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USE OF CHELATING AGENT AND PEPTIDE ANTIMICROBIAL COMPOUNDS

Field of the Invention

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The invention relates to the field of antimicrobial agents that target plant pathogens.

Introduction

20 The control of plant pathogens and the protection of crops is a serious worldwide issue that is becoming an increasing concern with recent increases in the world's population and the associated food shortages. In addition, modern agriculture practices in relation to harvest and storage tend to provide good conditions for pathogen growth.

25 The use of chemical pesticides has been the standard approach to pest control. However, many currently used pesticides display several serious disadvantages. For example, many have an adverse impact on the environment and many have low or decreasing efficacy. Of particular concern are the low potency and/or narrow spectrum of activity of some compounds, together with the development of pathogen resistance.

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It is among the objectives of the present invention to attempt a solution to these problems, and specifically for example to provide a means to improve the activity of antimicrobial agents that are effective against plant pathogenic microorganisms.

Summary of the Invention

The inventors have surprisingly found that a chelating agent can synergistically potentiate the effectiveness of antimicrobial agents that are effective against a plant pathogenic
5 microorganism.

Accordingly, the inventors provide:

- (i) the use of a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism to inhibit the growth of and/or kill a plant pathogenic
10 microorganism on a plant; and
- (ii) the use of a chelating agent to increase the activity of an antimicrobial that is effective against a plant pathogenic microorganism.

In preferred embodiments said chelating agent and said antimicrobial are applied to a
15 plant in need thereof, preferably wherein said antimicrobial agent and said chelating agent are administered to said plant:

- a) as part of the same composition; or
- b) separately, either sequentially or simultaneously.

20 In preferred embodiments the antimicrobial agent is effective against a plant pathogenic bacterium or fungus. In preferred embodiments the antimicrobial agent comprises a polypeptide, preferably wherein said polypeptide comprises Blad or an active variant thereof. In preferred embodiments the chelating agent is a polyamino carboxylate, preferably EDTA.

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The inventors also provide:

- a method of inhibiting the growth of and/or killing a plant pathogenic microorganism comprising administering to a plant in need thereof a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism; and
- 30 - a method of increasing the activity of an antimicrobial that is effective against a plant pathogenic microorganism comprising using said antimicrobial with a chelating agent.

Further, the inventors provide a composition comprising a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism. Preferably the antimicrobial agent is effective against a plant pathogenic bacterium or fungus, preferably a fungus. In preferred embodiments the antimicrobial agent comprises a polypeptide, preferably wherein said polypeptide comprises Blad or an active variant thereof. In preferred embodiments the chelating agent is a polyamino carboxylate, preferably EDTA.

The inventors additionally provide the use of a composition of the invention to inhibit the growth of and/or kill a plant pathogenic microorganism on a plant, and a method of inhibiting the growth of and/or killing a plant pathogenic microorganism comprising administering to a plant in need thereof a composition of the invention.

The inventors have also surprisingly found that the Blad polypeptide from *Lupinus* shows potent antimicrobial activity against a number of diverse bacterial organisms that are pathogenic to plants.

Accordingly, the inventors provide the use of a composition comprising an antimicrobial polypeptide comprising Blad or an active variant thereof to kill, or inhibit the growth of, a plant pathogenic bacterium on a plant. Preferably said composition further comprises a chelating agent. In preferred embodiments, said bacterium is a pathogenic species from one of the following genera: *Pseudomonas*, *Erwinia* and *Streptomyces*. In preferred embodiments, the composition is applied to a plant in need thereof.

The inventors also provide a method of killing, or inhibiting the growth of, a plant pathogenic bacterium on a plant, said method comprising administering to said plant a composition comprising an effective amount of an antimicrobial polypeptide comprising Blad or an active variant thereof.

Brief Description of the Drawings

Figure 1 shows the *Lupinus albus* β -conglutin precursor encoding sequence (SEQ ID NO: 1); and

5 Figure 2 shows the internal fragment of the β -conglutin precursor encoding sequence that corresponds to Blad (SEQ ID NO: 3).

Detailed description of the Invention

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The inventors have found that, when using a combination of a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism, said combination is particularly effective at inhibiting the growth of and/or killing a plant pathogenic microorganism. In one aspect, therefore, the inventors provide a composition comprising, or consisting essentially of, a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism. A composition comprising a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism may also be a formulation comprising another compound(s) added to the composition by the skilled person.

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Antimicrobial agents

The antimicrobial agent is any agent that reduces the growth of or kills a microorganism that is pathogenic to a plant. Said microorganism is preferably a bacterium (Gram-positive or Gram-negative) or a fungus, preferably a fungus (which may be a yeast or a mold). Preferably, the antimicrobial agent comprises (or consists essentially of) a polypeptide, such as an antifungal protein. Suitable examples of antifungal proteins include chitinases, chitin-binding proteins, chitosanases, β -1,3-glucanases, and β -N-acetyl-D-glucosaminidases. Particular examples of microorganisms that may be targeted by the antimicrobial agent are given below.

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Blad polypeptide

In preferred embodiments, where the antimicrobial agent comprises (or consists essentially of) a polypeptide, said polypeptide comprises (or consists essentially of) Blad
5 or an active variant thereof.

Blad ("banda de *Lupinus albus* doce" - band from sweet *L. albus*) was the name given to a stable and intermediary breakdown product of β -conglutin, the major storage protein present in seeds of the *Lupinus* genus. It was characterised as a 20 kD polypeptide,
10 composed of 173 amino acid residues, and encoded by an internal fragment (519 nucleotides, deposited in GenBank under the accession number ABB13526) of the gene encoding the precursor of β -conglutin from *Lupinus* (1791 nucleotides, published in GenBank, under the accession number AAS97865). When primers encoding Blad terminal sequences are used to amplify a sequence from genomic *Lupinus* DNA, a ~620 bp
15 product is obtained, indicating the presence of an intron in the gene fragment encoding Blad. Naturally-occurring Blad is the main component of a 210 kD glycooligomer which accumulates exclusively (following intensive limited proteolysis of β -conglutin) in the cotyledons of *Lupinus* species, between days 4 and 12 after the onset of germination. Whilst said oligomer is glycosylated, naturally-occurring Blad is non-glycosylated. The
20 Blad-containing glycooligomer is composed of several polypeptides, the major ones exhibiting molecular masses of 14, 17, 20, 32, 36, 48 and 50 kD. The 20 kD polypeptide, Blad, is by far the most abundant polypeptide within the oligomer and appears to be the only one with lectin activity. Naturally-occurring Blad constitutes approximately 80% of the total cotyledonary protein in 8-day old plantlets.

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The *L. albus* β -conglutin precursor encoding sequence (SEQ ID NO: 1) is given in Figure 13. The β -conglutin parent subunit coding sequence is located at residues 70 to 1668. The encoded, 533 amino acid residue β -conglutin parent subunit (SEQ ID NO: 2) is:

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MGKMRVRFPTLVVLGIVFLMAVSIGIAYGEKDVLSHERPEEREQE EWQPRRQR
 PQSRREEREQE EQGSPSYPRRQSGYERRQYHERSEQREEREQEQQGSPSYRR
 QRNPYHFSSQRFQTLTKNRNGKIRVLERFDQRTNRLNLQNYRIVEFQSKPNTLI
 LPKHSADADYVLVVLNGRATITIVNPDRRQAYNLEYGDALRIPAGSTSYILNPDDN
 5 QKLRVVKLAIPINNPGYFYDFYPSSTKDQQSYFSGFSRNTLEATFNTRYEEIQRI
 ILGNEDEQEYEEQRRGQE QSDQDEGVIVIVSKKQIQKLTKHAQSSSGKDKPSDSG
 PFNLRSNAPIYSNKYGNFYEITPDRNPQVQDLNISLTYIKINEGALLLPHYNSKA
 IYVVVVDEGEGNYELVGIRDQQRQDEQEEKEEEVIRYSARLSEGDIFVIPAGYP
 ISINASSNLRLLLGFGINADENQRNFLAGSKDNVIRQLDRAVNELTFPGSAEDIER
 10 LIKNQQQSYFANGQPQQQQQQQSEKEGRRGRRGSSLPF

The internal fragment of the β -conglutin precursor encoding sequence that corresponds to Blad (SEQ ID NO: 3) is given in Figure 14. The Blad polypeptide (SEQ ID NO: 4) is:

15 RRQRNPYHFSSQRFQTLTKNRNGKIRVLERFDQRTNRLNLQNYRIVEFQSKPNT
 LILPKHSADADYVLVVLNGRATITIVNPDRRQAYNLEYGDALRIPAGSTSYILNP
 DNQKLRVVKLAIPINNPGYFYDFYPSSTKDQQSYFSGFSRNTLEATFNTRYEEIQ
 RIILGNED

20 Therefore, when the antimicrobial agent comprises (or consists essentially of) a
 polypeptide comprising (or consists essentially of) Blad or an active variant thereof, said
 agent comprises (or consists essentially of) a polypeptide sequence comprising (or
 consisting essentially of) of SEQ ID NO: 4 or an active variant thereof.

25 An active variant of Blad is a variant of Blad that retains the ability to act as an
 antimicrobial (i.e. has antimicrobial activity - see below for a description of the level of
 such activity and how to measure it). "An active variant of Blad" includes within its scope
 a fragment of SEQ ID NO: 4. In preferred embodiments, a fragment of SEQ ID NO: 4 is
 selected that is at least 10% of the length of SEQ NO: 4, preferably at least 20%,
 30 preferably at least 30%, preferably at least 40%, preferably at least 50%, preferably at
 least 60%, preferably at least 70%, preferably at least 80%, preferably at least 90% and
 most preferably at least 95% of the length of SEQ NO: 4. Blad or a variant thereof
 generally has a length of at least 10 amino acid residues, such as at least 20, 25, 30, 40,
 50, 60, 80, 100, 120, 140, 160 or 173 amino acid residues.

"An active variant of Blad" also includes within its scope a polypeptide sequence that has homology with SEQ ID NO: 4, such as at least 40% identity, preferably at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 85%, preferably at least 90%, preferably at least 95%, preferably at least 97%, and most preferably at least 99% identity, for example over the full sequence or over a region of at least 20, preferably at least 30, preferably at least 40, preferably at least 50, preferably at least 60, preferably at least 80, preferably at least 100, preferably at least 120, preferably at least 140, and most preferably at least 160 or more contiguous amino acid residues. Methods of measuring protein homology are well known in the art and it will be understood by those of skill in the art that in the present context, homology is calculated on the basis of amino acid identity (sometimes referred to as "hard homology").

The homologous active Blad variant typically differs from the polypeptide sequence of SEQ ID NO: 4 by substitution, insertion or deletion, for example from 1, 2, 3, 4, 5 to 8 or more substitutions, deletions or insertions. The substitutions are preferably 'conservative', that is to say that an amino acid may be substituted with a similar amino acid, whereby similar amino acids share one of the following groups: aromatic residues (F/H/W/Y), non-polar aliphatic residues (G/A/P/I/L/V), polar-uncharged aliphatics (C/S/T/M/N/Q) and polar-charged aliphatics (D/E/K/R). Preferred sub-groups comprise: G/A/P; I/L/V; C/S/T/M; N/Q; D/E; and K/R.

A polypeptide comprising Blad or an active variant thereof (as described above) may consist of Blad or an active variant thereof with any number of amino acid residues added to the N-terminus and/or the C-terminus provided that the polypeptide retains antimicrobial activity (again, see below for a description of the level of such activity and how to measure it). Preferably, no more than 300 amino acid residues are added to either or both ends of Blad or an active variant thereof, more preferably no more than 200 amino acid residues, preferably no more than 150 amino acid residues, preferably no more than 100 amino acid residues, preferably no more than 80, 60 or 40 amino acid residues, most preferably no more than 20 amino acid residues.

A polypeptide comprising (or consisting essentially of) Blad or an active variant thereof (as described above) may be utilised in the invention in the form of a purified (e.g. removed from a plant, animal or microbial source) or isolated form, and/or may be recombinant. Production of a recombinant form enables the production of active variants
5 of Blad.

Methods of purifying naturally-occurring Blad are already described in the art (e.g. Ramos *et al* (1997) *Planta* 203(1): 26-34 and Monteiro *et al* (2010) *PLoS ONE* 5(1): e8542). A suitable source of naturally-occurring Blad is a plant of the *Lupinus* genus,
10 such as *Lupinus albus*, preferably a cotyledon of said plant, preferably harvested between about 4 to about 14 days after the onset of germination, more preferably harvested 6 to 12 days after the onset of germination (such as 8 days after the onset of germination). Methods are disclosed in the art for a total protein extraction leading to a crude extract comprising Blad, and for a protein purification of such an extract leading to a partially
15 purified extract e.g. comprising the Blad-containing glycooligomer that comprises Blad.

To isolate Blad itself one can then use SDS-PAGE and/or, preferably, reverse phase (RP)-HPLC on a C-18 column.

20 An alternative way of obtaining a partially purified extract comprising the glycooligomer that comprises Blad is to utilise the chitin binding activity of Blad. The glycooligomer binds in a very strong manner to a chitin column as part of a chitin affinity chromatography purification, being eluted with 0.05 N HCl. Details of an example of this purification method are as follows:

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Cotyledons from eight-day old lupin plants were harvested and homogenized in Milli-Q plus water (pH adjusted to 8.0), containing 10 mM CaCl₂ and 10 mM MgCl₂. The homogenate was filtered through cheesecloth and centrifuged at 30,000 g for 1 h at 4 °C. The pellet was subsequently suspended in 100 mM Tris-HCl buffer, pH 7.5,
30 containing 10% (w/v) NaCl, 10 mM EDTA and 10 mM EGTA, agitated for 1 h at 4 °C, and centrifuged at 30,000 g for 1 h at 4 °C. The total globulin fraction, contained in the supernatant, was precipitated with ammonium sulphate (561 g/l), left

stirring in the cold for 1 h and centrifuged at 30,000 g for 30 min at 4 °C. The pellet obtained was dissolved in 50 mM Tris-HCl buffer, pH 7.5, desalted in PD-10 columns equilibrated in the same buffer and passed through a chitin-affinity chromatography column pre-equilibrated in the same buffer. The column was washed
5 with 50 mM Tris-HCl buffer, pH 7.5, and the bound proteins eluted with 0.05 N HCl. The eluted fractions were immediately neutralized with 2 M Tris and the peak fractions pooled, lyophilized and analyzed by SDS-PAGE.

For the preparation of the chitin column, crude chitin was obtained from Sigma and
10 processed as follows: the chitin sample was washed extensively with Milli-Q plus water, followed by 0.05 N HCl. It was then washed with 1% (w/v) sodium carbonate and then with ethanol, until the absorbance of the wash was less than 0.05. Chitin was then packed into a pipette tip and equilibrated with 50 mM Tris-HCl buffer, pH 7.5.

15 Methods of producing recombinant proteins are well known in the art. Such methods as applied here will involve inserting the polynucleotide encoding a polypeptide comprising Blad or an active variant thereof into a suitable expression vector - enabling the juxtaposition of said polynucleotide with one or more promoters (e.g. an inducible promoter, such as T7lac) and with other polynucleotides or genes of interest - introducing
20 the expression vector into a suitable cell or organism (e.g. *Escherichia coli*), expressing the polypeptide in the transformed cell or organism and removing the expressed recombinant polypeptide from that cell or organism. To assist such purification the expression vector may be constructed such that the polynucleotide additionally encodes, for example, a terminal tag that can assist purification: e.g., a tag of histidine residues for
25 affinity purification. Once the recombinant polypeptide is purified, the purification tag may be removed from the polypeptide, e.g., by proteolytic cleavage.

In a composition of the invention that comprises an antimicrobial agent that comprises (or consists essentially of) a polypeptide, said polypeptide is preferably in partially purified
30 form, more preferably in purified form. Said polypeptide is partially purified when it is present in an environment lacking one or more other polypeptides with which it is naturally associated and/or is represented by at least about 10% of the total protein

present. Said polypeptide is purified when it is present in an environment lacking all, or most, other polypeptides with which it is naturally associated. For example, purified Blad means that Blad represents at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the total protein in a composition.

In a composition of the invention that comprises an antimicrobial agent, the *Lupinus* protein content may consist essentially of the Blad-containing glycooligomer that comprises a polypeptide that comprises (or consist essentially of) Blad or an active variant thereof.

Plant pathogenic microorganisms

The plant pathogenic microorganism against which the antimicrobial agent is effective is any microorganism capable of causing disease on or in a plant. Particularly preferred bacterial targets include pathogenic *Pseudomonas* species, such as *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas tolaasii* and *Pseudomonas agarici* (preferably *P. syringae*); pathogenic *Erwinia* species, such as *Erwinia persicina*, *Pectobacterium carotovorum*, *Erwinia amylovora*, *Erwinia chrysanthemi*, *Erwinia psidii* and *Erwinia tracheiphila*, and pathogenic *Streptomyces* species such as *Streptomyces griseus*.

Particularly preferred fungal targets include pathogenic *Alternaria* species, such as *Alternaria alternata*, *Alternaria arborescens*, *Alternaria arbusti*, *Alternaria brassicae*, *Alternaria brassicicola*, *Alternaria carotiincultae*, *Alternaria conjuncta*, *Alternaria dauci*, *Alternaria euphorbiicola*, *Alternaria gaisen*, *Alternaria infectoria*, *Alternaria japonica*, *Alternaria petroselini*, *Alternaria selini*, *Alternaria solani* and *Alternaria smyrnii*, pathogenic *Fusarium* species, such as *Fusarium oxysporum* and *Fusarium graminearum* (preferably *F. oxysporum*); pathogenic *Botrytis* species, such as *Botrytis cinerea*; and pathogenic *Colletotrichum* species, such as *Colletotrichum actuatatum*, *Colletotrichum coccodes*, *Colletotrichum capsici*, *Colletotrichum crassipes*, *Colletotrichum gloeosporioides*, *Colletotrichum graminicola*, *Colletotrichum kahawae*,

Colletotrichum lindemuthianum, *Colletotrichum musae*, *Colletotrichum nigrum*,
Colletotrichum orbiculare, *Colletotrichum pisi* and *Colletotrichum sublineolum*.

Chelating agents

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The chelating agent (also known as a chelant, a chelator or a sequestering agent) is any compound that binds to a metal ion to form a non-covalent complex and reduce the ion's activity. Suitable chelating agents include polyamino carboxylates, such as EDTA (ethylenediaminetetraacetic acid) and EGTA (ethyleneglycol bis(β -aminoethyl ether)-
10 *N,N,N',N'*-tetraacetic acid). Preferably, EDTA is used as the chelating agent, preferably at a concentration of at least 10 μ g/ml, at least 50 μ g/ml, or at least 100 μ g/ml, and up to 500 μ g/ml, up to 1 mg/ml, up to 5 mg/ml, up to 10 mg/ml, or up to 20 mg/ml. Preferably, EDTA is used at a concentration of between 0.1 mg/ml and 20 mg/ml, more preferably between 1 mg/ml and 20 mg/ml.

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Outcomes

The antimicrobial agent, in combination with the chelating agent, may be used to inhibit the growth of a plant pathogenic microorganism (meaning that it has microbistatic
20 activity) and/or to kill said microorganism (meaning that it has microbicidal activity).

The skilled person will be able to identify, through routine methods, a suitable dose and/or concentration of the antimicrobial agent, at a particular concentration of the selected chelating agent, to obtain a particularly desired growth inhibition or killing of the microorganism. Preferably, the combination of the antimicrobial agent and chelating
25 agent is non-toxic to humans or animals.

Preferably, when a combination of the antimicrobial agent and chelating agent (e.g. a composition of the invention) is used as a microbistatic, the combination reduces the rate of growth by 10%, more preferably by 50%, more preferably by 75%, more preferably by
30 90%, more preferably by 95%, more preferably by 98%, more preferably by 99%, and even more preferably by 99.9% in comparison to equivalent conditions where the

combination is not present. Most preferably the combination prevents any growth of the microorganism.

Preferably, when a combination of the antimicrobial agent and chelating agent (e.g. a composition of the invention) is used as a microbicidal, the combination kills 10% of the population of the microorganism, more preferably 50% of said population, more preferably 75% of said population, more preferably 90% of said population, more preferably 95% of said population, more preferably 98% of said population, more preferably 99% of said population, and even more preferably by 99.9% of said population in comparison to equivalent conditions where the combination is not present. Most preferably the combination kills all of the population of the microorganism.

When used to prevent or inhibit infection of a plant by a microorganism said combination is preferably used in an effective amount, that is to say an amount that provides a level of growth inhibition and/or killing of a microorganism such that a detectable level of infection prevention or inhibition is achieved (e.g. a detectable level of prevention or inhibition of plant tissue damage is achieved), preferably in comparison to equivalent conditions where the combination is not present.

If the antimicrobial chosen comprises a polypeptide comprising Blad or an active variant thereof then, in combination with a chelating agent (e.g. EDTA at any of the concentrations described above), suitable concentrations with which to use said polypeptide include at least 5 µg/ml, at least 10 µg/ml, at least 20 µg/ml, at least 50 µg/ml, at least 100 µg/ml or at least 500 µg/ml, and up to 1 mg/ml, up to 2.5 mg/ml, up to 5 mg/ml or up to 10 mg/ml. Preferably the concentration of said polypeptide is between 50 µg/ml and 10 mg/ml, more preferably between 500 µg/ml and 5 mg/ml, and even more preferably between 1 mg/ml and 5 mg/ml (such as about 2.5 mg/ml).

For example, at 50 mM (i.e. about 15.8 mg/ml) EDTA a concentration of Blad of 1 mg/ml or 2.5 mg/ml can be used e.g. to inhibit the growth of *B. cinerea* or *F. oxysporum* respectively. This is a surprising finding given that Blad on its own needs to be used at about 5 mg/ml or 10 mg/ml (respectively) to inhibit the growth of these pathogens. The inventors' findings enable a) effective use of antimicrobial agents (that are effective

against plant pathogens) at lower concentrations through the use of a chelating agent or b) increased efficacy of antimicrobial agents (that are effective against plant pathogens) at standard concentrations through the use of a chelating agent. This enables more economical/effective use of such antimicrobials to prevent or treat infection of a plant (or
5 part thereof) by a microorganism.

Uses and methods

The inventors also provide the use of a composition of the invention to inhibit the growth
10 of and/or kill a plant pathogenic microorganism on a plant. To this end they also provide a method of inhibiting the growth of and/or killing a plant pathogenic microorganism comprising administering to a plant in need thereof a composition of the invention (e.g. an effective amount of said composition).

15 The inventors also provide the use of a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism to inhibit the growth of and/or kill a plant pathogenic microorganism on a plant. They also provide the use of a chelating agent to increase the activity of an antimicrobial that is effective against a plant pathogenic microorganism. To this end they also provide:

- 20 a) a method of inhibiting the growth of and/or killing a plant pathogenic microorganism comprising administering to a plant in need thereof a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism (e.g. an effective amount of a combination of said agents); and
- b) a method of increasing the activity of an antimicrobial that is effective against a
25 plant pathogenic microorganism comprising using said antimicrobial with a chelating agent.

In these embodiments said antimicrobial agent and said chelating agent may be administered to said plant as part of the same composition or separately. If these two agents are administered separately then the administration may be sequential (with either
30 agent being administered first) or simultaneous. Administration to a plant may be achieved for example by applying (e.g. spraying) the agents (or composition comprising

said agents) onto a plant (or any part thereof) or immersing the plant or part thereof (e.g. seed) in appropriate solution(s).

When a chelating agent is used to increase the activity of an antimicrobial that is effective
5 against a plant pathogenic microorganism said chelating agent (and concentration thereof) is preferably selected such that the combination of said antimicrobial agent and said chelating agent provides an increased level of growth inhibition and/or killing of a plant pathogenic microorganism (e.g. such that an increased level of plant infection prevention or inhibition is achieved e.g. an increased level of prevention or inhibition of plant tissue
10 damage is achieved), preferably in comparison to equivalent conditions where the chelating agent is not present.

In the use/method embodiments of the invention the antimicrobial agent and chelating agent are as described in detail above.

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The plant in need of a combination of an antimicrobial that is effective against a plant pathogenic microorganism and a chelating agent may be any plant that is at risk of acquiring an infection or that has an infection, wherein said infection is caused by a plant pathogenic microorganism. Preferably, the plant is a crop plant (e.g. any plant that is
20 grown to be harvested to provide food, livestock fodder, fuel, fibre, or any other commercially valuable product). Preferably, said crop plant is a food crop plant, such as a plant providing a sugar (e.g. sugar beet, sugar cane), a fruit (including a nut), a vegetable or a seed. Particular plants that provide seeds include cereals (e.g. maize, wheat, barley, sorghum, millet, rice, oats and rye) and legumes (e.g. beans, peas and lentils).

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The inventors also provide the use of a composition comprising (or consisting essentially of) an antimicrobial polypeptide comprising (or consisting essentially of) Blad or an active variant thereof to kill, or inhibit the growth of, a plant pathogenic bacterium on a plant. To this end the inventors further provide a method of killing, or inhibiting the
30 growth of, a plant pathogenic bacterium on a plant, said method comprising administering to said plant a composition comprising (or consisting essentially of) an effective amount of an antimicrobial polypeptide comprising (or consisting essentially of) Blad or an active

variant thereof. Optionally the antimicrobial polypeptide comprising (or consisting essentially of) Blad or an active variant thereof may be used in isolated form.

In preferred embodiments, said composition is applied to a plant in need thereof. The
5 plant in need of said composition may be any plant that is at risk of acquiring an infection
or that has an infection, wherein said infection is caused by a plant pathogenic bacterium.
Preferred plants are as described above. The meanings of 'killing/inhibiting growth of a
bacterium', and 'effective amount', are as described above in relation to the combination of
antimicrobial agent and chelating agent.

Examples

In the following Examples **BLAD** denotes the naturally-occurring Blad-containing glycooligomer comprising the 20 kD Blad polypeptide, purified as per Ramos *et al* (1997)

5 Planta 203(1): 26-34: see "Plant material and growth conditions" and "Purification of proteins" parts of the Materials and Methods section of that document.

Definitions:

10 **MIC** - Minimum Inhibitory Concentration: the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism.

MFC/MBC - Minimum Fungicidal/Bactericidal Concentration (or Minimal Lethal Concentration): the lowest concentration of an antimicrobial agent needed to kill 99.9% of the initial inoculum after 24 h under a standardized set of conditions.

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Example 1 - Bactericidal activity of BLAD and synergistic effect of EDTA thereon.

A. BLAD was found to be bacteriostatic at 100 µg/ml and bactericidal at 250 µg/ml against *P. aeruginosa*. Against *P. aeruginosa* BLAD at 50 µg/ml or EDTA at 1 mg/ml
20 inhibits growth (i.e. both are bacteriostatic) but a combination of the two is bactericidal.

B. Against *Erwinia pirsicina* BLAD has an MIC of 32 µg/ml and EDTA has an MIC of 15 mM. However, in the presence of sub-inhibitory amounts of EDTA (0.75 mM) the MIC for BLAD is lowered to 16 µg/ml.

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C. Against *Streptomyces griseus* BLAD has an MIC of 1024 µg/ml and EDTA has an MIC of 16 mM. However, in the presence of sub-inhibitory amounts of EDTA (8 mM) the MIC for BLAD is lowered to 256 µg/ml.

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Example 2 - Synergistic effect of EDTA on fungicidal activity of BLAD.

Inhibition halo data for BLAD with and without EDTA against *Botrytis cinerea* on Potato Dextrose Agar (PDA) at 1.2% w/v of agar (incubation 3 days at 25 °C):

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Agent(s)	Inhibition halo diameter (mm)			Average inhibition halo diameter (mm)
Blad (200 µg)	21	21	22	21
Blad (100 µg)	16	16	15	16
Blad (50 µg)	0	0	0	0
Blad (20 µg)	0	0	0	0
EDTA (50 mM)	0	0	0	0
Blad (200 µg) + EDTA (50 mM)	23	24	25	24
Blad (100 µg) + EDTA (50 mM)	19	19	18	19
Blad (50 µg) + EDTA (50 mM)	13	15	14	14
Blad (20 µg) + EDTA (50 mM)	10	12	12	11

Growth of *B. cinera* was inhibited with 200 µg and 100 µg of BLAD. This inhibition was potentiated with the addition of 50 mM (approximately 15.8 mg/ml) EDTA.

- 10 No growth inhibition was observed using BLAD alone at 20 µg or 50 µg or using EDTA alone at 50 mM. However, inhibition was observed when BLAD at either concentration was combined with 50 mM EDTA.

Inhibition halo data for BLAD with and without EDTA against *Fusarium oxysporum* on Potato Dextrose Agar (PDA) at 1.2% w/v of agar (incubation 3 days at 25 °C):

Agent(s)	Inhibition halo diameter (mm)			Average inhibition halo diameter (mm)
Blad (200 µg)	25	22	22	23
Blad (100 µg)	0	0	0	0
Blad (50 µg)	0	0	0	0
Blad (20 µg)	0	0	0	0
EDTA (50 mM)	0	0	0	0
Blad (200 µg) + EDTA (50 mM)	28	27	30	28
Blad (100 µg) + EDTA (50 mM)	22	21	26	23
Blad (50 µg) + EDTA (50 mM)	24	20	15	20
Blad (10 µg) + EDTA (50 mM)	0	0	0	0

- 5 Growth of *F. oxysprum* was inhibited with 200 µg of BLAD. This inhibition was potentiated with the addition of 50 mM EDTA.

No growth inhibition was observed using BLAD alone at 20 µg, 50 µg or 100 µg, or using EDTA alone at 50 mM. However, inhibition was observed when 50 µg or 100 µg of

- 10 BLAD was combined with 50 mM EDTA.

We claim:

1. Use of a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism to inhibit the growth of and/or kill a plant pathogenic microorganism on a plant, wherein the antimicrobial agent is an antimicrobial polypeptide comprising the Blad sequence shown in SEQ ID NO:4 or an active variant thereof which has antimicrobial activity and which comprises a sequence which has at least 70% identity to either SEQ ID NO:4 or a fragment of SEQ ID NO:4 which is at least 60 amino acids in length.
2. Use of a chelating agent to increase the activity of an antimicrobial agent that is effective against a plant pathogenic microorganism, wherein the antimicrobial agent is as defined in claim 1.
3. A use according to claim 1 or claim 2 wherein said chelating agent and said antimicrobial agent are applied to a plant in need thereof.
4. A use according to claim 3 wherein said antimicrobial agent and said chelating agent are administered to said plant:
 - a) as part of the same composition; or
 - b) separately, either sequentially or simultaneously.
5. A use according to any one of the preceding claims wherein the antimicrobial agent is effective against a plant pathogenic bacterium or fungus.
6. A use according to any one of the preceding claims wherein the chelating agent is a polyamino carboxylate.
7. A use according to claim 6, wherein the polyamino carboxylate is EDTA.
8. A method of inhibiting the growth of and/or killing a plant pathogenic microorganism comprising administering to a plant in need thereof a chelating agent and an antimicrobial agent

that is effective against a plant pathogenic microorganism, wherein the antimicrobial agent is as defined in claim 1.

9. A method of increasing the activity of an antimicrobial agent that is effective against a plant pathogenic microorganism comprising using said antimicrobial with a chelating agent, wherein the antimicrobial agent is as defined in claim 1.
10. A composition comprising a chelating agent and an antimicrobial agent that is effective against a plant pathogenic microorganism, wherein the antimicrobial agent is as defined in claim 1.
11. A composition according to claim 10 wherein the antimicrobial agent is effective against a plant pathogenic bacterium or fungus, preferably a fungus.
12. A composition according to claim 10 or claim 11 wherein the chelating agent is a polyamino carboxylate.
13. A composition according to claim 12, wherein the polyamino carboxylate is EDTA.
14. Use of a composition according to any one of claims 10 to 13 to inhibit the growth of and/or kill a plant pathogenic microorganism on a plant.
15. A method of inhibiting the growth of and/or killing a plant pathogenic microorganism comprising administering to a plant in need thereof a composition according to any one of claims 10 to 13.
16. Use of a composition comprising an antimicrobial agent as defined in claim 1 to kill, or inhibit the growth of, a plant pathogenic bacterium on a plant.
17. A use according to claim 16 wherein said composition further comprises a chelating agent.

18. A use according to claim 16 or claim 17 wherein the bacterium is a pathogenic species from one of the following genera: *Pseudomonas*, *Erwinia* and *Streptomyces*.
19. A use according to any one of claims 16 to 18 wherein the composition is applied to a plant in need thereof.
20. A method of killing, or inhibiting the growth of, a plant pathogenic bacterium on a plant, said method comprising administering to said plant a composition comprising an effective amount of an antimicrobial agent as defined in claim 1.
21. A use according to claim 1 or 16, wherein the plant is sugar beet, sugar cane, maize, barley, wheat, sorghum, millet, rice, oats, rye, beans, peas or lentils.
22. A use according to any one of claims 1, 2, 14 or 16, substantially as herein described with reference to any example thereof.
23. A method according to any one of claims 8, 9, 15 or 20, substantially as herein described with reference to any example thereof.
24. A composition according to claim 10, substantially as herein described with reference to any example thereof.