or more fungi and often many different fungi may cause disease in one plant species. Fungal infections cause pre-harvest damage to crops by killing them outright or weakening them so as to decrease yields and render the plants susceptible to other infections. Post-harvest, fungal infection also results in significant loss of agricultural products during storage, processing and handling. Clearly, there is a significant need to control the fungal infection of plants and plant products and a number of chemical agents have been developed for this purpose, but to date no fully satisfactory agents, i.e. agents that completely control the fungus while at the same time being devoid of undesirable side effects, have been found.

[0004] Use of chemical fungicides is the primary method to prevent fungal diseases in plants. Excessive use of synthetic fungicides, however, may cause development of fungicide resistance in the pest population, resulting in the need for higher quantities of the pesticide for effective control. Fungicide residues have been found, for example, in groundwater, animal feed and food for human consumption as a result of pesticide use and can be harmful to animals, including humans. Fungicides may also eliminate beneficial microorganisms which again may result in emergence of "new" diseases. Alternative ways to control plant pathogens are therefore needed so that reduced amounts of chemical fungicides are used while maintaining the same protection against pre- and post-harvest loss caused by fungi and FLOs.

[0005] Chitin and chitin-derived molecules such as polymeric and oligomeric chitosan are

known to possess antifungal properties. Chitin is a linear polysaccharide consisting of β(1→4) linked N-acetyl-D-glucosamine residues and occurs mainly as a structural component in the cell walls of fungi and yeasts and in the exoskeletons of insects and arthropods (e.g., crabs, lobsters and shrimps). Chitosan can be prepared from chitin by partial deacetylation and is a heteropolymer of N-acetyl-D-glucosamine and D-glucosamine residues. Unlike chitin, chitosan is soluble in water or in dilute aqueous acid solutions. The name chitosan refers to a continuum of soluble polymeric chitin derivatives that can be described and classified according to the fraction of N-acetylated residues (F_A) or degree of N-acetylation (DA), the average degree of polymerization (DP_n) or the average molecular weight (M_{Wn}), the molecular weight distribution (PD, PolyDispersity) and the pattern of N-acetylation (P_A) or sequence. Chitosan is non-toxic, biocompatible and biodegradable.

[0006] Chitooligosaccharides (CHOS) which encompass chitopoly- or chitooligo-saccharides are oligomers prepared from chitosan either chemically or enzymatically. Chitosan can be converted to CHOS by acid hydrolysis or by enzymatic hydrolysis with glycosyl hydrolases like chitinases or chitosanases. The F_A, Mw, PD, DP_n and P_A of the resulting CHOS-mixture depend on the chitosan starting material and the specificity of the enzyme used, as well as on reaction conditions such as reaction time, reaction temperature and reaction pH.

[0007] Low molecular weight CHOS have been found to be effective against *Candida krusei* and to inhibit spore germination in *Fusarium oxysporum* (Tikhonov, Carbohydr. Polym. 2006, p66-72). Without wishing to be bound by theory it is believed that the anti-fungal effect of CHOS is dependent on its interaction with lipids in the plasma membrane, leading to morphological changes and cell surface disruption (Palma-Guerrero et al., Fungal Genet. Biol. 2009, p585-594; Park et al., J. Microbiol. Biotechnol. 2008, p1729-1734). The composition of the fungal plasma-membrane seems to be important for the sensitivity against chitosan and a higher content of polyunsaturated fatty acids makes the fungi more sensitive (Palma-Guerrero et al., Mol. Microbiol. 2010, p1021-1032).

[0008] Antifungal effects of low molecular weight chitin or chitosan have been published (Kendra & Hadwiger, Experimental Mycology, 1984, p276-281; Ghaouth et al., Mycol. Res. 1992, p769-779; US Patent 5,965,545; U.S. Patent 5,374,627; Japanese Patent Application 62-198604; U.S. Patent Application Serial No. 08/453,651 and International Publication No. WO 00/59949). Furthermore, a synergistic combination of essential oils and chitosan to control post-harvest diseases is described in US Patent 2003/0113421.

[0009] Fungicide combinations have been used in the art. The present inventors have identified that the use of chitosan and its chitopoly- or chitooligo-saccharides are particularly useful in a composition also containing a fungicide not containing chitopoly- or chitooligo-saccharides for use as an anti-fungal agent and that a low dose of that latter fungicide may be used. The compositions were found to have broad efficacy.

[0010] In particular, as will be described in detail below, preferred chitopoly- or chitooligo-

saccharides from chitosan (also referred to as CHOS or chitooligosaccharides herein) were derived from chitosan by specific enzymatic hydrolysis to obtain a defined average oligomeric size (DP_n). The fraction of acetylation (F_A) of the CHOS is dependent of the F_A of the chitosan from which it is produced. In work leading to the present invention, the present inventors found that chitosan-derived chitopoly- or chitooligo-saccharides, with a specified chemical composition comprising β(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, boosted the activity of commercial plant protection fungicides leading to a very significant reduction in the amount of the commercial pesticides needed to control both pre- and post-harvest plant diseases. The synergistic effect observed by the inventors from the combination of the chitosan or its chitopoly- or chitooligo-saccharides described herein and the commercial fungicides was much greater than could be expected based on previously reported combinations involving different pesticides. This finding has allowed the development of a more efficient and robust method for protecting plants against air- and soil-borne pathogenic fungi, while at the same time reducing the use of chemical pesticides. The present disclosure is concerned with a composition comprising (i) chitosan or chitopoly- or chitooligo-saccharides thereof, according to claim 1, and (ii) a fungicide not containing chitopoly- or chitooligo-saccharides.

[0011] In a first embodiment, the present invention provides a composition comprising (i) chitooligosaccharides, wherein said chitooligosaccharides comprise β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine monomers and have a degree of acetylation between a) 0.01 and 0.40, or b) 0.05 and 0.20, and an average degree of polymerization of 20-60 as assessed by measurement with ^1H NMR spectroscopy, and (ii) a fungicide not containing chitopoly- or chitooligo-saccharides, wherein the chitooligosaccharides are present in the composition at a concentration in the range of 1-1000 µg/ml and the chitooligosaccharides and fungicide are present in a ratio of 1000:1 to 1:10 (w/w); and the fungicide is

1. a) selected from an anilide fungicide; an anilinopyrimidine fungicide; a pyrrole fungicide; a methoxyacrylate strobilurin fungicide; a carbanilate fungicide; a pyrazole fungicide; a pyridine fungicide; a methoxycarbanilate strobilurin fungicide and a naphthoquinone fungicide, and combinations thereof, or
2. b) selected from a benzamide fungicide, a strobilurin fungicide, a conazole fungicide, a carboxamide fungicide, a triazole fungicide and a dicarboximide fungicide, and combinations thereof; or
3. c) not an inorganic fungicide; and the chitooligosaccharides have a D-glucosamine sugar unit at (i) ≥ 50% of their reducing ends, ii) ≥ 85% of their reducing ends, or iii) ≥ 95% of their reducing ends.

[0012] The components of the composition may be provided separately or together, e.g. in a product or kit. The present disclosure provides a kit or product comprising (i) chitosan or chitopoly- or chitooligo-saccharides thereof, wherein said chitosan or chitopoly- or chitooligo-

saccharides thereof comprise β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine monomers and have a degree of acetylation between 0.01 and 0.40, preferably between 0.05 and 0.20, especially preferably 0.10 to 0.20, e.g. 0.15, and an average degree of polymerization according to claim 1, and (ii) a fungicide not containing chitopoly- or chitooligo-saccharides, wherein said components (i) and (ii) are presented separately.

[0013] In a second embodiment, the present invention provides a kit comprising the chitooligosaccharides and fungicide of the composition of the invention.

[0014] Preferred aspects of the invention, as described below, as they relate to the composition of the invention also apply to the product or kit of the invention.

[0015] "Chitosan" as referred to herein is a linear soluble polysaccharide composed of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) which can be produced by deacetylation of chitin. Chitosan also encompasses chitosan salts. Chitin is a polymer containing β(1-4)-linked N-acetyl-D-glucosamine residues that are linked in a linear fashion. The deacetylation reaction which produces chitosan is rarely conducted to full completion and therefore the chitosan polymeric chain is generally described as a copolymeric structure comprised of D-glucosamine along with N-acetyl-D-glucosamine residues.

[0016] The fine structure of chitosan may be defined by the molar fraction of residual N-acetyl-D-glucosamine groups in chitosan expressed as a degree of N-acetylation (DA) or fraction of acetylation (F_A). Alternatively the molar fraction of D-glucosamine residues, deacetylation degree (DD), may be used. The degree of deacetylation can be controlled during the chitosan production process.

[0017] In contrast to chitin, the presence of free amine groups along the chitosan chain allows this macromolecule to dissolve in water or in dilute aqueous acidic solvents due to the protonation of these groups, rendering the corresponding chitosan salt in solution.

[0018] As described in more detail hereinafter, chitosan and the chitosan-derived chitopoly- or chitooligo-saccharide molecules disclosed herein may be described herein in terms of their molecular weight (Mw) expressed in Daltons (Da) or kilodaltons (kDa) or average degree of polymerization (DP_n). The invention is concerned with chitooligosaccharides with a DP_n of 20-60 as assessed by measurement with ^1H NMR spectroscopy.

[0019] The chitosan-derived "chitopoly- or chitooligo-saccharides" refer to cleaved portions of larger chitosan molecules. Generally they have a DP_n less than 60, as described hereinafter and preferably are prepared as described hereinafter. Nevertheless, it will be appreciated that the terms "chitosan" and its "chitopoly- or chitooligo-saccharides" are overlapping in scope as smaller chitosan molecules are also chitopoly- or chitooligo-saccharides from larger chitosan molecules. Thus, their molecular weight and DP_n are the determinative factors in terms of size. The chitopoly- or chitooligo-saccharides are also referred to herein collectively as

chitooligosaccharides (CHOS) or chitooligomers, poly- or oligo-saccharides derived from chitosan, or simply poly- or oligo-saccharides. Furthermore, it will be appreciated that structurally and functionally equivalent poly- or oligo-saccharides i.e. comprising deacetylated and acetylated units in the preparations described herein, may be derived from sources other than chitosan (or chitin), or they may be produced synthetically.

[0020] "Degree of acetylation" (DA) (i.e. N-acetylation, expressed as a percentage) and "fraction of acetylated residues" (F_A, expressed as a value between 0 and 1) are used interchangeably herein and describe the molar fraction of residual N-acetyl-D-glucosamine groups in chitosan or the chitopoly- or chitooligo-saccharides derived from chitosan. Chitin generally has a degree of N-acetylation (DA) of more than 70% (i.e. the number of N-acetyl-D-glucosamine monomers is more than 70% and consequently the number of D-glucosamine monomers is less than 30%) and is insoluble in water and weak acidic solutions. Chitosan on the other hand generally has less than 70% DA (the fraction of N-acetyl-D-glucosamine monomers is less than 70% and consequently the fraction of D-glucosamine monomers is more than 30%) and is soluble in dilute acid. Degree of N-acetylation refers to the fraction of N-acetyl-D-glucosamine sugars in the molecule (i.e. 10% N-acetylation is reflected as 0.10); the other sugars are D-glucosamine units. As referred to herein "monomers", "units" and "residues" are used interchangeably to refer to individual saccharide molecules that are linked to form the polymer.

[0021] The "average degree of polymerization" (DP_n) of a chitosan or chitopoly- or chitooligo-saccharide derived therefrom is defined herein as the average number of D-glucosamine and N-acetyl-D-glucosamine monomeric units in a chitosan or polysaccharide or oligosaccharide molecule derived from chitosan. The DP_n of those of the invention are 20-60 (DP denotes the degree of polymerization of individual molecules or a pure composition with identical molecules, i.e. a non-averaged size).

[0022] The DP_n values recited herein represent the DP_n of a chitosan/polysaccharide/oligosaccharide preparation and thus encompass both variability in the range of molecules present in the preparation and furthermore in the detection accuracy. In relation to the latter, variation in determining the DP_n may be ±10, e.g. ±5%. Thus for example, a preparation with a DP_n of 30 may have a DP_n ranging from 28.5 to 31.5 depending on the measurement system used. The measurement is in accordance with the methods used in the Examples (i.e. by ^{1}H-NMR spectroscopy).

[0023] As mentioned above, variation in the range of molecules present in the preparation exists and the extent of variation may vary. Thus, depending on the preparation method, the molecules in the preparation may be widely divergent in size or the molecules may be of a similar size (e.g. when a size exclusion separation step is used). Preferably the preparation includes molecules of a similar size, e.g. more than 50%, e.g. more than 70, 80, 90, 95 or 99% of the chitosan or chitosan-derived molecules in the preparation have a size that varies no more than 20% (e.g. less than 15, 10, 5 or 1%) from the average DP_n. Thus, for example, in a

preparation with a DP_n of 30 more than 50% of the molecules preferably have a size (DP) between 24 and 36 (20% deviation).

[0024] A "fungicide" as referred to herein is a pesticide that controls fungal disease resulting from infection by a fungus or fungus-like organisms by specifically inhibiting or killing fungi or fungal spores or their growth or spread. As referred to herein a fungicide is a fungicide not containing chitopoly- or chitooligo-saccharides unless explicitly stated otherwise. The "fungicide not containing chitopoly- or chitooligo-saccharides" comprised in the composition of the present invention contains no chitopoly- or chitooligo-saccharides (preferably no chitopoly- or chitooligo-saccharides containing D-glucosamine or N-acetyl-D- glucosamine residues) such as those from chitins, chitosans, or CHOS, e.g. chitopoly- or chitooligo-saccharides from chitin or chitosan with a DP_n from 5 to 250. Preferably the fungicide is a chemical molecule and is not naturally occurring. Chemicals used to control oomycetes are also referred to as fungicides since oomycetes use the same mechanisms as fungi to infect plants. Fungicides can either be contact, translaminar or systemic fungicides. Contact fungicides are not taken up into the plant tissue and only protect the plant where the spray is deposited; translaminar fungicides redistribute the fungicide from the upper, sprayed leaf surface to the lower, unsprayed surface; systemic fungicides are taken up and redistributed through the xylem vessels to the upper parts of the plant.

[0025] A "fungus" as referred to herein is any member of a kingdom of organisms (Fungi) that lack chlorophyll, leaves, true stems and roots, reproduce by spores and live as saprotrophs or parasites. The group includes moulds, mildews, rusts, yeasts and mushrooms.

[0026] The term "fungus-like organisms" encompasses myxomycetes (slime moulds) and oomycetes which were formerly classified in the kingdom Fungi. Unlike true fungi, the cell walls of these organisms contain cellulose and lack chitin. Oomycetes are a group of heterotrophic organisms generally known as the water moulds and downy mildews. Although oomycetes have similarities to fungi through convergent evolution, they are not fungi (as previously thought); instead, the oomycetes are part of the kingdom *Stramenopiles* and are thereby distinct from plants, fungi and animals.

[0027] The chitopoly- or chitooligo-saccharides used in accordance with the invention may be prepared by various means. CHOS may be prepared from chitosan by using physical methods such as hydrothermal, microwave, ultrasonication and gamma-rays, but these methods are not amenable to the creation of well-defined CHOS mixtures. Chemical methods using acid, H_2O_2 or $NaNO_2$ can also yield CHOS. However, enzymatic production of CHOS allows for production of well-defined CHOS mixtures and are more preferred. Such processes are described in more detail below. Chemoenzymatic synthesis may also be used to produce pure CHOS of defined DP_n, F_A and P_A.

[0028] Chitosan may be isolated from the cell walls of certain fungi (e.g. *Mucoraceae*), but is generally prepared from chitin by homogeneous or heterogeneous deacetylation. Examples of

commercially available chitins are those available from sources such as France Chitin, Hov-Bio, Sigma, Sekagaku Corp, Chitinor amongst others. Methods for chitin de-acetylation are well known in the art and some are described in Aranaz, Current Chemical Biology, 2009, 3, p203-230. The extent of deacetylation may be affected by factors including concentration of the alkali, previous treatment, particle size and density of chitin.

[0029] Enzymatic depolymerization of chitin and chitosan involves chitinases and chitosanases, respectively. These methods are described in detail in Aam et al., Mar. Drugs, 2010, 8, p1482-1517, whereas the various enzyme families comprised by the terms chitinases and chitosanases are described in Hoell et al., Biotechnology and Genetic Engineering, 2010, 27, p331-366. Chitinases which may be used include those in the glycoside hydrolase (GH) families 18 (e.g. ChiA, B, C) and 19 (e.g. ChiG). Chitinases are also capable of hydrolyzing chitosan, albeit to different extents. Enzymes with chitosanase activity have been found in GH families 5, 7, 8, 46, 75 and 80.

[0030] Chitinases are found in plants, microorganisms and animals. Chitinases have been cloned from various species of microorganisms and may be obtained from commercial sources, i.e. companies such as Sigma. Alternatively chitinases may be produced using standard recombinant techniques for protein expression. The scientific literature contains numerous examples of the cloning, overexpression, purification and subsequent application of all types of chitinases (e.g. Horn et al., FEBS J. 2006, p491-503 and references therein, as well as Hoell *et al.,* 2010, *supra*).

[0031] Unspecific enzymes such as papain and cellulases may also be used to degrade chitosans. These enzymes may be present in mixtures containing minor fractions of chitinases or chitosanases, as well as containing enzymes that do not act on chitin, chitosan or CHOS.

[0032] CHOS produced enzymatically or chemically normally consist of a mixture of oligomers differing in DP, F_A and P_A. Several techniques for separation and purification of CHOS may be used such as gel filtration, ultrafiltration and ion exchange and metal affinity chromatography. Conveniently size exclusion chromatography (SEC) may be used and oligomers may be detected using an online refractive index detector and this allows separation of CHOS with similar DP values, independently of F_A and P_A (see for example Sørbotten et al., FEBS J., 2005, 272, p538-549). Further separation of CHOS can be achieved using cation-exchange chromatography. With this method, CHOS of identical DP can be separated based on the number of deacetylated units.

[0033] The percent of deacetylated reducing ends generated by the enzymatic cleavage of chitosan may be controlled by selection of the appropriate enzyme as described in the Examples. Published data show that the selection of the enzyme may also be used to affect the percentage of deacetylated residues at other positions in the cleavage products, i.e. positions that were close to the cleavage point, preferably, the newly generated reducing end, the newly generated non-reducing end and the sugars next to each of these two newly generated ends (Horn et al., FEBS J., 2006, p491-503; Aam et al., Mar. Drugs, 2010, 8,

p1482-1517).

[0034] In order to characterize CHOS in terms of DP_n, F_A and P_A, several techniques known in the art may be used, primarily nuclear magnetic resonance (NMR) and mass spectrometry.

[0035] The DP_n of chitooligosaccharides in compositions of the invention is between 20 and 60. More preferably the DP_n of the chitosan polysaccharides and oligosaccharides comprised in the composition of the present invention is between 20 to 40, especially preferably 20 to 35 (molecular weight between 3,320 - 5,900 Da) or 25 to 35 or 30 to 40. Further preferably the DP_n of the chitosan polysaccharides and oligosaccharides may by any real number between 20 and 60, e.g. 23, 28, 30, 33.5, 34, 34.6, 37, 40, 41, 48, 49, 50 and58 (preferably 23, 28, 30, 34, 37, 40, 41 and 50) and optionally a range of ±1, 2 or 3 relative to that number, e.g. 23±2, i.e. 21 to 25. As discussed above, experimental variation may account for up to 5 or 10% variation to the values given above. As discussed previously, in a preferred feature the preparation includes molecules of a similar size, e.g. more than 50%, e.g. more than 70, 80, 90, 95 or 99% of the chitosan or chitosan-derived molecules in the preparation have a size that varies no more than 20% (e.g. less than 15, 10, 5 or 1%) from the DP_n.

[0036] The degree of acetylation of the chitooligosaccharides in compositions of the invention is between a) 0.01 and 0.40, or b) 0.05 and 0.20. In one aspect the degree of acetylation is between 0.05 and 0.40, i.e. from 0.05 to 0.40. Preferably the degree of acetylation is from 0.05 to 0.20, preferably from 0.1 to 0.20, e.g. 0.15.

[0037] Enhanced performance is observed when the chitosan or chitopoly- or chitooligo-saccharides therefrom have deacetylated reducing ends. Thus, the chitosan or chitopoly- or chitooligo-saccharides therefrom described herein and used in compositions of the invention have a D-glucosamine sugar unit at ≥ 50% of the reducing ends of the chitosan or chitopoly- or chitooligo-saccharides therefrom, more preferably ≥ 85%, ≥ 90%, or ≥ 95%. The "reducing end" of a chitooligomer comprises a carbon atom that can be in equilibrium with the open-chain aldehyde or keto form.

[0038] As described in the Examples, the methods used produce chitooligosaccharides which show high synergy in terms of fungicidal activity when used with fungicides not containing chitopoly- or chitooligo-saccharides. Thus, preferably the chitosan-derived chitopoly- or chitooligo-saccharides are prepared as described in the Examples. Preferably said method comprises acid hydrolysis, or dissolving chitosan in water or a weak acidic solution (optionally followed by adjustment of the pH) and then enzymatic cleavage using an enzyme capable of catalysing degradation of chitosan into chitopoly- or chitooligo-saccharides, preferably an enzyme that cleaves the glycosidic bonds after a deacetylated residue (e.g. using a chitosanase such as a family 46 chitosanase, e.g. ScCsn46A, or another non-processive endo-chitosanase that preferably cleaves the glycosidic bond after the deacetylated residue). Site-directed mutants of such enzymes may also be used, which carry mutations that may affect the type or activity of the chitopoly- or chitooligo-saccharides produced and/or the efficiency of the

degradation process. Optionally the resultant chitopoly- or chitooligo-saccharide mix may be further separated based on the size of the molecules, e.g. by size exclusion chromatography.

[0039] Fungicides which do not contain chitopoly- or chitooligo-saccharides and which may be comprised in the composition of the present invention are selected from

1. a) an anilide fungicide; an anilinopyrimidine fungicide; a pyrrole fungicide; a methoxyacrylate strobilurin fungicide; a carbanilate fungicide; a pyrazole fungicide; a pyridine fungicide; a methoxycarbanilate strobilurin fungicide and a naphthoquinone fungicide, and combinations thereof, or
2. b) a benzamide fungicide, a strobilurin fungicide, a conazole fungicide, a carboxamide fungicide, a triazole fungicide and a dicarboximide fungicide, and combinations thereof; or
3. c) not an inorganic fungicide.

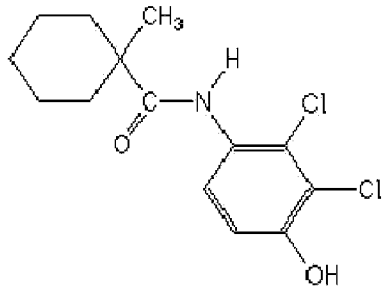
[0040] Fungicides for use in compositions of the invention or in methods or uses of the invention (as described hereinafter) may be selected from one or more of the following groups of fungicides: aliphatic nitrogen fungicides; amide fungicides; acylamino acid fungicides; anilide fungicides; benzanilide fungicides; furanilide fungicides; sulfonanilide fungicides; benzamide fungicides; furamide fungicides; phenylamide fungicides; phenylsulfamide fungicides; sulfonamide fungicides; valinamide fungicides; antibiotic fungicides; strobilurin fungicides; methoxyacrylate strobilurin fungicides; methoxycarbanilate strobilurin fungicides; methoxyiminoacetamide strobilurin fungicides; methoxyiminoacetate strobilurin fungicides; aromatic fungicides; arsenical fungicides; aryl phenyl ketone fungicides; benzimidazole fungicides; benzimidazole precursor fungicides; benzothiazole fungicides; botanical fungicides; bridged diphenyl fungicides; carbamate fungicides; benzimidazolylcarbamate fungicides; carbanilate fungicides; conazole fungicides; copper fungicides; cyanoacrylate fungicides; carboxamide fungicides; dicarboxamide fungicides; dicarboximide fungicides; dichlorophenyl dicarboximide fungicides; phthalimide fungicides; dinitrophenol fungicides; dithiocarbamate fungicides; cyclic dithiocarbamate fungicides; polymeric dithiocarbamate fungicides; dithiolane fungicides; fumigant fungicides; hydrazide fungicides; imidazole fungicides; inorganic fungicides; mercury fungicides; inorganic mercury fungicides; organomercury fungicides; morpholine fungicides; organophosphorus fungicides; organotin fungicides; oxathiin fungicides; oxazole fungicides; polysulfide fungicides; pyrazole fungicides; pyridine fungicides; pyrimidine fungicides; anilinopyrimidine fungicides; pyrrole fungicides; quinoline fungicides; quinone fungicides; quinoxaline fungicides; thiadiazole fungicides; thiazole fungicides; thiazolidine fungicides; thiocarbamate fungicides; thiophene fungicides; triazine fungicides; triazole fungicides; triazolopyrimidine fungicides; urea fungicides; and zinc fungicides. The fungicide may also be a naphthoquinone fungicide. One set of preferred fungicides (not containing chitopoly- or chitooligo-saccharides) may be selected from the above-described groups which excludes antibiotic fungicides (preferably strobilurin fungicides, especially preferably methoxyiminoacetate strobilurin fungicides) and/or thiazole fungicides (preferably conazole fungicides).

[0041] The fungicide comprised in the composition of the present invention is preferably selected from one or more fungicides comprised in the following groups: an anilide fungicide, e.g. benalaxyl, benalaxyl-M, bixafen, boscalid, carboxin, fenhexamid, fluxapyroxad, isotianil, metalaxyl, metalaxyl-M, metsulfovax, ofurace, oxadixyl, oxycarboxin, penflufen, pyracarbolid, sedaxane, thifluzamide, tiadinil and vangard; an anilinopyrimidine fungicide, e.g. cyprodinil, mepanipyrim and pyrimethanil; a pyrrole fungicide, e.g. dimetachlone, fenpiclonil, fludioxonil and fluoroimide; a methoxyacrylate strobilurin fungicide, e.g. azoxystrobin, bifujunzhi, coumoxystrobin, enestroburin, jiaxiangjunzhi (coumethoxystrobin), picoxystrobin and pyraoxystrobin; a carbanilate fungicide, e.g. diethofencarb, triclopyricarb, pyraclostrobin and pyrametostrobin; a pyrazole fungicide, e.g. bixafen, fenpyrazamine, fluxapyroxad, furametpyr, isopyrazam, penflufen, penthiopyrad, pyraclostrobin, pyrametostrobin, pyraoxystrobin, rabenzazole and sedaxane; a pyridine fungicide, e.g. boscalid, buthiobate, pyrisoxazole, dipyrithione, fluazinam, fluopicolide, fluopyram, triclopyricarb, parinol, pyribencarb, pyridinitril, pyrifenox, pyroxychlor and pyroxyfur; and a methoxycarbanilate strobilurin fungicide, e.g. triclopyricarb, pyraclostrobin and pyrametostrobin, and combinations thereof. Also preferred are quinone fungicides, e.g. naphthoquinone fungicides, e.g. dithianon, and combinations thereof with the above described fungicides.

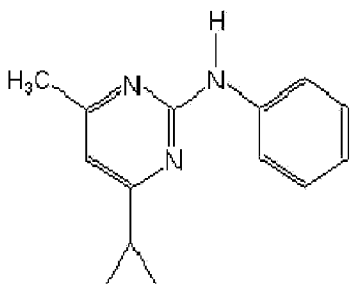
[0042] Other preferred fungicides are phenylsulfamide, strobilurin, benzimidazole, phthalimide, dithiocarbamate, morpholine, phenylamide, carboxamide, dicarboxamide and anilinopyrimidine fungicides, and combinations thereof.

[0043] Preferably the fungicide comprised in the composition of the present invention is selected from one or more of fenhexamid, cyprodinil, fludioxonil, azoxystrobin, boscalid and pyraclostrobin and combinations thereof. Another preferred fungicide is dithianon, and combinations with the above described preferred specific fungicides. The chemical structure of these fungicides is provided below.

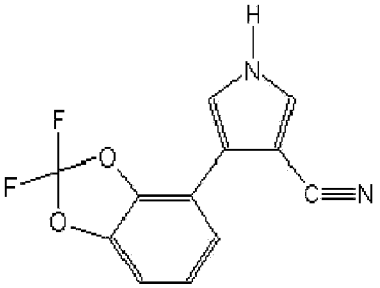
Fenhexamid:



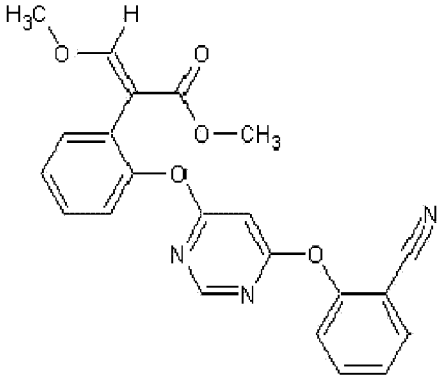
Cyprodinil:



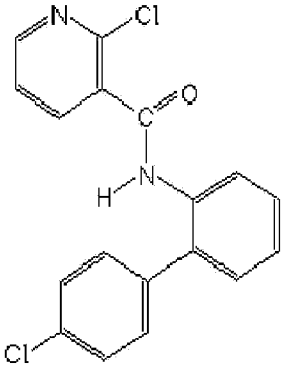
Fludioxonil:



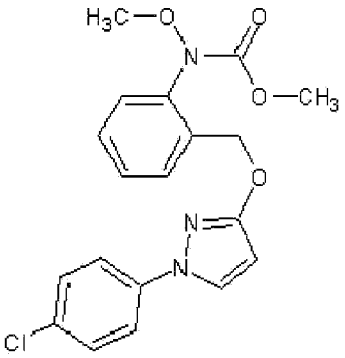
Azoxystrobin:



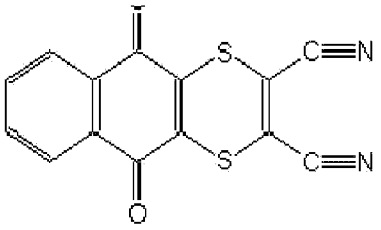
Boscalid:



Pyraclostrobin:



Dithianon:



[0044] In a preferred embodiment the fungicide comprised in the composition of the present invention is preferably selected from commercially available brands including Teldor® WG 50 (fenhexamid; manufactured by Bayer Crop Science Pty Ltd.), Switch® 62.5 WG (cyprodinil and fludioxonil; manufactured by Syngenta Crop Protection Pty Ltd.), Amistar® (azoxystrobin; manufactured by Syngenta Crop Protection Pty Ltd.), Signum® WG (boscalid and pyraclostrobin; manufactured by BASF) and Stratego® (trifloxystrobin and propiconazole; manufactured by Bayer Crop Science Pty Ltd.). Another preferred fungicide is Delan® WG (dithianon, manufactured by BASF).

[0045] Alternative fungicides that may be used include mancozeb and chlorothalonil.

[0046] The fungicides described herein are readily available through various commercial sources.

[0047] In a further preferred aspect, more than one fungicides may be comprised in the composition together with the chitosan or chitosan-derived molecules. Thus the composition may comprise 1 or alternatively 2, 3, 4 or 5 or more different fungicides which do not contain chitopoly- or chitooligo-saccharides. The preferred aspects of the fungicide as referred to herein apply to all the fungicides (which do not contain chitopoly- or chitooligo-saccharides) which might be present and for the purposes of calculating ratios and optimal concentrations, as described hereinafter, where more than one fungicide which does not contain chitopoly- or chitooligo-saccharides is used it is considered as though it were a single fungicide.

[0048] Where more than one fungicide which does not contain chitopoly- or chitooligo-saccharides is present, each fungicide may be present in equal or different proportions relative to one another. Preferably when two fungicides are present they are present in a similar w/w amount, e.g. have a ratio of from 1:1 to 5:1 for the major:minor fungicide. In a preferred example the following combinations of fungicides may be used:

cyprodinil and fludioxonil, preferably at a ratio of 2:1 to 1:1 e.g. 1.5:1; or

boscalid and pyraclostrobin, preferably at a ratio of 5:1 to 1:1 e.g. 4:1.

[0049] Optionally, other than the one or more fungicides referred to above, no further

fungicides which do not contain chitopoly- or chitooligo-saccharides are present in the composition.

[0050] The chitosan or chitopoly- or chitooligo-saccharides thereof and the fungicide may be provided in any suitable proportion relative to one another. This is largely dependent on the fungicide to be used and on the pre- or post-harvest plant fungal disease that is being targeted. In compositions of the invention the ratios of chitooligosaccharides: fungicide ranges from 1000:1 to 1:10 based on a w/w basis in the final composition. Preferably the ratio is greater than 1:1, i.e. from 1000:1 to 1:1. In an alternative preferred aspect similar proportions of the components are provided, e.g. a ratio of 10:1 to 1:10 or 50:1 to 1:2.

[0051] As described in the Examples the compositions described herein lead to synergistic fungicidal effects. This allows the dosage of one or both of the components to be reduced, e.g. to a suboptimal concentration relative to the concentration required for fungicidal activity when used alone.

[0052] Thus, in a preferred aspect, one or both of the components, preferably the fungicide component (i.e. the component not containing the chitopoly- or chitooligo-saccharides of the present invention) of the composition of the present invention is present in the composition at a suboptimal concentration. A "suboptimal concentration" as referred to herein is any concentration of a fungicide that is lower than the concentration of a particular fungicide which produces the maximum fungicidal effect when used without other fungicidal active ingredients. The optimal working concentration of a fungicide (when used alone) may be lower than the concentration that produces the maximum fungicidal effect for a given fungicide and is often determined and provided by the manufacturer of the fungicide taking other factors into account. Costs as well as regulatory provisions may be taken into account in determining the optimal concentration of a fungicide and thus the recommended optimal concentration may not be the same as that which produces the maximum fungicidal effect, e.g. the optimal concentration may be lower. Consequently, a "suboptimal concentration" of a fungicide as defined herein is lower than the concentration which produces the maximum fungicidal effect (which latter concentration may be the same as the optimal working concentration which is recommended for a particular fungicide or it may be different) when the fungicide is used alone. Preferably the suboptimal concentration is also lower than the optimal working concentration. Thus the optimal working concentration is the concentration used to obtain the best possible fungicidal effect for a fungicide when used alone taking into account all factors influencing the amount of the fungicide that may be used. The concentration of a fungicide achieving the maximum fungicidal effect may be readily determined by the skilled person using one or more methods described in the art, such those described below.

[0053] Fungicidal efficacy may be determined by a variety of mechanisms, e.g. as described in the Examples. Thus, for example the inhibition of fungal germination, fungal growth, fungal sporulation, disease severity on the target organism or fungal infection may be used as the determinant of fungicidal activity. Such data may also be obtained from field tests. The result may be expressed as %, IC_{50} and so forth, depending on the test. The concentration at which

the optimum fungicidal activity is achieved, taking into account side-effects such as toxicity, may be based on any of these measurements, e.g. disease severity.

[0054] Thus a suboptimal concentration of a fungicide (not containing chitopoly- or chitooligo-saccharides) to be comprised in the composition of the present invention may be 90% of the concentration of the fungicide which produces the maximum fungicidal effect (or the optimal concentration of the fungicide) when it is used alone. Alternatively, the concentration of the fungicide may be 80, 70, 60, 50 , 40 , 30, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1% or less (or less than 1%) of the optimal concentration of the fungicide or the concentration which produces the desired fungicidal effect, e.g. the maximum fungicidal effect. In a preferred embodiment of the invention, the composition comprises 1-20% (or less) of the optimal concentration of the fungicide or the concentration which produces the desired fungicidal effect, e.g. ≤ 1 (e.g. 0.1 to 1%) or 10% (or less). The above values refer to the amount of the fungicide in its final formulation (e.g. Teldor, Switch etc. as described hereinafter), or the amount of the fungicide alone. When the optimal concentration of a fungicide fails to provide the maximum fungicidal effect, e.g. due to regulatory limitations on the concentration of the fungicide that may be used, the present invention is particularly advantageous as the combination of components of the composition of the invention boosts the activity of the fungicide even at lower concentrations allowing it to reach its maximum fungicidal effect within allowable concentration limits.

[0055] The optimal concentration of the fungicide or fungicide mixes used in preferred compositions of the invention are, for example:

Teldor (containing fenhexamid) : 1500 µg/ml

Switch (containing cyprodinil + fludioxonil) : 500 µg/ml

Amistar (containing azoxystrobin): 1000 µg/ml

Signum (containing boscalid + pyraclostrobin): 1000 µg/ml

Delan (containing dithianon): 800 µg/ml.

[0056] Thus sub-optimal concentrations are preferably, respectively, ≤ 150, 50, 100 and 100 µg/ml respectively (10% of the optimal concentration), or for Delan, ≤ 80 µg/ml (10% of the optimal concentration), or ≤ 15, 5, 10 and 10 µg/ml respectively (1% of the optimal concentration), or for Delan, ≤ 8 µg/ml (1% of the optimal concentration).

[0057] The above optimal concentrations may also be expressed by virtue of the constituent fungicide as follows:

Fenhexamid, present at 500g/kg in Teldor: 750 µg/ml (optimum); 75 µg/ml (10%, sub-optimum); 7.5 µg/ml (1%, sub-optimum),

Cyprodinil, present at 375g/kg in Switch: 187.5 µg/ml (optimum); 18.8 µg/ml (10%, sub-optimum); 1.9 µg/ml (1%, sub-optimum),

Fludioxonil present at 250g/kg in Switch : 125 µg/ml (optimum); 12.5 µg/ml (10%, sub-optimum); 1.3 µg/ml (1%, sub-optimum),

Azoxystrobin, present at 500g/kg in Amistar: 500 µg/ml (optimum); 50 µg/ml (10%, sub-optimum); 5 µg/ml (1%, sub-optimum),

Boscalid present at 26.7% in Signum; 267 µg/ml (optimum); 26.7 µg/ml (10%, sub-optimum); 2.7 µg/ml (1%, sub-optimum),

Pyraclostrobin present at 6.7% in Signum: 67 µg/ml (optimum); 6.7 µg/ml (10%, sub-optimum); 0.7 µg/ml (1%, sub-optimum),

Dithianon present at 70% w/w in Delan: 560 µg/ml (optimum); 56 µg/ml (10%, sub-optimum); 5.6 µg/ml (1%, sub-optimum).

[0058] Preferably the fungicidal activity of said chitosan or chitopoly- or chitooligo-saccharides thereof and said fungicide are synergistic, i.e. they have more than additive effects than when used in the same test alone. The presence or absence of synergy can be determined as described in the Examples, and E_{obs}/E_{exp} is >1, preferably >2, >5 or >10.

[0059] The composition of the present invention has use in various preventative and therapeutic treatments in plants and thus may be formulated accordingly.

[0060] Thus, the composition of the invention may also comprise one or more of the following but not limited to: a stabilizing agent (e.g. by the use of salts or non-electrolytes, acetate, SDS, EDTA, citrate or acetate buffers, mannitol, glycine, HSA or polysorbate), adhesive, anti-foam agent, surface-active agent, chelating agent, dye or colourant and nutrient.

[0061] The composition of the invention may be combined with one or more conventional carriers, diluents and/or excipients appropriate for the particular use for the composition, e.g., agriculturally acceptable carriers for agricultural uses, to produce conventional preparations which are suitable or can be made suitable for administration such as powders, sachets, suspensions, emulsions, solutions, aerosols, and the like. They may be formulated as liquids (solutions or suspensions) or as solids.

[0062] Examples of suitable carriers, excipients and diluents are lactose, dextrose, sucrose, maltose, glucose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, calcium carbonate, calcium lactose, corn starch, aglinates, tragacanth, gelatin, calcium silicate, polyvinylpyrrolidone, cellulose, water syrup, water, water/ethanol, water/ glycol, water/polyethylene, glycol, propylene glycol, methylhydroxybenzoates, propyl hydroxybenzoates, talc, magnesium stearate, mineral oil or fatty substances or suitable

mixtures thereof. The compositions may additionally include lubricating agents, wetting agents, emulsifying agents, viscosity increasing agents, granulating agents, disintegrating agents, binding agents, osmotic active agents, suspending agents, preserving agents, adsorption enhancers (e.g. surface penetrating agents, e.g. bile salts, lecithins, surfactants, fatty acids, chelators), organic solvent, antioxidant, stabilizing agents, anti-foaming agent, ionic or non-ionic thickeners, surfactants, filler, ionic or non-ionic thickener, sequestrant, polymer, propellant, alkalinizing or acidifying agent, fatty compounds and the like. The compositions of the invention may be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the plant by employing procedures well known in the art.

[0063] The use of solutions, suspensions, gels and emulsions are preferred, e.g. the active ingredient may be carried in water, a water-based liquid, an oil, a gel, an emulsion, an oil-in-water or water-in-oil emulsion, a dispersion or a mixture thereof.

[0064] The composition of the present invention is preferably water soluble, if necessary by addition of a chemical component(s) such as components to lower the pH, for example organic acids such as acetic acid. The composition may be provided as a wettable (or water soluble) powder or any kind of liquid or mixed with a commercial pesticide as a wettable powder or any kind of liquid. Powders may be formed with the aid of any suitable powder base. Drops and solutions may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing, solubilising or suspending agents. Aerosol sprays are conveniently delivered from pressurised packs, with the use of a suitable propellant.

[0065] The components of the composition may be present as the sole active ingredients or may be combined with other ingredients, particularly other active ingredients, e.g. to augment the fungicidal affect of the composition or the components of the composition. The compositions can be used as described above for the prevention or treatment of fungal infection in plants.

[0066] Also disclosed herein is a method for treating, preventing or controlling fungal disease, damage or infection in a plant caused by a fungus or fungus-like organism, comprising contacting the plant or part thereof which is affected or to be protected from the fungus with (i) chitosan or chitopoly- or chitooligo-saccharides thereof, wherein said chitosan or chitopoly- or chitooligo-saccharides thereof comprise β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine monomers and have a degree of acetylation between 0.01 and 0.40, preferably between 0.05 and 0.20, and an average degree of polymerization according to claim 1, and (ii) a fungicide not containing chitopoly- or chitooligo-saccharides.

[0067] The chitooligosaccharides are present at a concentration in the range of 1-1000 µg/ml and the chitooligosaccharides and fungicide are present in a ratio of 1000:1 to 1:10 (w/w). Components (i) and (ii) are preferably as described hereinbefore. Alternatively expressed the chitooligo-saccharides of the composition of the inventionand a fungicide not containing chitopoly- or chitooligo-saccharides as described hereinbefore may be used for treating,

preventing or controlling fungal disease, damage or infection in a plant caused by a fungus or fungus-like organism. As used herein "treating" refers to the reduction, alleviation or elimination, preferably to normal levels, of one or more of the symptoms of disease, damage or infection, relative to the symptoms or effects present on a different, normal part of the plant or a corresponding normal plant. Such symptoms include levels or extent of fungal germination, fungal growth, fungal sporulation, disease severity (e.g. necrosis or death) on the target organism or level or extent of fungal infection. The severity, level or extent of these symptoms may be determined as described herein. Preferably, where the reduction or alleviation is quantifiable, the symptom(s) is reduced by more than 50%, e.g. > 60, 70, 80 or 90%.

[0068] "Preventing" refers to absolute prevention, reduction or alleviation of the occurrence of any symptoms of disease, damage or infection, e.g. absence of detectable fungus and fungus-like organism or their parts and/or maintenance of normal levels of detectable fungus and fungus-like organism, or their parts or reduction or alleviation of the extent or timing (e.g. delaying) of the infection with said fungus and fungus-like organism.

[0069] Plants treated with the composition described herein preferably have improved or enhanced resistance to fungi and fungal-like organisms in that they show reduced rates of infectivity when compared to non-treated plants. Alternatively, improved resistance to fungi and fungal-like organisms can be apparent in diminished disease symptoms and/or growth, viability, reproduction and dispersal of the pathogen when compared to non-treated plants. The complexity of the mode of action of the components of the composition of the present invention lower the probability of the pathogen developing resistance to the composition, relative to the probability of developing resistance to individual components of the composition.

[0070] "Controlling" refers to maintaining the extent of the disease, damage or infection, e.g. reducing or preventing the spread of the infection to other organisms or parts of the plant.

[0071] As referred to herein "disease" caused by the fungus or fungus-like organism refers to any adverse effect caused by the fungus or fungus-like organism which affects the function of the plant or part thereof, said function including the quality and commercial value of the possible food or other products derived from the plant.

[0072] "Damage" refers to damage to one or more areas or parts of the plant affected by the fungus or fungus-like organism, e.g. localized necrotic damage.

[0073] "Infection" refers to invasion of and multiplication in plant tissues by the fungus or fungus-like organism and is evident from the presence of the fungus or fungus-like organism or its parts (e.g. spores) or damage or disease resulting from its presence.

[0074] Preferably, the fungus to be treated, prevented or controlled in plants is a fungal species from genera including *Fusarium, Gliociadium, Rhizoctonia, Trichoderma, Mycosphaerella, Phytophthora, Plasmopora, Leptosphaeria, Cercospora, Rhizoctonia, Cochliobolus, Pseudocercosporella, Pyricularia, Pseudoperonospora, Alternaria* (e.g. *Alternaria*

brassicicola), Pytium, Colletotrichum, Mucor (e.g. *Mucor piriformis), Microdochium (*e.g. *Microdochium majus), Uncinula, Ustilago, Erysiphe, Botrytis (*e.g. *Botrytis cineria), Saccharomyces, Sclerotium, Candida, Aspergillus* and *Alternaria.* A further preferred fungus is from the genus *Venturia.* Preferably the fungus is *Botrytis cineria, Alternaria brassicicola, Mucor piriformis* or *Microdochium majus.* Another preferred fungus is *Venturia inaequalis.*

[0075] Preferably the method is carried out on plants that may be infected or at risk of being infected by plant pathogenic fungi or fungal-like organisms.

[0076] The plant to be treated is preferably a cereal (e.g. maize, rice, triticale, sorghum, millet, wheat, oats, barley, rye or spelt), a pseudocereal (e.g. buckwheat or quinoa), forage grass, turf grass, grape, root or tuber crop plant (such as potato and carrots), or fruit (e.g. pome fruit), berry, vegetable or pulse crop plant (such as soybeans, peas, chickpeas). Preferred examples include strawberry, chickpea and bean plants. Further preferred examples are pomaceous fruit e.g. apple trees (e.g. *Malus domestica),* stone fruit and vines.

[0077] As referred to herein a "part" of a plant refers to seeds, roots, stems, leaves, flowers and fruits or a portion of that part.

[0078] The "contacting" step requires that the active ingredient is brought into contact with the plant for an appropriate period of time. Conveniently the two components may be used together and may be used as a single composition (a composition as described herein) or applied simultaneously. In the alternative the two components may be applied sequentially.

[0079] Conveniently the components of the composition of the present invention can be applied to all or part of a plant, e.g. the seeds, roots, stems, leaves, flowers and fruits. Alternatively or additionally the treatment can be applied to the soil in which said plant is growing or is to be grown or to the fungus (or fungus-like organism) itself. Normally, application is topical. However, any other administration strategies known to the skilled person can be used.

[0080] The components of the composition of the present invention may be applied to the plant or plant part by spraying, dipping or drenching. Alternatively or additionally, they may be applied directly to the fungus or fungus-like organisms by these methods. Effective concentrations of the chitosan or chitosan-derived molecules are in the range of 1-1000 µg/ml, in liquid preparations for treatment of plants, e.g. 50-150 µg/ml, e.g. 100 µg/ml. Compositions, methods and uses of the invention contain or use chitooligosaccharides at a concentration in the range 1-1000 µg/ml. Effective amounts of the fungicide which does not contain chitopoly- or chitooligo-saccharides, e.g. a commercial pesticide, are in the range of 1 - 100%, e.g. 1-20%, of the recommended concentration for the plant/pathogen in question, with 10% (or less) being preferred. Lower concentrations such as ≤1%, e.g. 0.1 to 1% may also be used. Generally the fungicide not containing chitopoly- or chitooligo-saccharides is present at a concentration of 1 to 300 µg/ml, e.g. 1 to 150 µg/ml, preferably 1 to 100 µg/ml. Even lower concentrations may also be used, e.g. ≤1 µg/ml, e.g. 0.1 to 1 µg/ml. These concentrations act

as a guide for developing comparable non-liquid preparations. It is understood, however, that the most favourable concentration of the ingredients will vary depending on the pathogen, its host plant, the disease present, and the administration route. It is well within the level of skill of those in the art to find the most favourable concentrations by following adequate testing procedures.

[0081] The determination of an effective amount to be used is well within the scope of the practitioner. An "effective amount" is an amount effective to inhibit the infection, germination or growth of a fungus and fungus-like organism, relative to the infection, germination or growth that is seen in the absence of any such treatment.

[0082] Exemplary chitosan or chitosan-derived products and fungicide combinations are as follows:
the fungicides fenhexamid (Teldor), cyprodinil and fludioxonil (Switch), azoxystrobin (Amistar) and boscalid and pyraclostrobin (Signum) in combination with chitosan or chitopoly- or chitooligo-saccharides of chitosan with a DP_n of 9, 9.5, 15, 23, 28, 30, 33.5, 34, 34.6, 37, 40, 41, 48, 49, 50, 58, for example 15, 23, 28, 30, 34, 37, 40, 41, 50. The DP_n ranges from 20 to 60, particularly 20 to 40, especially preferably 20 to 35 are used in aspects of the invention. Also preferred is the use of the fungicide dithianon (Delan) in combination with such chitosan or chitopoly- or chitooligo-saccharides of chitosan.

[0083] These combinations may be used to treat or prevent fungi selected from *Botrytis cineria, Alternaria brassicicola, Mucor piriformis* and *Microdochium majus.* They may also be used to treat *Venturia inaequalis.*

[0084] In another preferred combination, the DP_n is 20 to 40, e.g. 30 to 40, e.g. 37 and the fungicide not containing chitopoly- or chitooligo-saccharides is fenhexamid, cyprodinil and fludioxonil, azoxystrobin or boscalid and pyraclostrobin (preferably boscalid and pyraclostrobin) and preferably the fungus is *Botrytis cineria.*

[0085] In a yet further preferred combination, the DP_n is 15 to 35, e.g. 20 to 35, e.g. 23 and the fungicide not containing chitopoly- or chitooligo-saccharides is fenhexamid, cyprodinil and fludioxonil, azoxystrobin or boscalid and pyraclostrobin and preferably the fungus is *Botrytis cineria,* e.g. on strawberries.

[0086] A further preferred combination is provided by a DP_n of 20 to 40, e.g. 25 to 35, e.g. 30 and the fungicide not containing chitopoly- or chitooligo-saccharides is cyprodinil and fludioxonil or boscalid and pyraclostrobin and preferably the fungus is *Botrytis cineria,* e.g. on chickpeas or beans.

[0087] A yet further preferred combination is provided by a DP_n of 20 to 40, e.g. 25 to 35, e.g. 30 and the fungicide not containing chitopoly- or chitooligo-saccharides is dithianon and preferably the fungus is *Venturia inaequalis,* e.g. on pomaceous fruits (such as apples), stone

fruits or on vines.

[0088] The above described DP_n ranges may also be applied to broader aspects of the invention described hereinbefore.

[0089] As described above, the components in the compositions described herein for the indications described above may be used separately.

[0090] Also disclosed herein is a product comprising (i) chitosan or chitopoly- or chitooligo-saccharides thereof as described herein and (ii) a fungicide not containing chitopoly- or chitooligo-saccharides as described herein as a combined preparation for simultaneous, separate or sequential use in the treatment or prevention of fungal infection in plants as described herein. In an aspect of the invention, the product comprises chitooligosaccharides and fungicides of the composition of the invention as a combined preparation for simultaneous, separate or sequential use in plant therapy, optionally for treating, preventing or controlling fungal disease, damage or infection in a plant caused by a fungus or fungus-like organism.

[0091] Optionally the compositions and products described herein may contain one or more additional active ingredients.

[0092] Also disclosed herein is the use of chitosan or chitopoly- or chitooligo-saccharides thereof and a fungicide not containing chitopoly- or chitooligo-saccharides as described herein (or a product, composition or kit as described herein) as a fungicide, preferably for treating, preventing or controlling fungal disease, damage or infection in a plant caused by a fungus or fungus-like organism. A further aspect of the invention provides use of chitooligosaccharides of the composition of the invention and a fungicide not containing chitopoly- or chitooligo-saccharides as a fungicide, wherein the chitooligosaccharides are present at a concentration in the range of 1-1000 µg/ml and the chitooligosaccharides and fungicide are present in a ratio of 1000:1 to 1:10 (w/w).

[0093] The invention will now be described by way of the following Examples in which:

Figure 1 shows a size exclusion chromatogram of chitopoly- or chitooligo-saccharides (CHOS) with DP_n30. The material in the DP >12 areas was fractionated into several samples. The DP_n of the fractions was found using ^{1}H-NMR and the material was further used in the biological assays.

Figure 2 shows the effect on germination of *Botrytis cinerea* 101 of CHOS mixtures with a DP_n of 30, produced by hydrolysis of chitosan DP_n 206 (F_A 0.15) with ScCsn46A, (95% deacetylated reducing ends). Subsequently, subfractions were prepared by separating this hydrolysis mixture using a SEC column as described in the Materials and Methods section. The resulting sub-fractions had DP_n values ranging from 34 to 163.

Figure 3 shows the effect of acetylation of reducing end sugars on the ability of CHOS to inhibit

germination of *B. cinerea* 101. CHOS with 95% deacetylated reducing ends was obtained by hydrolyzing chitosan (F_A 0.15, DP_n=206) with ScCsn46A, whereas CHOS with 35 % deacetylated reducing ends was obtained by hydrolyzing the same chitosan with ChiA.

Figure 4 shows the results of an experiment to assess dose-response relationships of CHOS DP_n 37 (prepared by hydrolyzing with ScCsn46A) on germination of B. *cinerea* 101, *Alternaria brassicicola* A 328 and *Mucor piriformis* M 119.

Figure 5 shows the results of an experiment to assess the effect of combinations of chitosan DP_n 206, CHOS DP_n 30 (F_A 0.15 and 95% deacetylated reducing ends) and Switch on the cumulative infection of detached chickpea leaves by *B. cinerea* 101.

Figure 6 shows the results of an experiment to assess the effect of the combination of chitosan DP_n 206, CHOS DP_n 30 (F_A 0.15 and 95% deacetylated reducing ends) and Signum (Sig) on infection of detached chickpea leaves by *B. cinerea* 101.

Figure 7 shows the results of an experiment to assess the effect of the combination of chitosan DP_n 206, CHOS DP_n 30 and Switch on *B. cinerea* 101 infection of bean leaves.

Figure 8 shows the results of an experiment to assess the effect of chitosan DP_n 206, the CHOS DP_n 30 and Signum (Sig) on *B. cinerea* 101 infections of bean leaves.

EXAMPLES

MATERIALS AND METHODS

Fungal cultures

[0094] *Botrytis cinerea* isolate BC 101, *B. cinerea* BD, *Alternaria brassicicola* A 328, *Microdochium majus* and *Mucor piriformis* M 119 were obtained from the culture collection at the Norwegian University of Life Sciences (UMB). For the *in vitro* and *in vivo* bioassays, conidia were collected from cultures grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) under regular laboratory light for 2 weeks at 23±1°C. Concentrations of conidia in aqueous suspensions were determined by haemocytometer count at 400X magnification and adjusted to 4 $\times 10^4$ conidia/ml with sterile water.

Fungicides

[0095] Five fungicides not containing chitopoly- or chitooligo-saccharides were used:

1. 1. Teldor® WG 50 (Bayer Crop Science Pty Ltd.): 500 g/kg fenhexamid. Recommended concentration of Teldor®; 150g/100L water
2. 2. Switch® 62.5 WG (Syngenta Crop Protection Pty Ltd.): 375 g/kg cyprodinil, 250 g/kg fludioxonil. Recommended concentration of Switch® 50g/100L
3. 3. Amistar® (Syngenta Crop Protection Pty Ltd.): 500 g/kg azoxystrobin. Recommended concentration of Amistar® 100g/100L
4. 4. Signum® WG (BASF): 26.7% w/w boscalid and 6.7% w/w pyraclostrobin. Recommended concentration of Signum® 100g/100L
5. 5. Delan® WG (BASF): 70% w/w dithianon.
   Recommended concentration of Delan® 80g/100L.

Chitosan and chitooligosaccharide (CHOS) production

[0096] Chitosan KitoNor (DP_n 206, F_A 0.15, M_{Wn} 34kDa); was produced by acid hydrolysis from chitin from Snow crab (*Chionoecetes opilio*) by Norwegian Chitosan, Gardermoen, Norway. The average molecular weight and DP_n was calculated from viscosimetric measurements. CHOS with lower DP_n were produced by enzymatic hydrolysis of chitosan (DP_n 206) using a chitosanase, ScCsn46A (Heggset et al., Biomacromolecules, 2010, p2487-2497), or a chitinase, Chi A (Brurberg et al., FEMS Microbiol Lett., 1994, p399-404; Horn et al., FEBS J., 2006, p491-503.).

[0097] KitoNor (20 mg/mL) was dissolved in water with 0.5 % (v/v) acetic acid. After all of the chitosan was dissolved the pH was adjusted with 0.1N NaOH to 5.5. Recombinant chitosanase ScCsn46A from *Streptomyces coelicolor* A3(2) (Heggset et al., Biomacromolecules, 2010, p2487-2497) or chitinase A (ChiA) from *Serratia marcescens* (Brurberg et al., FEMS Microbiol Lett., 1994, p399-404) was added to the chitosan solutions to a final concentration of 0.5 µg/mg chitosan and the reaction was incubated with shaking (225 rpm) at 37°C. The reaction was stopped by decreasing the pH to 2.5 with 0.1N HCl and by keeping the tube with the reaction mixture at boiling temperature for ten minutes to permanently inactivate the enzymes. The DP_n of the resulting CHOS sample was determined by ^{1}H-NMR analysis on a Varian 300 mHz instrument, as described in Sørbotten et al. FEBS J., 2005, p538-549.

Separation of CHOS by Size Exclusion Chromatography (SEC)

[0098] The CHOS were separated by SEC on three XK 26 columns packed with Superdex™ 30 prep grade (GE Healthcare) coupled in series with an overall dimension of 2.6 cm × 180 cm. The mobile phase (150 mM NH_4Ac pH 4.6) was run at a constant flow of 0.4 mL/min (Sørbotten et al. 2005, FEBS J., p538-549). The signals were read on a RI detector (Gilson model 133). In each run 100 mg of CHOS was applied (i.e. 5 mL) and fractions were collected. Identification of DP_n of the fractions was performed with ^{1}H-NMR. The fractions were not baseline separated.

[0099] The fractions were dialyzed with Float-A-Lyzers (MWCO 100-500 Da, SpectrumLabs) to remove salts, sterile filtrated through Filtropur S 0.2 µm sterile filters (Sarstedt, Germany) and lyophilized, prior to use.

***In vitro* bioassay of chitosan and fungicides not containing chitopoly- or chitooligo-saccharides**

[0100] The antifungal effects of chitosan and CHOS samples and fungicides not containing chitopoly- or chitooligo-saccharides were investigated in a synthetic medium (2.5 mM NH_4NO_3; 0.28 mM $CaCl_2•2H_2O$; 0.16 mM $MgSO_4•7H_2O$; 0.002 mM $MnSO_4•4H_2O$; 0.002 mM $ZnSO_4•7H_2O$; 1 mM KH_2PO_4; 0.06 mM $FeC_6H_5O_7•5H_2O$ and 55.5 mM glucose, pH 5.2 - 5.3) in a flat-bottom 96-well microtiter plate (Nunc™, Roskilde, Denmark), 200µL/well with $2×10^4$ conidia/mL. There were 4 replicate wells of each treatment. The microtiter plates were incubated at 23±1 °C for up to 72 h. An invert microscope (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) was used to visually estimate the germination percentage at 400X magnification after 24h and these estimates were used to express anti-microbial activity as half maximal inhibitory concentrations (IC_{50}) or minimum inhibitory concentrations (MIC). Mycelial growth following germination was measured as absorbance at 595 nm in a microtiter plate reader 72 h after inoculation.

[0101] Synergistic effects were calculated as the ratio between observed efficacy, E_{obs} (% inhibition) and the expected efficacy, E_{exp} (calculated by Abbott's formula) (Levy et al. Eppo Bulletin, 1986, p651-657) % E_{exp} = a+b - (ab/100). Here a = % germination inhibition by that concentration of the fungicide alone, b = % germination inhibition by that concentration of the chitosan alone. An E_{obs}/E_{exp} ratio of 1 indicates additivity, ratios >1 indicates synergy and ratios <1 indicates antagonistic interactions.

***In vivo* bioassay of chitosan and fungicides not containing chitopoly- or chitooligo-saccharides on plants**

[0102] The flower infection test was performed on detached, newly opened strawberry

(*Fragaria* × *ananassa*) flowers (cv. Corona) from the greenhouse (Hjeljord et al. Phytopathology, 2001, p1172-1180). Eighteen flowers per treatment (six replications of three flowers) were cut off with a 1½ -2 cm stem and placed in empty pipette tip racks set in plastic containers filled with 1-2 cm water. Conidia suspensions of the pathogen (final concentration: 1×10^6 conidia/ml) were mixed with test ingredients (i.e. fungicides, CHOS, chitosan or mixtures thereof, as well as control ingredients) and 10 µl drops of each mixture were placed at the base of three petals on each flower using an automatic pipette (Finnpipette 4027, Thermo Labsystems, Finland). All flowers were then incubated at 23±1°C in large trays covered with aluminium foil. The relative humidity around the flowers was 90-95%, measured by a thermo-hygrometer (Lambrecht, Germany).

[0103] The necrotic regions on the abaxial surface of the flowers under the inoculation point were recorded daily for 8 days and the area under the disease progress curve (AUDPC) was calculated on the basis of the cumulative infection percentage by the following equation:

$$AUDPC = \Sigma\ [(D_i-D_i-1) \times \{S_i-1+0.5\ (S_i-S_i-1)\}\ ]$$

[0104] Where, D_i = Days of the i^{th} assessment, S_i = Proportion of the i^{th} infected inoculation point.

[0105] The protection index was calculated by using the AUDPC values in the following equation (Bardin et al., Biological Control, 2008, p476-483).

$$100 \times (AUDPC_{control}-AUDPC_{treatment})/AUDPC_{control}$$

where $AUDPC_{control}$ represents flowers inoculated with only B. *cinerea* conidia and $AUDPC_{treatment}$ represents flowers treated with the conidia applied in solutions containing pesticides and/or chitooligosaccharides to be tested.

[0106] The interaction (synergy) between fungicides and the chitosan products in the flower assay was determined by Abbott's equation as above.

[0107] Similar tests were performed on detached chickpea leaves (*Cicer arientinum*) (3 × 6 leaves per treatment and 3 parallels), or on 30 day-old bean leaves (*Vicia faba*) (6 inoculation drops on each leaf and 3 parallels). The leaves were inoculated with 10 µL drops with 2×10^6/ml B. *cinerea* 101 conidia. The development of the disease and the amount of sporulation were recorded every 24 hours up to 8 days. The experiment was repeated at least twice.

Field trials with chitosan and the fungicides not containing chitopoly- or chitooligo-saccharides

[0108] Apple trees (*Malus domestica* Broch) of the cultivar Aakerø in the apple orchard at the Norwegian University of Life Sciences, Aas , Norway were used. There were three replicates of each treatment and three trees in each replicate. The trees were sprayed to runoff once in the

flowering period (28Th of May) and three times in the fruiting season (24th of June, 7th of July and 17th of August). At harvest (3rd of September) the number of apples with infection of apple scab (*Venturia inaequalis*) was recorded.

Determination of average degree of polymerization (DP_n) with ^{1}H-NMR spectroscopy

[0109] The chitooligosaccharide (CHOS) samples were analysed by ^{1}H-NMR spectroscopy on a Varian Gemini 300MHz instrument. The average degree of polymerization (DP_n) was calculated by the equation (Dα+Dβ+D+Aα+Aβ+A)/(Dα+Dβ+Aα+Aβ), where Dα, Dβ, Aα and Aβ are the integral of the reducing end signals of the α and β anomers of the deacetylated (D) and acetylated (A) units, D is the integral of the signals from the internal and nonreducing end deacetylated units and A is the integral of the signals from the internal and nonreducing end acetylated units (Sørbotten et al. FEBS J., 2005, p538-549).

Data analysis

[0110] The % inhibition data for the fungal germination were transformed using arcsine transformation and tested by one-way ANOVA (analysis of variance). Non-transformed data are presented. When appropriate, means were separated by Tukey's method (Fenech, J Am. Stat. Ass., 1979, p881-884). All calculations were done using Microsoft Office Excel 2007 and Minitab 16 (MINITAB, USA).

EXAMPLE 1: Production of chitooligosaccharides (CHOS)

[0111] Chitosan KitoNor (DP_n 206, F_A 0.15 and M_W 34 kDa) was hydrolyzed with chitosanase ScCsn46A, which primarily cleaves after deacetylated units and under the conditions of the assay produced 95% deacetylated reducing ends, as determined by ^{1}H-NMR). The chitooligomers were separated by SEC (see Materials and Methods) and the results are shown in Fig. 1. These chitooligosaccharides of varying DP_n were used in Example 2. For details see Materials and Methods: "Chitosan and chitooligosaccharide (CHOS) production".

EXAMPLE 2: Effect of DP_n of CHOS on fungicidal activity (inhibition of germination)

[0112] The effect of chitosan (F_A 0.15, DP_n 206, not part of the invention), or chitooligosaccharides (CHOS), obtained by hydrolysis of this chitosan with ScCsn46A to DP_n values varying from 9.5 to 96 (95% deacetylated reducing ends, as determined by ^{1}H-NMR) on

spore germination of *Botrytis cinerea* 101 was evaluated. The experiment was conducted as described in the Materials and Methods section under "*In vitro* bioassay".

[0113] The DP_n of the chitosan/CHOS influences the inhibitory effect on the germination of B. *cinerea* 101. The most active fractions have DP_n values in the order of 30, but the data also show that DP_n values in the range of 10-40 have useful activities (Table 1).

Table 1. Effect of DP_n of CHOS on fungicidal activity (inhibition of germination)

DP_n of chitosan/CHOS	**MIC (µg ml^{-1})**	**IC_{50} (µg ml^{-1})**
206	5000	2500
96	2500	1230
62	2500	630
49	2500	630
40	1200	250
37	310	80
28	310	80
15	310	120
9.5	>2500	2500
IC_{50}= half maximal inhibitory concentrations. MIC= minimum inhibitory concentrations.		

EXAMPLE 3: Effect of deacetylated reducing ends in CHOS on fungicidal activity (inhibition of germination)

[0114] An *in vitro* assay of the fungicidal activity of chitosan and CHOS with >95% (chitosan hydrolysed by chitosanase ScCsn46A from *Streptomyces coelicolor*) or 35% (chitosan hydrolysed by chitinase A from *Serratia marcescens*) deacetylated reducing ends was carried out as described in the Materials and Methods section under "*In vitro* bioassay". The results are shown in Figure 3.

[0115] Given the same DP_n, CHOS with deacetylated reducing ends are much more inhibitory to spore germination than CHOS with acetylated reducing ends (Fig. 3). For example, 80 µg ml^{-1} CHOS with 95% deacetylated reducing ends prevented further hyphal growth, whereas 310 µg ml^{-1} CHOS with 35% deacetylated reducing ends was needed to attain the same effect (data not shown).

EXAMPLE 4: Effect of partial purification of CHOS with various DP_n on fungicidal activity (inhibition of germination)

[0116] This experiment assessed the effect of partially purified chitooligosaccharides (CHOS) with varying chain lengths on the germination inhibition of B. *cinerea* 101, assessed 24 hours after inoculation. Purification was by SEC, see the Materials and Methods section. The results are shown in Figure 2 and demonstrate that sub-fractions with high DP_n (DP_n 78-163) are less inhibitory than the original non-hydrolyzed chitosan DP_n 206, probably due to the removal of low molecular weight oligomers from those fractions. The CHOS DP_n 30 hydrolysate that had not been separated further on the SEC column had about the same inhibitory effect as the DP_n 34 SEC fraction. The non-purified DP_n 30 and the purified DP_n 34 fractions were the most inhibitory of the fractions tested.

EXAMPLE 5: Effect of chitosan or CHOS with various DP_n on germination or growth of different fungi

[0117] An experiment was designed to assess effects of chitosan and CHOS with various DP_n on germination and mycelial growth of two strains of B. *cinerea* and a strain of *Mucor piriformis.* This experiment assessed germination inhibition (GI) and growth inhibition (Gr.I) of B. *cinerea* 101 (BC 101), *B.cinerea* BD (BC BD) and *Mucor piriformis* M 119 caused by 80 µg ml^{-1} non-hydrolyzed chitosan and chitooligosaccharides (CHOS) (F_A 0.15) with different DP_n, produced by enzymatic hydrolysis with ScCsn46A (95% deacetylated reducing ends). Growth inhibition was measured by absorbance reading at 595 nm 72 hours after inoculation.

[0118] The results, depicted in Table 2, show that CHOS with average DP_n between 23 and 40 were generally more inhibitory to spore germination and growth than CHOS with higher or lower average DP_n. The data further show that the inhibitory effect of the compounds varies depending on the target organism.

Table 2. Effect of chitosan or CHOS with various DP_n on germination inhibition (GI%) or growth inhibition (Gr.I %) of different fungi

		BC 101		BC BD		*M. piriformis*	
Chitosan/ CHOS	**DP_n**	**GI % 24hrs**	**Gr.I % 72hrs**	**GI % 24hrs**	**Gr.I % 72hrs**	**GI% 24hrs**	**Gr.I % 72hrs**
	206	0 c	14 e	0 c	7e	0 g	23 c
	75±6.8	4 c	36 d	0 c	25 d	26 e	48 b
	58±2.7	0 c	50 cd	0 c	42 c	50 d	53 ab
	48±3.0	4 c	54 c	0 c	46 c	48 d	51 ab
	40±1.4	77 a	99 a	100 a	99 a	90 a	57 a
	23±1.3	72 b	76 b	100 a	78b	81 b	57 a
	15±1.4	0 c	ND	100 a	ND	58 c	ND

		BC 101		BC BD		*M. piriformis*	
Chitosan/ CHOS	**DP_n**	**GI % 24hrs**	**Gr.I % 72hrs**	**GI % 24hrs**	**Gr.I % 72hrs**	**GI% 24hrs**	**Gr.I % 72hrs**
	9±0.8	0 c	6 e	0 c	3 e	0 g	22 c
Means in columns without common letters are significantly different according to Tukey's method at P≤ 0.01. ND =not determined.							

EXAMPLE 6: Effect of chitosan or CHOS with various DP_n on disease severity caused by two strains of B. *cinerea*

[0119] A bioassay was designed to assess effects of non-hydrolyzed chitosan and CHOS with various DP_n on infection of strawberry flowers by two strains of B. *cinerea.* In this experiment the effect on the disease severity caused by *B. cinerea* 101 and B. *cinerea* BD on detached strawberry flowers treated with 500 µg/mL chitosan or chitooligosaccharides (CHOS) (F_A 0.15 and 95% deacetylated reducing ends) with different DP_n was assessed. The CHOS fractions with lower DP_n were produced by enzymatic hydrolysis of chitosan (DP_n 206) using a chitosanase, ScCsn46A. For details see Materials and Methods section: "Chitosan and chitooligosaccharide (CHOS) production". The disease severity was assessed as area under the disease progress curve (AUDPC), calculated from cumulative disease incidence at 23±1°C, 1 to 8 days after inoculation.

[0120] The results in Table 3 show that CHOS with DP_n 23 are more protective against B. *cinerea* infection of strawberry flowers than CHOS with higher and lower DP_n. Of the other products tested, the DP_n 40 material clearly shows the best results.

Table 3. Effect of chitosan or CHOS with various DP_n on disease severity caused by two strains of B. *cinerea* on strawberry flowers

Chitosan / CHOS	***B. cinerea* 101**		***B. cinerea* BD**	
	AUDPC	**Protection index (%)**	**AUDPC**	**Protection index (%)**
Control	4.3 a	-	4.3 a	-
DP_n 206	4.0 ab	7.8 c	3.5 a	19.8 d
DP_n 48	3.4 b	20.3 c	2.8 c	34.5 c
DP_n 40	1.9 c	55.2 b	1.7 d	61.5 b
DP_n 23	0.9 d	79.8 a	0.7 e	83.6 a
DP_n 9	4.0 ab	7.0 c	3.5 a	19.0 d
Means in columns without common letters are significantly different according to Tukey's method at P≤ 0.01.				

EXAMPLE 7: Effect of CHOS with DP_n 37 on germination of different fungi

[0121] An experiment was designed to compare effects of CHOS (DP_n 37) on three genera of plant pathogenic fungi. As seen in Figure 4, the results show a dose-response relationship for all genera tested, but these did respond somewhat differently. *B. cinerea* and *M. piriformis* showed decreasing germination over a broad CHOS concentration range (0.002 - 0.25%), whereas *A. brassicicola* showed complete germination at 0.002% and none at ≥ 0.008%.

COMPARATIVE EXAMPLE 8: Fungicidal activity of chitosan and the fungicide Switch (inhibition of germination) on two strains of *B. cinerea*

[0122] Non-hydrolyzed chitosan was mixed with a fungicide (Switch) to compare the effects of the mixture and the components separately on germination of two strains of *B. cinerea.* This experiment evaluated germination inhibition of *B. cinerea* 101 or *B. cinerea* BD recorded 24 hours after inoculation with chitosan (DP_n 206) and/or the fungicide Switch.

[0123] The results are provided in Table 4 and show that Switch applied at 25 µg ml^{-1}, a concentration that is only 1/20 of the recommended concentration (the recommended concentration, 500 µg ml^{-1}, are according to the standard product leaflets provided by the manufacturers of this product) together with 640 µg/ml chitosan DP_n 206 (not part of the invention) completely inhibited spore germination of both *Botrytis* strains. The combinations were clearly synergistic.

Table 4. Fungicidal activity of chitosan and the fungicide Switch (inhibition of germination) on two strains of *B. cinerea*

Treatment	**% inhibition of *B. cinerea* 101**	**% inhibition of *B. cinerea* BD**
Chitosan 640 µg ml^{-1}	32 d	17 d
Chitosan 80 µg ml^{-1}	11 c	5 e
Switch 25 µgml^{-1}	82 b	43 c
Chitosan 640 µg ml^{-1} + Switch 25 µg ml^{-1}	100 a	100 a
Chitosan 80 µg ml^{-1}+ Switch 25 µg ml^{-1}	100 a	90 b
Means in columns without common letters and related to the same fungicide, are significantly different according to Tukey's method at P≤ 0.01.		

COMPARATIVE EXAMPLE 9: Fungicidal activity of chitosan and the fungicides Switch or Signum (inhibition of germination) on *Microdochium majus*

[0124] Further experiments assessed the effects of combining chitosan with fungicides (Switch or Signum) on germination of the plant pathogenic fungus *Microdochium majus.* This experiment evaluated germination inhibition of *M. majus* recorded 24 h after inoculation with chitosan (DP_n 206) and fungicides. For details see Materials and Methods: "*In vitro* bioassay of chitosan and fungicides not containing chitopoly- or chitooligo-saccharides" .

[0125] The results in Table 5 show that combination of 640 µg ml^{-1} chitosan DP_n 206 (not part of the invention) with Switch at 1/50 of recommended concentration (recommended concentration is 500 µg ml^{-1}) or Signum at 1/1000 of recommended concentration (recommended concentration is 1000 µg ml^{-1}), completely inhibited spore germination of *M. majus.* Recommended concentrations of Switch and Signum are according to the standard product leaflets provided by the manufacturers of these products.

Table 5. Fungicidal activity of chitosan and the fungicides Switch or Signum (inhibition of germination) on *Microdochium majus*

Treatme nt	**% inhibition of *M. majus***
Chitosan 640 µg ml^{-1}	3 d
Switch 10 µg ml^{-1}	71 b
Signum 1 µg ml^{-1}	26 c
Chitosan 640 µg ml^{-1} + Switch 10 µg ml^{-1}	100 a
Chitosan 640 µg ml^{-1}+ Signum 1 µg ml^{-1}	100 a
Means in columns without common letters and related to the same fungicide, are significantly different according to Tukey's method at P≤ 0.01.	

EXAMPLE 10: Fungicidal activity of CHOS with DP_n23 and the fungicides Teldor, Switch, Amistar and Signum (inhibition of germination) on *B. cinerea*

[0126] Possible synergism between CHOS (DP_n 23) and fungicides (Teldor, Switch, Amistar and Signum) in inhibition of germination of *B. cinerea* was investigated in this experiment. The effect of combination of chitooligosaccharides (CHOS) DP_n 23 (F_A 0.15 and 95% deacetylated reducing ends) and fungicides in inhibiting germination (assessed 24 hours after inoculation) of *B. cinerea* BC 101 was assessed.

[0127] The results are shown in Table 6 which shows that high synergistic effects are seen when combining 5 µg ml^{-1} DP_n 23 with 1 % of the recommended concentration of Switch, Amistar and Signum and 10% of the recommended concentration of Teldor. Comparison of the data in Table 6 with the data in Table 4 further shows that the DP_n 23 product is more powerful than the non-hydrolyzed chitosan with DP_n 206.

Table 6. Fungicidal activity of CHOS with DP_n23 and the fungicides Teldor, Switch, Amistar and Signum (inhibition of germination) on *B. cinerea* 101

Treatment (µg/ml)		**Germination inhibition (%)**	**E_{obs}/E_{exp}**
Control		0 b	-
DP_n 23	5 µg ml^{-1}	1 b	-
Teldor	150 µg ml^{-1}	0 b	-
DP_n 23	5 µg ml^{-1}+ Teldor 150 µg ml^{-1}	20 a	20
DP_n23	5 µg ml^{-1}	1 c	-
Switch	5 µg ml^{-1}	30 b	-
DP_n 23 5 µg ml^{-1} + Switch 5 µg ml^{-1}	94 a	3	
DP_n 23 5 µg ml^{-1}	1 b	-	
Amistar 10 µg ml^{-1}	2 b	-	
DP_n 23 5 µg ml^{-1} + Amistar 10 µg ml^{-1}	92 a	31	
DP_n 23 5 µg ml^{-1}	1 b	-	
Signum 10 µg ml^{-1}	1 b	-	
DP_n23 5 µg ml^{-1} + Signum 10 µg ml^{-1}	98 a	49	

Means in columns without common letters and related to the same fungicide, are significantly different according to Tukey's method at P≤ 0.01. Synergism is calculated by the E_{obs}/E_{exp} ratio, 1 indicates additivity, ratios >1 indicate synergy and ratios <1 indicate antagonistic interactions. The recommended concentrations for application of Teldor, Switch, Amistar and Signum are 1500, 500, 1000, 1000 µg/ml, respectively, according to the standard product leaflets provided by the manufacturers of these products.

COMPARATIVE EXAMPLE 11: Fungicidal activity of chitosan and the fungicides Teldor,

Switch, Amistar and Signum (inhibition of infection of strawberry flowers) on *B. cinerea*

[0128] A bioassay was designed to evaluate effects of the combination of chitosan and fungicides (Teldor, Switch, Amistar or Signum) on flower infection by *B. cinerea* 101. For experimental details see: *In vivo* bioassay of chitosan and fungicides on plants, infection on strawberry flowers in Materials and Methods section. This experiment assessed the effect of combination of chitosan with DP_n 206 (not part of the invention) and fungicides in μg ml^{-1} in inhibiting *B. cinerea* BC 101 infection of detached strawberry flowers. Disease severity was measured as the area under the disease progress curves (AUDPC), with AUDPC values calculated from the cumulative disease incidence at 23±1°C, recorded up to 8 days after inoculation. Percent protection index is % reduction in AUDPC by the treatment compared with the control.

[0129] The results are shown in Table 7 which shows that 1% of the recommended concentration of Teldor, Switch and Amistar (15, 5 and 10 μg /ml respectively) in combination with 1000 μg ml^{-1} chitosan DP_n 206 and 5000 μg ml^{-1} chitosan in combination with 0.2% of the recommended concentration of Signum (2 μg/ml), gave the same level of protection against infection by *B. cinerea* as the recommended concentration of the respective fungicides alone. are 1500, 500, 1000 and 1000 μg ml^{-1} respectively, according to the standard product leaflets provided by the manufacturers of these products.

EXAMPLE 13: Fungicidal activity of chitosan or CHOS with DP_n 30 and the fungicide Switch (reduction of infection on chickpea leaves) on *B. cinerea*

[0130] Possible synergism between non-hydrolyzed chitosan, CHOS (DP_n 30) and Switch in reducing infection of chickpea leaves by *B. cinerea* 101 was investigated. For experimental details see: *In vivo* bioassay of chitosan and fungicides not containing chitopoly- or chitooligo-saccharides on plants, infection on chickpea leaves in the Materials and Methods section.

[0131] The results, depicted in Fig. 5, show that, when combined with Switch, CHOS (DP_n 30) was much more effective than the non-hydrolyzed chitosan (DP_n 206). The figure also illustrates the power of CHOS: the combination of 1/50 of the recommended Switch concentration (10 μg ml^{-1}) (recommended concentration is 500 μg ml^{-1}) with 320 μg ml^{-1} CHOS (DP_n 30) was as protective as the recommended concentration of Switch.

EXAMPLE 14: Fungicidal activity of chitosan or CHOS with DP_n30 and the fungicide Signum (reduction of infection on chickpea leaves) on *B. cinerea* 101

[0132] This experiment was similar to that described in Example 13, except that the fungicide Signum was tested. For experimental details see: *In vivo* bioassay of chitosan and fungicides not containing chitopoly- or chitooligo-saccharides on plants, infection on chickpea leaves in the Materials and Methods section.

[0133] The results are shown in Figure 6. Figure 6 shows that all combinations of chitosan DP_n 206 and the CHOS DP_n 30 with Signum showed a better effect in reducing disease severity than each component alone. 1000 µg ml^{-1} Signum, 2500 µg ml^{-1} chitosan DP_n 206 and 2500 µg ml^{-1} DP_n 30 completely controlled infection, whereas the combination of 10 µg ml^{-1} Signum and 320 µg ml^{-1} CHOS DP_n 30 resulted in only 5 % infection after 8 days. These results illustrate the synergistic effect of CHOS with a reduced concentration of Signum.

EXAMPLE 15: Fungicidal activity of chitosan or CHOS with DP_n 30 and the fungicide Signum (reduction of sporulation on infected chickpea leaves) on *B. cinerea* 101

[0134] Sporulation of the plant pathogenic fungus on infected plant parts is a source of secondary inoculum and an important factor in disease epidemiology. An experiment was designed to assess the effects of the combination of chitosan or CHOS with the fungicide Signum on sporulation of *B. cinerea* on infected chickpea leaves. This experiment assessed the effect of combination of chitosan DP_n 206, or chitooligosaccharide (CHOS) DP_n 30 and Signum on the average number of spores produced after 8 days by *B. cinerea* 101 in each inoculation spot on chickpea leaves. For experimental details see: *In vivo* bioassay of chitosan and fungicides on plants not containing chitopoly- or chitooligo-saccharides, infection on chickpea leaves in the Materials and Methods section.

[0135] The results in Table 9 show that the combination of chitosan and Signum reduced sporulation of *B. cinerea* 101 more than each component alone and CHOS DP_n 30 was more effective than chitosan DP_n 206 when each were combined with Signum in reducing the sporulation of *B. cinerea* 101.

Table 9. Fungicidal activity of chitosan or CHOS with DP_n 30 and the fungicide Signum (reduction of sporulation on infected chickpea leaves) on *B. cinerea* 101

Treatment	**Average number of conidia produced in inoculation point**
Untreated control	2.1×10^5
Signum 10 µg ml^{-1}	3.6×10^4
Chitosan DP_n 206, 320 µg ml^{-1}	7.8×10^4
CHOS DP_n 30, 320 µg ml^{-1}	4.1×10^4
Signum 10 µg ml^{-1} + Chitosan DP_n 206,	7.8×10^3

Treatment	Average number of conidia produced in inoculation point
320 µg ml^{-1}	
Signum 10 µg ml^{-1} + CHOS DP_n 30, 320 µg ml^{-1}	2.9×10^2

EXAMPLE 16: Fungicidal activity of chitosan or CHOS with DP_n30 and the fungicide Switch (reduction of infection on bean leaves) on *B. cinerea*

[0136] An experiment was designed to assess the effects of the combination of chitosan or CHOS with the fungicide Switch on *B. cinerea* 101 infection of bean leaves. For experimental details see: *In vivo* bioassay of chitosan and fungicides not containing chitopoly- or chitooligo-saccharides on plants, infection on bean leaves in the Materials and Methods section.

[0137] The results are shown in Figure 7. In this assay 500 µg ml^{-1} Switch (recommended concentration) and the combination of 2.5 µg ml^{-1} Switch and 160 µg ml^{-1} of DP_n 30 or DP_n 206 chitosan completely controlled infection, while 2.5 µg ml^{-1} Switch, or 160 µg ml^{-1} chitosan DP_n 206 or CHOS DP_n 30 separately were less effective.

EXAMPLE 17: Fungicidal activity of chitosan or CHOS with DP_n30 and the fungicide Signum (reduction of infection on bean leaves) on *B. cinerea* 101.

[0138] This experiment was similar to that described in Example 16, except that the fungicide Signum was tested. For experimental details see: *In vivo* bioassay of chitosan and fungicides not containing chitopoly- or chitooligo-saccharides on plants, infection on bean leaves in the Materials and Methods section.

[0139] The results are shown in Figure 8 which shows that in this assay, complete control of infection was obtained by 1000 µg ml^{-1} Signum (recommended concentration), or the combination of 5 µg ml^{-1} Signum + 160 µg ml^{-1} chitosan DP_n 206.

EXAMPLE 18: Field experiment for testing the fungicidal activity of CHOS with DP_n 30 and the fungicide Delan against apple scab (*Venturia inaequalis*)

[0140] In a field trial the effect of 0.1% CHOS and recommended (0.8%) and 1/10 concentration (0.08%) of Delan on the infection of *Venturia inaequalis* on apples was

investigated. For experimental details see: Field trials with chitosan and the fungicides not containing chitopoly- or chitooligo-saccharides in the Materials and Methods section.

[0141] The results in Table 10 shows that the combination of CHOS and 1/10 of the recommended concentration of Delan was more effective than the recommended concentration of Delan

Table 10. Fungicidal activity of CHOS with DP_n30 and the fungicide Delan (reduction of apples with apple scab) on *Venturia inaequalis.*

Treatment	% apple with apple scab
Untreated control	31,2 ± 9,7[a]
Delan 0.8g/L (800 µg ml^{-1})	20,9 ± 9,5
Delan 0.08g/L (80 µg ml^{-1})	27,5 ± 12,0
CHOS DP_n 30, 1.0 g/L (1000 µg ml^{-1})	25,9 ± 13,3
Delan 80 µg ml^{-1} + Chitosan DP_n 30, 1000 µg ml^{-1}	16,7 ± 5,2
[a]Standard deviation	

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Patentkrav

1. Sammensætning omfattende (i) chitooligosaccharider, hvor nævnte chitooligosaccharid omfatter β-(1-4)-bundet D-glucosamin og N-acetyl-D-glucosaminmonomerer og en acetyleringsgrad mellem a) 0,01 og 0,40, eller b) 0,05 og 0,20, og en gennemsnitlig polymerisationsgrad på 20-60 som bedømt ved måling med ^{1}H NMR-spektroskopi, og (ii) et fungicid ikke indeholdende chitopoly- eller chitooligo-saccharider,

hvor

chitooligosaccharide rne er til stede i sammensætningen i en koncentration i området fra 1-1000 µg/ml, og chitooligosaccharide rne og fungicidet er til stede i et forhold på 1000:1 til 1:10 (vægt/vægt); og

fungicidet er

a) valgt fra et anilid-fungicid; et anilinopyrimidin-fungicid; et pyrrol-fungicid; et methoxyacrylatstrobilurin-fungicid; et carbanilat-fungicid; et pyrazol-fungicid; et pyridin-fungicid; et methoxycarbanilatstrobilurin-fungicid og et naphthoquinon-fungicid og kombinationer deraf, eller

b) valgt fra et benzamid-fungicid, et strobilurin-fungicid, et conazol-fungicid, et carboxamid-fungicid, et triazol-fungicid og et dicarboximid-fungicid og kombinationer deraf; eller

c) ikke et uorganisk fungicid; og

chitooligosaccharide rne har en D-glucosaminsukkerenhed ved (i) ≥ 50% af deres reducerende ender, ii) ≥ 85% af deres reducerende ender, eller iii) ≥ 95% af deres reducerende ender.

2. Sammensætning ifølge krav 1, hvor DP_n er

i) mellem 20 til 40;

ii) mellem 20 til 35; eller

iii) 23, 30 eller 40.

3. Sammensætning ifølge krav 1 eller 2, hvor nævnte chitooligosaccharider er opnåelige ved

a) syrehydrolyse, eller

b) en fremgangsmåde omfattende opløsning af chitosan i vand eller en svag sur opløsning efterfulgt af enzymatisk spaltning under anvendelse af

et enzym, som kan katalysere nedbrydning af chitosan til chitooligosaccharider, eventuelt a) et enzym som spalter glycosidbindingerne efter en deacetyleret rest eller b) en familie 46 chitosanase, hvor eventuelt den resulterende chitooligosaccharidprodukt-blanding separeres ved størrelsesekskluderingskromatografi.

4. Sammensætning ifølge et hvilket som helst af kravene 1 til 3, hvor chitooligosaccharide rne er til stede i sammensætningen i en koncentration på

a) mellem 10 og 1000 µg/ml, eller

b) 50-150 µg/ml.

5. Sammensætning ifølge et hvilket som helst af kravene 1 til 4, hvor fungicidet er valgt fra fenhexamid, cyprodinil, fludioxonil, azoxystrobin, boscalid, pyraclostrobin og dithianon og kombinationer deraf.

6. Sammensætning ifølge et hvilket som helst af kravene 1 til 5, hvor mindst 2 fungicider ikke indeholdende chitopoly- eller chitooligo-saccharider er til stede.

7. Sammensætning ifølge et hvilket som helst af kravene 1 til 6, hvor fungicidet er til stede i sammensætningen i en suboptimal koncentration, hvor en suboptimal koncentration er lavere end koncentrationen, som genererer den maksimale fungicideffekt, når fungicidet anvendes alene, hvor koncentrationen af fungicidet i sammensætningen eventuelt er a) 1-20% af koncentrationen som er nødvendig for at opnå den maksimale fungicideffekt af fungicidet, eller b) <10% af koncentrationen som er nødvendig for at opnå den maksimale fungicideffekt af fungicidet.

8. Sammensætning ifølge et hvilket som helst af kravene 1 til 7, hvor fungicidet er valgt fra (i) fenhexamid; (ii) cyprodinil og fludioxonil; (iii) azoxystrobin; (iv) boscalid og pyraclostrobin og (v) dithianon, og chitooligosaccharider har en DP_n på a) 20 og 60, b) 20 til 40, c) 20 til 35, eller d) 23, 30 eller 37.

9. Kit omfattende (i) chitooligosaccharider som defineret i et hvilket som helst af kravene 1 til 8 og (ii) et fungicid som defineret i et hvilket som helst af kravene 1

til 8.

10. Fremgangsmåde til behandling, forebyggelse eller bekæmpelse af svampesygdom, svampeskade eller svampeinfektion i en plante forårsaget af en svamp eller svampelignende organisme, omfattende at bringe planten eller en del deraf, som er angrebet eller som skal beskyttes fra svampen, i kontakt med (i) chitooligosaccharider som defineret i et hvilket som helst af kravene 1 til 8 og (ii) et fungicid ikke indeholdende chitopoly- eller chitooligo-saccharider, hvor chitooligosaccharide rne er til stede i en koncentration i området fra 1-1000 µg/ml, og chitooligosaccharide rne og fungicidet er til stede i et forhold på 1000:1 til 1:10 (vægt/vægt).

11. Fremgangsmåde ifølge krav 10, hvor nævnte fungicid er som defineret i et hvilket som helst af kravene 1, 5, 7 eller 8.

12. Fremgangsmåde ifølge krav 10 eller 11, hvor fremgangsmåden udføres på planter, og a) svampen er valgt fra *Botrytis cineria, Alternaria brassicicola, Mucor piriformis, Microdochium sp* og *Venturia inaequalis,* og/eller b) planten er korn, plænegræs, ris, vindrue eller rod, rodknold, frugt, bær, grøntsag eller bælgfrugt.

13. Produkt omfattende (i) chitooligosaccharider som defineret i et hvilket som helst af kravene 1 til 8 og (ii) et fungicid som defineret i et hvilket som helst af kravene 1 til 8 som et kombineret præparat til samtidig, separat eller sekventiel anvendelse i planteterapi, eventuelt til behandling, forebyggelse eller bekæmpelse af svampesygdom, svampeskade eller svampeinfektion i en plante forårsaget af svamp eller svampelignende organisme.

14. Anvendelse af chitooligosaccharider som defineret i et hvilket som helst af kravene 1 til 8 og et fungicid ikke indeholdende chitopoly- eller chitooligo-saccharider som et fungicid, hvor chitooligosaccharide rne er til stede i en koncentration i området fra 1-1000 µg/ml, og chitooligosaccharide rne og fungicidet er til stede i et forhold på 1000:1 til 1:10 (vægt/vægt).

15. Anvendelse ifølge krav 14, hvor nævnte fungicid er som defineret i et hvilket som helst af kravene 1, 5, 7 eller 8.

DRAWINGS

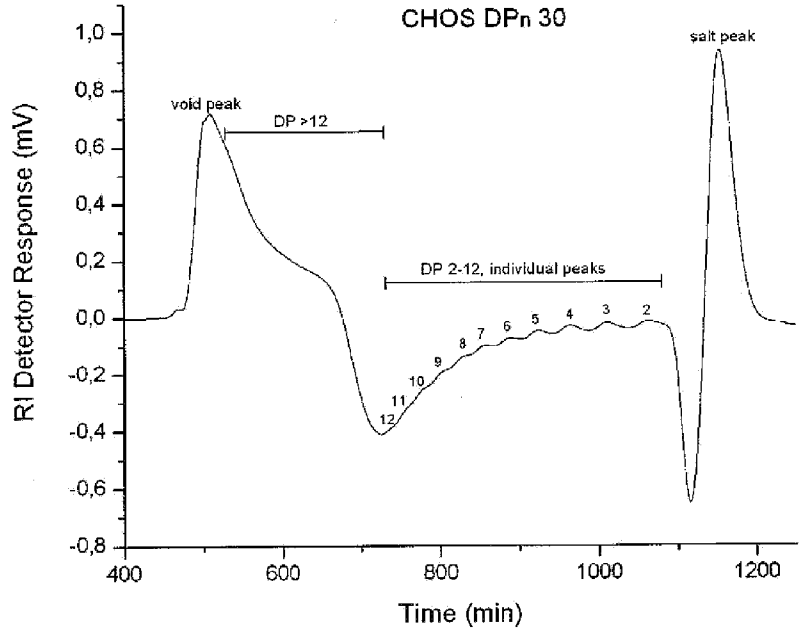

Figure 1

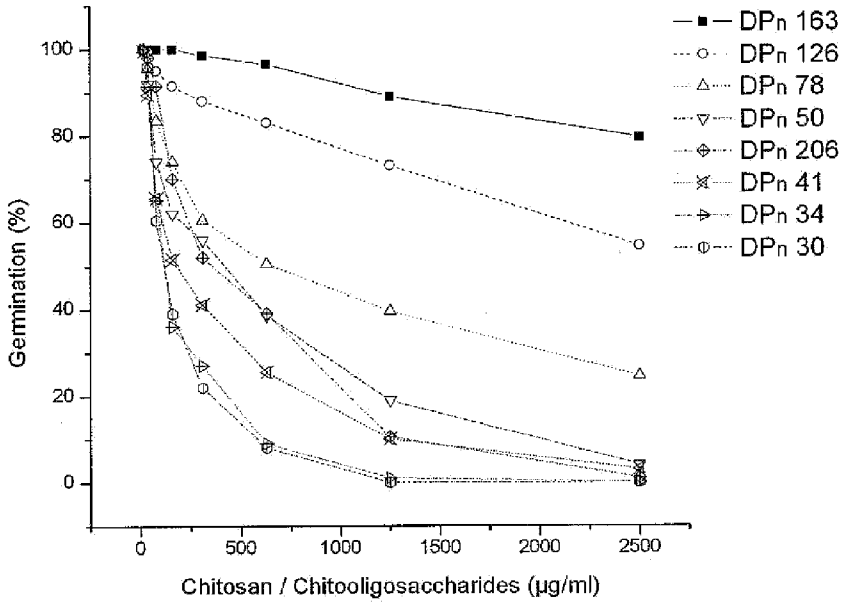

Figure 2

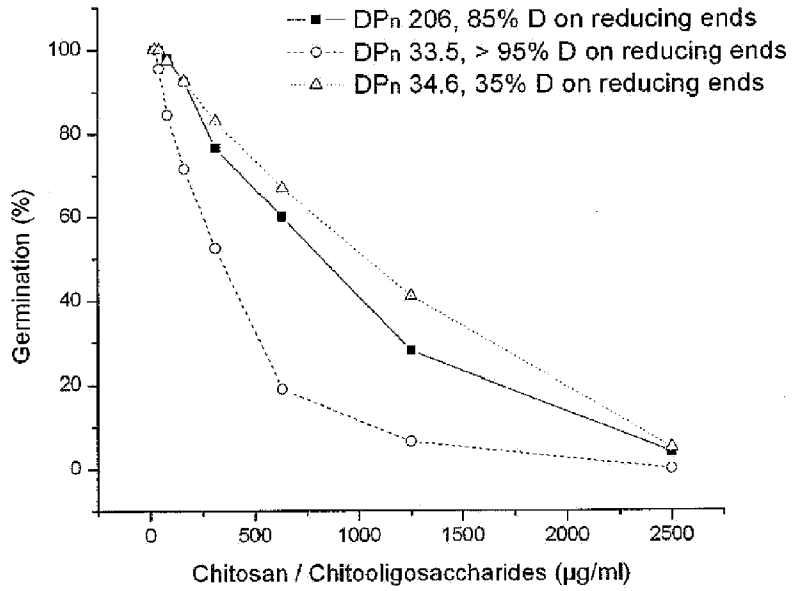

Figure 3

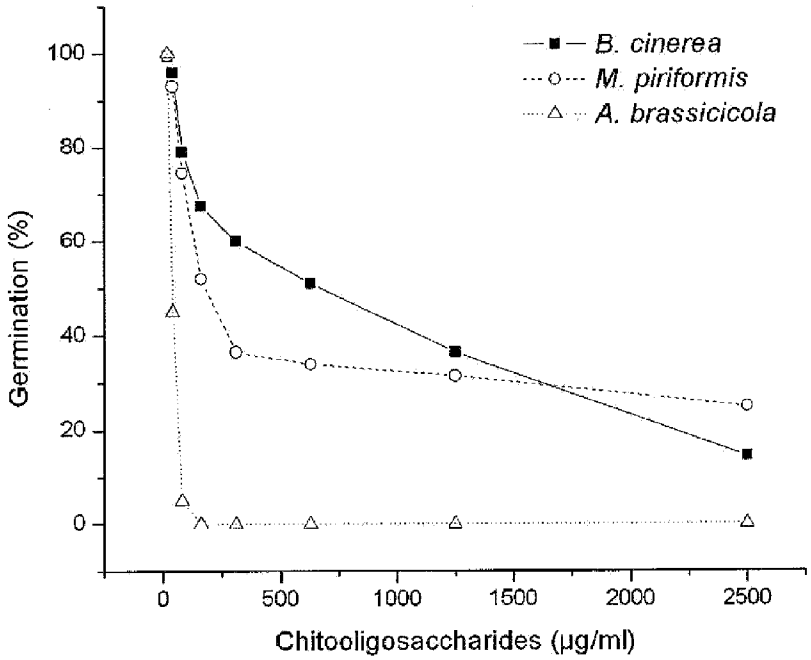

Figure 4

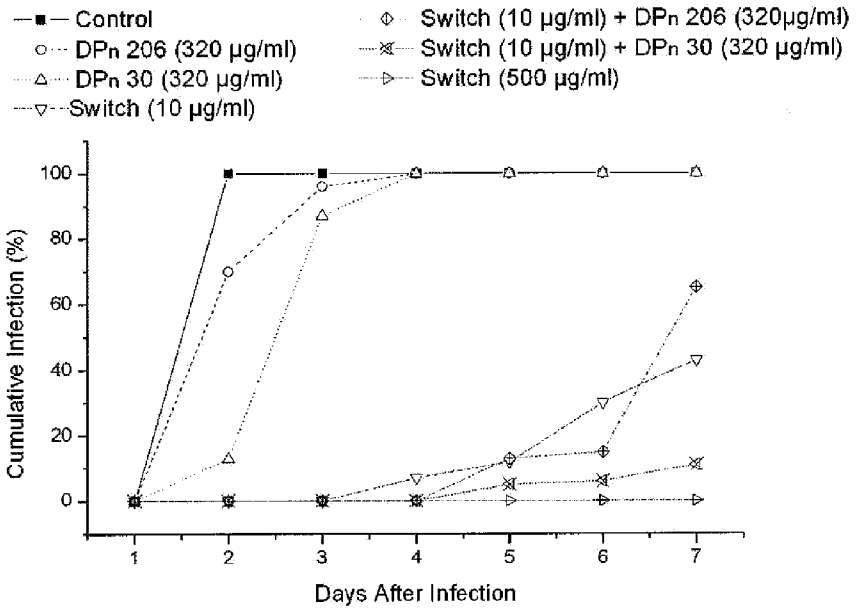

Figure 5

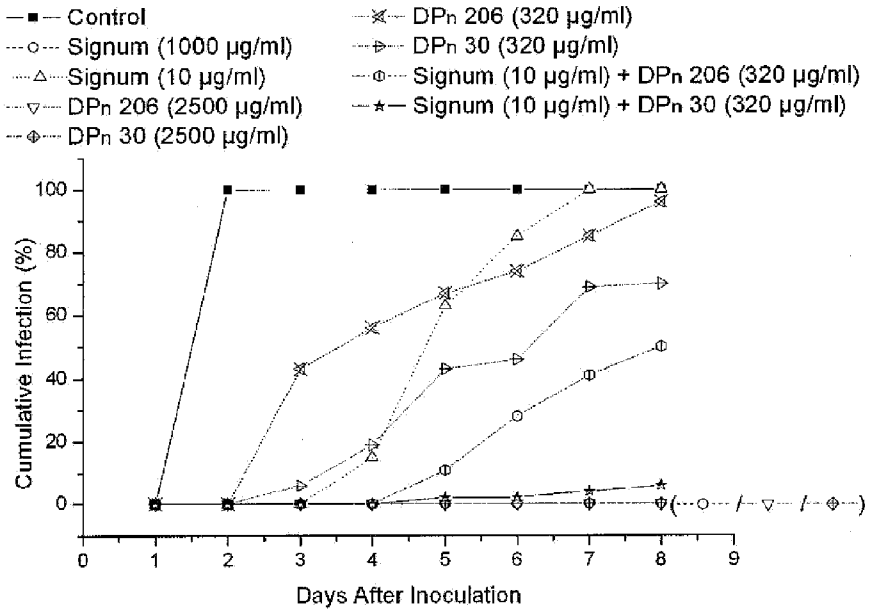

Figure 6

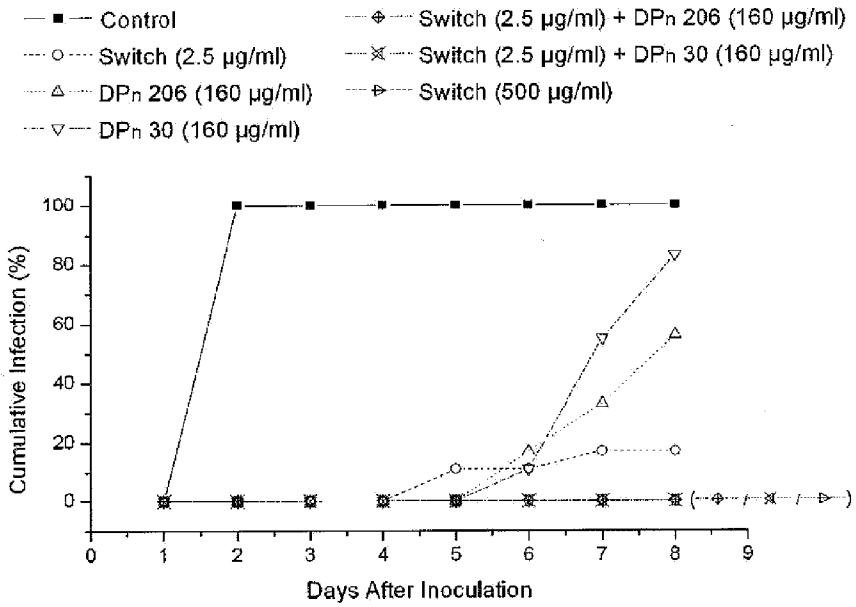

Figure 7

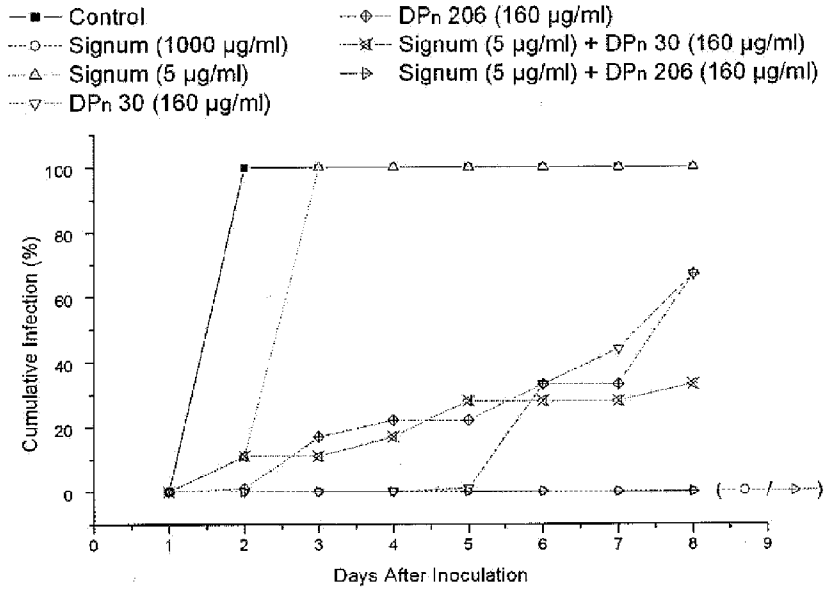

Figure 8