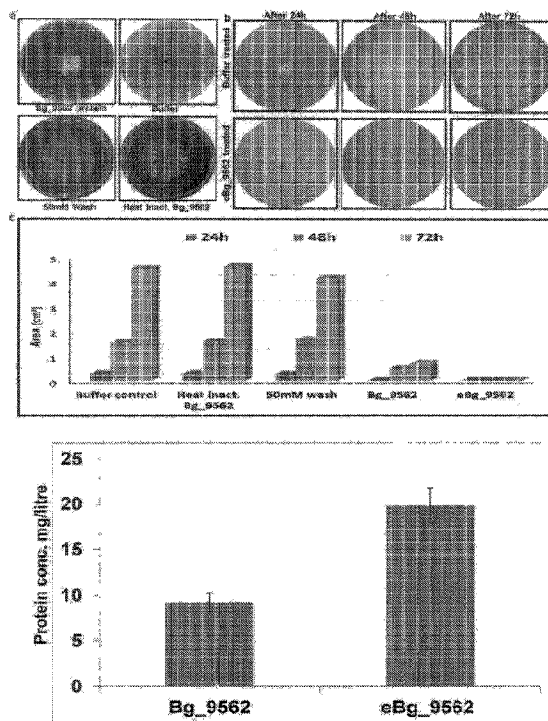




(86) Date de dépôt PCT/PCT Filing Date: 2017/07/19
(87) Date publication PCT/PCT Publication Date: 2018/01/25
(45) Date de délivrance/Issue Date: 2023/01/17
(85) Entrée phase nationale/National Entry: 2019/01/11
(86) N° demande PCT/PCT Application No.: IB 2017/054354
(87) N° publication PCT/PCT Publication No.: 2018/015895
(30) Priorité/Priority: 2016/07/19 (IN201611024726)

(51) Cl.Int./Int.Cl. *C07K 14/195* (2006.01)
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(54) Titre : PROTEINE MUTÉE DE LA LIGNEE DE BURKHOLDERIA GLADIOLI NGJ1 CONTRE LES PATHOGENES FONGIQUES
(54) Title: MUTATED PROTEIN FROM BURKHOLDERIA GLADIOLI STRAIN NGJ1 AGAINST FUNGAL PATHOGENS



(57) Abrégé/Abstract:

The present invention relates to a novel protein comprising novel genes that is extracted from Burkholderia gladioli strain NGJ1. A nucleotide sequence encoding the novel protein is represented by sequence SEQ ID No. 1 and amino acid sequence of the novel protein is represented by the sequence SEQ ID No. 2 is further provided. A nucleotide sequence and the amino acid sequence are obtained from genetically engineered Bg_9562 gene. The novel protein as well as encoding gene is adapted for broad spectrum anti-fungal and mycophagous activities.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau

(43) International Publication Date
25 January 2018 (25.01.2018)



(10) International Publication Number
WO 2018/015895 A1

- (51) International Patent Classification:
C07K 14/195 (2006.01) A01N 63/02 (2006.01)
- (21) International Application Number:
PCT/IB2017/054354
- (22) International Filing Date:
19 July 2017 (19.07.2017)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
201611024726 19 July 2016 (19.07.2016) IN
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- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).
- Declarations under Rule 4.17:**
- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
 - as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

(54) Title: NOVEL PROTEIN AGAINST FUNGAL PATHOGENS

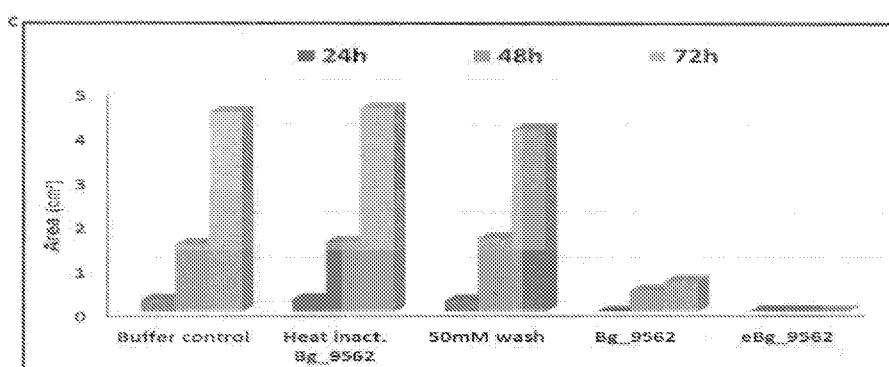


FIG 3.

(57) Abstract: The present invention relates to a novel protein comprising novel genes that is extracted from *Burkholderia gladioli* strain NGJ1. A nucleotide sequence encoding the novel protein is represented by sequence SEQ ID No. 1 and amino acid sequence of the novel protein is represented by the sequence SEQ ID No. 2 is further provided. A nucleotide sequence and the amino acid sequence are obtained from genetically engineered Bg_9562 gene. The novel protein as well as encoding gene is adapted for broad spectrum anti-fungal and mycophagous activities.

[Continued on next page]



WO 2018/015895 A1

WO 2018/015895 A1

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

MUTATED PROTEIN FROM *BURKHOLDERIA GLADIOLI* STRAIN NGJ1 AGAINST FUNGAL PATHOGENS

FIELD OF INVENTION

5 This invention relates to a novel protein against fungal pathogens. More specifically, the present invention relates to the novel protein from *Burkholderia gladioli* strain NGJ1 against fungal pathogens for controlling wide variety of fungal diseases.

BACKGROUND OF THE INVENTION

10 Bacteria are omnipresent that are found in soil, water, air etc. and also within multicellular organisms that include insects, plants and animals. They have very active cell to cell communication system and sometimes can behave as multicellular organisms by forming biofilms. For survival, they need to compete with other co-habiting bacteria, fungi etc. and have to live within or in association with plants, animals etc (Frey-Klett et al, 2011; Reinhold-Hurek and Hurek, 2011). The interaction of
15 bacteria with other organisms can be mutualistic, commensalistic, antagonistic or parasitic (Haas and Défago, 2005; Frey-Klett et al, 2011). Conventionally, they directly promote plant growth by synthesizing phytohormones etc. or indirectly help plants by protecting them from potential pathogenic microorganisms (Haas and Défago, 2005; Lugtenberg and Kamilova, 2009; Beneduzi et al, 2012).

Bacteria demonstrating antagonistic interactions are being considered as potential biocontrol
20 agents. The mechanism of biocontrol is generally attributed to production of antifungal metabolites (bacillaene, difficidin, fengycin, macrolactin, surfactin etc.), chitinolytic enzymes, siderophores, toxins etc. by the bacteria (Huang et al, 2005; Tortora et al, 2011; Raaijmakers and Mazzola, 2012). Interestingly, among anti-fungal bacteria, there are few species that can grow and multiply at the cost of growing fungal biomass. Such interaction is called bacterial mycophagy (Leveau and Preston, 2008).
25 One of the first direct evidence of bacterial mycophagy has been reported for *Collimonas* which without any added nutrients could grow in the autoclaved soil, in the presence of actively growing mycelium of three common dune soil invading fungi i.e. *Chaetomium globosum*, *Fusarium culmorum*, and *Mucorhiemalis* (Höppener-Ogawa et al, 2009).

Burkholderia spp. is rod-shaped Gram-negative bacteria that are found in diverse habitats. Few
30 among them have been reported to be pathogenic to plants and

humans; however, a few having potential activities towards plant growth-promoting, endophytic, and antifungal strains have also been reported. Recently, gene sequencing of bacterium *Burkholderia gladioli* strain NGJ1 from the rice seedlings has been carried out (Jha et al, 2015). *Rhizoctonia solani* is one of the important soil borne fungal pathogen which is causal agent of several economically important diseases in various crop plants, including rice (Zheng et al, 2013; Ghosh et al, 2014; Hane et al, 2014). It is considered to be a grave yard of rice cultivation. However, it is noteworthy that several bacterial species including *Burkholderia*, *Actinomycetes*, *Bacillus*, *Psuedomonas* etc. are known to be anti-fungal against *R. solani* (Andersen et al, 2003; Elshafie et al, 2012; Huang et al, 2012; Mela et al, 2012).

These biocontrol agents are potentially useful in providing environmental and eco-friendly alternatives of chemical fungicides for controlling various plant diseases; however there are yet limitations to utilize them directly. Some of biocontrol bacteria produce lytic compounds as extracellular lytic enzymes, siderophores, salicylic acid, antibiotics, and volatile metabolites, such as hydrogen cyanide (Nagarajkumar et al, 2004; Manwar et al, 2005; Compant et al, 2005; Kishore et al, 2005; Sharifi et al, 2005; Afsharmanesh et al, 2006) as antifungal agents. Apart from this, many novel antifungal proteins such as Baciamin, produced by *Bacillus amyloliquefaciens* (Wong et al, 2008), B29I, produced by *B. subtilis* strain B29 (Li et al, 2009), F2 protein, produced by *B. licheniformis* (Tang-Bing et al, 2012), PPEBL21 protein, produced by *Escherichia coli* BL21 (Yadav et al, 2012) and a hypothetical protein (gi154685475) produced by *B. subtilis* B25 (Tan et al, 2013) demonstrated inhibitory effect against various plant pathogens.

Korean published application KR 200641054 relates to a method for inhibiting anthracnose disease by using a culture solution of a strain of *Bacoliella gladiolis* NIAB 131-133 having antimicrobial activity and a method for inhibiting anthracnose causing fungi (*Colletotrichum gloeosporioides*) using culture solution of the bacterial strain *Bacillaria gladioli* NIAB 131-133. The bacterium strain works against the fungal strains but are not specific for rice plants and related diseases.

Japanese published application JP2010047532 provides a novel means using microorganisms of the genus *Burkholderia* as plant disease control method characterized by spraying a microorganism belonging to the genus *Burkholderia* and exhibiting an antibacterial action against plant pathogens on the foliage of a plant, and a plant disease control method for controlling the microorganisms of genus *Burkholderia*.

However, in spite of extensive global effort, till date no source of complete disease resistance has been identified for sheath blight disease of rice caused by *Rhizoctonia solani*. Further, no proper control measures are developed for *Rhizoctonia solani*, which can also be useful for controlling several other diseases caused by *R.*
 5 *solani* on various important crop plants. Moreover, besides *R. solani*, plants are susceptible to several other fungal pathogens. To control them in an environmental friendly manner still remains a challenge. Hence developing strategy for broad spectrum antifungal compounds would be helpful to control fungal diseases of diverse plants/crops.

10 To overcome the above mentioned problems, the present invention provides a genetically engineered gene sequence of *Burkholderia gladioli* strain NGJ1. The bacterial overexpressed and purified protein showed antifungal activity against *R. solani*. Furthermore, the purified protein exhibits broad-spectrum antifungal activity against several agriculturally important pathogens including *Magnaporthe*
 15 *oryzae*, *Venturia inaequalis*, *Alternaria brassicae*, *Fusarium oxysporum*, *Dedymella* *sp.*, *Phytophthora* *sp.*, *Colletotrichum* *sp.*, *Ascochyta rabiei*, *Neofusicoccum* *sp.*, *Alternaria* *sp.*, *Saccharomyces cerevisiae* and *Candida albicans*.

SUMMARY OF INVENTION

In one embodiment of the invention, a novel protein comprising novel genes
 20 that is extracted from *Burkholderia gladioli* strain NGJ1 is provided. A nucleotide sequence encoding the novel protein is represented by sequence SEQ ID No. 1 and amino acid sequence of the novel protein is represented by the sequence SEQ ID No. 2.

In another embodiment, the nucleotide sequence and the amino acid sequence are obtained from genetically engineered Bg_9562 gene. In an embodiment, the
 25 nucleotide sequence encoding the novel protein is at least 70% identical to the nucleotide sequence of SEQ ID No. 1 or the amino acid sequence represented by SEQ ID No. 2. In another embodiment, the nucleotide sequence of SEQ ID NO: 1 have at least 1 to 90 nucleotide acid substitutions, deletions, and/or insertions. In another embodiment, the amino acid sequence of SEQ ID NO: 2 have at least 1 to 30 amino acid
 30 substitutions, deletions, and/or insertions. In another embodiment, the amino acid sequence of SEQ ID NO: 2 varies by 11 amino acid substitutions.

In one embodiment, the novel protein is adapted for broad spectrum anti-fungal activities and produces high mass production of proteins. In another embodiment, the

amino acid sequence of the novel protein is of 111 amino acid residues long with molecular weight of ~13 kDa, a pI of about 4.65 and a pH optimum at about 7.4.

In another embodiment, the protein is adapted with anti-fungal and mycophagous activity that inhibits growth of fungal sclerotia and induces cell death in fungal mycelia. In another embodiment, the antifungal activity of Bg_9562 gene is useful for controlling sheath blight diseases of rice and other crops caused by *Rhizoctonia solani*.

In another embodiment, a method to prepare novel protein by SEQ ID No. 2 with antifungal activity is provided. The method comprises the steps of: (i) identification of the novel protein possessing anti-fungal and mycophagous activity from *Burkholderia gladioli* strain NGJ1, (ii) gene sequence and protein sequence is evolved by artificially synthesizing the identified protein of step (i) and incorporating the desired gene change in the identified protein to synthesize gene through gene synthesis, (iii) the evolved protein is over expressed in pET28a expression vector, (iii) the overexpressed protein is purified by Ni-NTA affinity chromatography, (iv) the purified protein is assessed for antifungal activity and (v) antifungal nature of the protein is established by reverse genetics approach. The recombinant expression of the novel protein prevents the growth of fungal sclerotia, induces cell death in fungal mycelia and treats some human/animal diseases.

In another embodiment, a composition from novel peptide is provided. The components of the composition comprises of oil that are selected from the group comprising of almond oil, rapeseed oil and sesame oil; thickeners that are selected from beeswax, cocoa butter and shea butter, emulsifiers selected from the group comprising of alcohols such as cetyl alcohol; and water.

In another embodiment, the composition encoding novel peptide is adapted to develop broad spectrum fungal disease resistance wherein the fungus is selected from the group comprising of *Rhizoctonia solani*, *Alternaria brassicae*, *Magnaporthe oryzae*, *Venturia inaequalis*, *Fusarium oxysporum*, *Dedymella sp.*, *Phytophthora sp.*, *Colletotrichum sp.*, *Ascochyta rabiei*, *Neofusicoccum sp.*, *Alternaria sp.*, *Saccharomyces cerevisiae* and *Candida albicans*

In another embodiment, a process to prepare the composition is provided. The process comprises the steps of: (i) Bg_9562 protein is produced in bulk quantities by fermentation using bioreactor, (ii) the fermented product obtained is encapsulated by chitosan or non-chitosan based nanoparticles; and (iii) the encapsulated

products of step (ii) is used to develop a water base or an oil base cream/ointment and, (iv) the product of step (iii) are further developed to prepare antifungal films comprising starch/ oils.

In another embodiment, the method to control fungal disease of plants or crops is provided by applying a polypeptide comprising the sequence of SEQ ID NO: 1 or 2, or a composition, to an infected plant, infected plant part, or infected crop, humans and animals that are at risk of fungal infection.

In another embodiment, the protein is produced by expressing its encoding nucleotide in the cells of bacteria, yeast, insects, plants, humans or animals using recombinant DNA technology.

In one aspect, the present invention provides a protein with a broad-spectrum antifungal activity, comprising an amino acid sequence of SEQ ID No. 2 wherein said amino acid sequence is encoded by the nucleotide sequence of SEQ ID No. 1.

In another aspect, the present invention provides a method to prepare a protein having antifungal activity and comprising an amino acid sequence of SEQ ID No. 2, wherein the method comprises the steps of:

- (i) identifying the protein that possesses antifungal activity and mycophagous activity from *Burkholderia gladioli* strain NGJ1,
- (ii) analyzing the amino acid sequence of the identified protein in step (i) to find critical residues associated with antifungal activity as well as mass production, and artificially designing a gene sequence that is SEQ. ID No. 1 by incorporating mutations at 11 positions and synthesizing the designed gene through gene synthesis,
- (iii) over expressing the protein with desired changes of step (ii) in an expression vector to get recombinant protein,
- (iv) purifying the over expressed protein of step (iii) by chromatography,
- (v) assessing the antifungal activity of the purified protein of step (iv), and
- (vi) establishing the role of antifungal protein by a reverse genetics approach to produce the protein from Bg_9562.

In another aspect, the present invention provides a composition for use as an antifungal comprising the protein of the invention, an oil selected from the group consisting of almond oil, rapeseed oil and sesame oil; thickeners selected from the group consisting of beeswax, cocoa butter and shea butter; emulsifiers selected from the group consisting of alcohols; and water.

In another aspect, the present invention provides a process for preparing an antifungal film, wherein the process comprises steps:

- (i) producing a protein comprising an amino acid sequence of SEQ ID No. 2 in bulk quantities

by fermentation in a bioreactor;

(ii) encapsulating the fermented product obtained from step (i) in chitosan or non-chitosan based nanoparticles;

(iii) using the encapsulated product obtained from step (ii) to develop water base or oil base cream/ointment; and

(iv) converting the product obtained from step (iii) into the antifungal film, wherein the antifungal film is made from starch/oils.

In another aspect, the present invention provides a method for controlling fungal disease of plants or crops, comprising: applying a polypeptide comprising the sequence of SEQ ID NO: 2, or the composition for use as an antifungal, to an infected plant, infected plant part, or infected crop.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Mycophagous and antifungal behavior of *Burkholderia gladioli* strain NGJ1 on *Rhizoctonia solani*. The *Burkholderia gladioli* strain NGJ1 inhibited fungal growth (formation of inhibition zone) during 3 dpi (days post inoculation) of co-cultivation. Subsequently at 8 dpi (days post inoculation), the bacterium is found growing over the entire fungal mycelia. Normal growth of the *R. solani* and NGJ1 (individually) was observed on PDA (Potato Dextrose Agar) plates. On CDA (Czapek Dox Agar) plates, *R. solani* grew (albeit slow) to cover the entire plate, but NGJ1 strain demonstrated very slow growth. However, upon confrontation with fungal mycelia, mycophagous behavior (bacterial spreading over fungi and degrading fungal mycelium) was very prolific on CDA plates.

Figure 2. Cloning and protein purification. (a) Restriction digestion conformation of Bg_9562 gene cloned in pET28a expression vector. (b) Purification of overexpressed protein by Ni²⁺-NTA-Agarose chromatography (L1- Marker, L2 – flow through, L3 – 50mM wash, L4 & L5- 100mM imidazole purification, L6 & L7- 200mM purification). (c) Western blotting of Bg_9562 protein with anti-His-antibody.

Figure 3. Effect of Bg_9562 protein on sclerotial growth pattern. (a) *Rhizoctonia solani* sclerotia were treated with 15µg/ml of Bg_9562 protein as well as different controls (Buffer control, 50mM wash and Heat inactivated protein). The representative pictures of fungal growth on PDA plates at 48h post treatment are depicted. (b) Fungal growth inhibition upon eBg_9562 protein treatment. 15 µg/ml of modified protein was

efficient in preventing growth of *R. solani* on PDA plates while buffer treated sclerotia showed proper growth. The representative pictures of fungal growth at different time intervals are depicted. (c) Area of mycelial growth of *R. solani* at different time intervals after treatment of sclerotia with 15µg/ml of Bg_9562 protein and eBg_9562 protein. The buffer, heat inactivated Bg_9562 and 50 mM wash were used as control (eBg_9562 protein is the evolved Bg_9562 protein). (d) protein estimation through Bradford suggesting enhanced production of eBg_9562 protein compared to wild Bg_9562.

Figure 4. MTT assay revealed Bg_9562 protein to induce cell death responses in fungi. (a) MTT assay of *R. solani* sclerotia after treatment with either 15µg/ml of Bg_9562 protein or PBS buffer. The presence of brown pigment in the control suggested the live cells while the lack of color formation in protein treated samples suggested cell death in fungi.

Figure 5. Generating mutant *B. gladioli* defective in production of Bg_9562 protein. (a) The partial gene fragment of Bg_9562 gene was cloned in pK18 mob vector, picture depicts release of insert upon restriction digestion (b) the pK18 mob-9562 plasmid were mobilized into the NGJ1 genome and the recombinants were selected on antibiotic plates. The PCR amplicon obtained through colony PCR using gene specific.

Figure 6. Antifungal activity of Bg_9562 mutant and complementing *B. gladioli* strains. (a) Two independent mutants (NGJ100, NGJ101) of Bg_9562 were found defective in mycophagous activity, as they failed to prevent fungal growth. Notably treatment with wild type NGJ1 could prevent the growth of fungal sclerotia. (b) The complements NGJ102 and NGJ103 (expressing full length copy of the Bg_9562 gene on a broad host range plasmid, pHM1) were proficient to the level of wild type NGJ1 in demonstrating antibacterial/mycophagous activity of *R. solani*. The pictures depict the sclerotial growth after 7 days of different treatments.

DESCRIPTION OF THE INVENTION

While the invention is susceptible to various modifications and/or alternative processes and/or compositions, specific embodiment thereof has been shown by way of

examples and tables and will be described in detail below. It should be understood, however that it is not intended to limit the invention to the particular processes and/or compositions disclosed, but on the contrary, the invention is to cover all modifications, equivalents, and alternative falling within the spirit and the scope of the invention as
5 defined by the appended claims.

The examples, tables, and protocols have been represented where appropriate, showing only those specific details that are pertinent to understanding the embodiments of the present invention so as not to obscure the disclosure with details that will be readily apparent to those of ordinary skill in the art having benefit of the description
10 herein.

The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration of the invention in any way. Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention. Various changes to the described
15 embodiments may be made in the function and arrangement of the elements described without departing from the scope of the invention.

The terms “comprises”, “comprising”, or any other variations thereof, are intended to cover a non-exclusive inclusion, such that one or more processes or composition/s or systems or methods proceeded by “comprises... a” does not, without
20 more constraints, preclude the existence of other processes, sub-processes, composition, sub-compositions, minor or major compositions or other elements or other structures or additional processes or compositions or additional elements or additional features or additional characteristics or additional attributes.

In this specification and in the claims that follow, reference will be made to a
25 number of terms that shall be defined to have the following meanings:

It must be noted that, as used in the specification/description and the appended claims and examples, the singular forms “a”, “an” and “the” may include plural referents unless the context clearly dictates otherwise.

Ranges may be expressed herein as from “about” one particular value, and or “to
30 about” another particular value. When such a range is expressed, another aspect

includes from the one particular value and or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about”, it will be understood that the particular value forms another aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the
5 other endpoint, and independently of the other endpoint.

The bacteria *Burkholderia gladioli* strain NGJ1 exhibiting fungal activity has been previously isolated from healthy rice seedling (Jha et al. 2015) and publically available expression vector pET28a that is commercially available from Novagen (Merck Life Science Private Limited) are used in the invention for expressing the novel
10 sequence of Bg_9562. Therefore, the *biological material is sufficiently described* in the present invention.

The present invention provides novel genes and proteins capable of broad spectrum anti-fungal activities obtained from *Burkholderia gladioli* strain NGJ1 and its expression in pET28a expression vector.

15 As stated before, there remains a need for gene sequence of that exhibits broad-spectrum antifungal activity against several agriculturally important pathogens, including *Rhizoctonia solani*, *Magnaporthe oryzae*, *Venturia inaequalis*, and *Fusarium oxysporum*. Also, antifungal activity must be useful to treat animals as well as human disease caused by fungi.

20 **Definitions:**

As used herein, the terms “Mycophagous behavior” when used in context of the present invention refers to the behavior of organisms that consume fungi.

As used herein, the terms “Antifungal behavior” when used in context of the present invention refers to treatment and prevention for mycoses such as athlete's
25 foot, ringworm, candidiasis (thrush), serious systemic infections such as cryptococcal meningitis, and others against fungi.

As used herein, the terms “mutant” when used in context of the present invention refers to resulting from or showing the effect of mutation.

As used herein, the terms “insertion mutagenesis” when used in context of the
30 present invention refers to mutagenesis of DNA by the insertion of one or more bases.

As used herein, the terms “reverse genetics approach” when used in context of the

present invention refers to an approach to discover the function of a gene by analyzing the phenotypic effects of specific engineered gene sequences.

In one aspect, the present invention provides identification of novel protein having antifungal and mycophagous activity from *Burkholderia gladioli* strain NGJ1.

5 In another aspect, the present invention provides overexpression and purification of potential antifungal protein.

In yet another aspect, the present invention provides the process for assessment of antifungal activity of the novel protein. Still another aspect of the present invention provides establishment of the role of the antifungal protein through reverse genetics
10 approach.

In one embodiment, the present invention relates to novel genes and proteins capable of broad spectrum anti-fungal activities. The novel genes expressing novel proteins from bacteria *Burkholderia gladioli* strain NGJ1 exhibit fungal eating (mycophagous) property. A nucleotide sequence encoding the novel protein is
15 represented by sequence SEQ ID No. 1 and amino acid sequence of the novel protein is represented by the sequence SEQ ID No. 2.

In another embodiment, the present invention provides novel genetically engineered nucleotide sequence and peptide sequence of Bg_9562 gene that is capable of strong anti-fungal activity that further inhibit the growth of fungal sclerotia, induces
20 cell death in fungal mycelia and treat humans/animals for fungal infections.

In an embodiment, the nucleotide sequence encoding the novel protein is at least 70% identical to the nucleotide sequence of SEQ ID No. 1 or the amino acid sequence represented by SEQ ID No. 2. In another embodiment, the nucleotide sequence of SEQ ID NO: 1 have at least 1 to 90 nucleotide acid substitutions, deletions, and/or insertions.
25 In another embodiment, the amino acid sequence of SEQ ID NO: 2 have at least 1 to 30 amino acid substitutions, deletions, and/or insertions. In another embodiment, the amino acid sequence of SEQ ID NO: 2 have at least 2 to 20 amino acid substitutions, deletions, and/or insertions. In another embodiment, the amino acid sequence of SEQ ID NO: 2 have at least 2 to 15 amino acid substitutions, deletions, and/or insertions. In another
30 embodiment, the amino acid sequence of SEQ ID NO: 2 have at least 5 to 15 amino acid substitutions, deletions, and/or insertions. In another embodiment, the amino acid sequence of SEQ ID NO: 2 varies by 11 amino acid substitutions. In another embodiment, in the amino acid sequence of SEQ ID no. 2 at least one or more Leucine, Valine, and/or Isoleucine residues are substituted.

In one embodiment, the novel protein imparts provides improved antifungal potency, broadened antifungal spectrum, improved solubility, improved thermos stability, and improved recombinant production compared to the wild type strain of *Burkholderia gladioli*. In one embodiment, the novel protein is adapted for broad
 5 spectrum anti-fungal activities and produces high mass production of proteins. In another embodiment, the amino acid sequence is of 111 residues long and has molecular weight of ~13kDa, a pI of about 4.65 and a pH optimum at about 7.4.

In one embodiment of the invention, mutation of Bg_9562 gene through insertion mutagenesis results in loss of the antifungal activity and the mycophagous activity of
 10 the bacteria *Burkholderia gladioli* strain NGJ1. In another embodiment, the antifungal activity of Bg_9562 gene is useful for controlling sheath blight diseases of rice and other crops caused by *Rhizoctonia solani*.

In another embodiment of the invention insertion of full length copy of the gene could complement the defect and restored anti-fungal and mycophagous activity.
 15 One more aspect of the present invention provides the Bg_9562 gene encoding an antifungal protein that can be potentially used for controlling fungal disease. In another embodiment of the present invention, the anti-fungal activity of Bg_9562 is particularly against *Rhizoctonia solani* sclerotia that cause sheath blight disease in rice. In another embodiment of the present invention, the Bg_9562 induces cell death response in
 20 *Rhizoctonia solani* sclerotia.

In another aspect of the present invention the novel nucleotide sequence and peptide sequence are adapted to control diseases caused by *R. solani* on rice (sheath blight disease) as well as other crops (damping off of soyabean/tomato; black scurf of potato; root rot of sugarbeet; belly rot of cucumber; bair patch of cereals), fungal
 25 diseases of rice (sheath blight as well as rice blast), fungal diseases of plants (as mentioned in Table 1) and used to treat fungal infections of human/animals. For example, to treat candidiasis in humans/animals. In another aspect of the present invention, the novel nucleotide and protein are adapted to be used as spray or ointment for varied applications, as a transgene for developing broad spectrum fungal disease
 30 resistant rice as well as other important crops, engineering disease resistance in rice as well as other crops against *R. solani* infections. Additionally, the transgene can be used to provide disease resistance against other fungal pathogen infections.

TABLE 1: The list of fungi used for testing antifungal activity of Bg_9562 protein

Sr. no.	Fungal strain	Disease
1	<i>Rhizoctonia solani</i>	Sheath blight of rice/ damping off of soyabean and tomato/ black scurf of potato/root rot of sugarbeet/ belly rot of cucumber/ bair patch of cereals 5
2	<i>Alternaria brassicae</i>	Black spot of crucifers
3	<i>Magnaporthe oryzae</i>	Blast/blight disease of cereals
4	<i>Venturia inaequalis</i>	Apple scab disease 10
5	<i>Fusarium oxysporum</i>	Vascular wilt of tomato, tobacco, sweet potatoes, banana, legumes
6	<i>Dedymella</i> sp.	gummy stem blight of Cucurbits
7	<i>Phytophthora</i> sp.7700	potato blight, soya bean root /stem rot
8	<i>Colletotrichum</i> sp.	black spot disease of Phaseolus
9	<i>Ascochyta rabiei</i>	Blight disease of chickpea 15
10	<i>Neofusicoccum</i> sp.	stem-end rot of mango
11	<i>Alternaria</i> sp.	Brown Leaf Streak on Sugarcane
12	<i>Saccharomyces cerevisiae</i>	Model fungi
13	<i>Candida albicans</i>	Model fungi

20 In another embodiment, the present invention further provides a method to prepare novel protein by SEQ ID No. 2 with antifungal activity. The method comprises the steps of: (i) protein possessing anti-fungal activity and mycophagous activity from *Burkholderia gladioli* strain is identified, (ii) gene and protein sequence from identified protein of step (i) is analysed by finding critical residues for antifungal as well as mass

25 production and are artificially synthesized by gene synthesis, (iii) the evolved protein is over expressed in pET28a expression vector, (iii) the overexpressed protein is purified by Ni-NTA affinity chromatography, (iv) the purified protein is assessed for antifungal activity and (v) antifungal protein is established by reverse genetics approach. The recombinant expression of novel protein prevents the growth of fungal sclerotia and

30 induces cell death in fungal mycelia.

In another embodiment of the present invention a composition comprising a novel peptide for use and development of water or oil based ointment or nano-encapsulated spray or antifungal film is provided. The components of the composition

comprises oil that are selected from the group comprising of almond oil, rapeseed oil and sesame oil; thickeners that are selected from beeswax, cocoa butter and shea butter, emulsifiers selected from the group comprising of alcohols such as cetyl alcohol; and water.

5 In another embodiment, the composition is adapted to develop broad spectrum fungal disease resistance wherein the fungus is selected from the group comprising of *Rhizoctonia solani*, *Alternaria brassicae*, *Magnaporthe oryzae*, *Venturia inaequalis*, *Fusarium oxysporum*, *Dedymella sp.*, *Phytophthora sp.*, *Colletotrichum sp.*, *Ascochyta rabiei*, *Neofusicoccum sp.*, *Alternaria sp.*, *Saccharomyces cerevisiae* and *Candida*
10 *albicans*.

In another embodiment, a process to prepare the composition is provided. The process comprises the steps of: (i) Bg_9562 protein is produced in bulk quantities by fermentation using bioreactor, (ii) the fermented product obtained is encapsulated by chitosan or non-chitosan based nanoparticles; and (iii) the encapsulated products of step
15 (ii) is used to develop a water base or an oil base cream/ointment and, (iv) the product of step (iii) are further developed to prepare antifungal films comprising starch/ oils.

In another embodiment, the method to control fungal disease of plants or crops is provided by applying a polypeptide comprising the sequence of SEQ ID NO: 1 or 2, or a composition of any one of claims 12-13, to an infected plant, infected plant part, or
20 infected crop that are at risk of fungal infection. The method also treat humans and animals. In another embodiment, the plant is a cereal, cucurbit, vegetable, root vegetable, or legume, or produces a fruit crop. In another embodiment, the plants or crops are selected from the group comprising of rice, soybean, tomato, potato, sugar beet, sugarcane, cucumber, apple, mango, phaseolus, tobacco, banana, legume, and
25 chickpea.

In another embodiment the present invention provides a composition comprising the gene encoding novel peptide for use in developing transgenic plants (including rice) with broad spectrum disease resistance. The composition is used in developing rice resistance against sheath blight disease (caused by *R. solani*). Further, transgenic rice
30 would also be resistant to blast disease (caused by *Magnaporthe oryzae*) as protein also shows antifungal activity against *Magnaporthe oryzae* (as illustrated in Table 1). The composition also treats fungal infections in human/animals.

In another embodiment, a method to control antifungal disease is provided. The

method comprises the steps of: (i) purified Bg_9562 protein is sprayed on the infected plants and nano-encapsulated form of the purified protein is sprayed onto the infected plants/fields; (ii) an antifungal cream is applied directly onto the disease lesion to control animal/human fungal infections and an antifungal film is applied as wound dressing to control fungal infections.

In another aspect of the present invention a non-naturally occurring novel protein or polypeptide of mutated sequence of Bg_9562 gene is provided. The novel proteins or polypeptide are adapted to exhibit antifungal activity by inhibiting growth of fungal sclerotia and inducing cell death in fungal mycelia. More specifically, the artificially developed novel nucleotide sequence of Bg_9562 gene and polypeptide or protein thereof is provided.

In another embodiment of the present invention a method to control sheath blight diseases of rice as well as other crops, method to control fungal diseases of rice, method to control fungal diseases of plants, method to treat fungal diseases of human/animal is provided.

In another aspect of the present invention method for development into spray or ointment for varied application, method for use as a transgene for developing disease resistance against fungal pathogens, method for controlling sheath blight disease of rice, as well as other important crops for developing broad spectrum fungal disease resistant plants.

In another embodiment, the protein is produced by expressing its encoding nucleotide in homologous or heterologous system in the cells of bacteria, yeasts, plants, humans and animals using recombinant DNA technology.

EXAMPLES:

The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration to the invention in any way. Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention various changes to the described embodiments may be made in the functions and arrangement of the elements described without departing from the scope of the invention.

Example 1: Identification of protein involved in antifungal and mycophagous activity of *B. gladioli* strain NGJ1

The present example provides identification of *Burkholderia gladioli* strain NGJ1 originated from healthy rice seedling (as described in Jha et al 2015) as a potent

antifungal and mycophagous bacterium. Initially, upto 3 dpi (days post incubation) the bacterium show antifungal activity and prevent the growth of fungi in its vicinity. While during 8 dpi (days post incubation) of confrontation, the bacterium started foraging over fungi and demonstrated mycophagous activity (*as shown in Figure 1*). Interestingly the mycophagous behavior (bacterial spreading over fungi and degrading fungal mycelium) was very prolific on minimal media CDA (Czapek Dox Agar) plates.

The inventors have found that some proteins of *Burkholderia gladioli* strain NGJ1 have potential signal for being targeted into host and one of such protein (Bg_9562) potentially encoding a phage tail protein was further characterized. The role of identification protein was studied with respect to mycophagous and antifungal behavior of *Burkholderia gladioli* strain NGJ1 on *Rhizoctonia solani*.

Some of the selected nucleotide sequences of Bg_9562 were modified to artificially synthesize evolved gene (eBg_9562) sequence having SEQ ID No. 1 made of 333 bp.

Bg_9562 (Nucleotide sequence): original

5'ATG AAC ACG GAA AAC CAG GAT CCG ACG AGC ACC AGC GAC AAC
GCC GCG AAC ACG CAC ACG CTC GAC ACG CCG ATC GCG CGC GGC GAG
CAG ACG ATC ACC CAG GTG ACG CTG GCC AAG CCC GAT GCC GGC GCG
CTG CGC GGC ACC TCG CTG TCG GCG CTC GTC AAC CTC GAC GTC GAC
GCG CTG TGC AAG GTG CTG CCG CGC ATC ACG AGC CCG GCG CTG ACC
GCG GCC GAC GTG CGC GCC ATG GAC CCC GCC GAC CTG GTC TCG CTG
GGA GGC ATC TTC GCC GGT TTT TTG ATG CCG AAG TCG CTG AAA GCG
AGC ATG GAA TCC CCG AGC GCG 3'

SEQ ID No. 1 (Nucleotide sequence of evolved - Bg_9562)

5'ATG AAC ACG GAA AAC CAG GAT CCG ACG AGC ACC AGC GAC AAC
GCC GCG AAC ACG CAC ACG CTC GAC ACG CCG CTC GCG CGC GGC GAG
CAG ACG ATC ACC CAG GTG ACG CTG GCC AAG CCC GAT GCC GGC GCG
CTG CGC GGC ACC TCG CTG TCG GCG CTC GTC AAC CTC GAC GTC GAC
GCG CTG TGC AAG GCA ACT CCG CGC GCT ACG AGC CCG GCG GTC ACC
GCG GCC GAC ATC CGC GCC ATG GAC CCC GCC GAC GCA ATC TCG GTC
GGA GGC ATC TTC GCC GGT TTT GTT ATG CCG AAG TCG ATC AAA GCG
AGC ATG GAA TCC CCG AGC GCG 3'

Further novel peptide sequence of the above novel protein having SEQ ID No.2 made of 111 amino acids is provided.

Bg_9562 (Amino acid sequence): original

5'MNTENQDPTSTSDNAANTHTLDTP**I**ARGEQTITQVTLA

KPDAGALRGTSLSALVNLDVDALCKVLPRITSPALTAAD
YRAMDPADLVSLGGIFAGFLMPKSLKASMESPSA3'

SEQ ID No.2 (Amino acid sequence of evolved- Bg_9562)

5

5' MNTENQDPTSTSDNAANTHTLDTPLARGEQTITQVTL
 AKPDAGALRGTSLSALVNLDVDALCKAIPRATSPAVTA
 ADIRAMDPADAISVGGIFAGFVMPKSIKASMESPSA3'

- 10 The changes *as highlighted* from the parent protein are adapted for high mass production, improved solubility and antifungal activity of the evolved Bg_9562.

Example 2: Overexpression and purification of potential antifungal protein

- The complete CDS of Bg_9562 gene (333 bp) was PCR amplified from *B. gladioli* strain NGJ1 genomic DNA using gene specific forward primer having SEQ ID
 15 No. 3 and reverse primer having SEQ ID No. 4 as disclosed in Table 2 and further cloned into pET28a bacterial expression vector (Novagen/ Merck Life Science Private Limited) to obtain pET28a -9562 (*as shown in Figure 2a*). The restriction sites of NdeI and HindIII had been added in the forward and reverse primer sequences, respectively.

- Upon sequence validation, the pET28a -9562 was transformed into *E. coli* (BL21
 20 strain, DE3-codon+) for recombinant protein production. The protein was purified using Ni²⁺-NTA-Agarose chromatography and was resolved on SDS PAGE (*as shown in Figure 2b*). Further it was electro-blotted onto polyvinylidene fluoride (PVDF) membrane and probed with mouse polyclonal antibodies (1:1000 dilutions) raised against anti-His-antibody. The presence of ~13 KDa band suggested the overexpression
 25 and purification of the desired protein (*as shown in Figure 2c*).

Table 2: Primer Sequences

Primer Number*	Sequence	Restriction sites
Forward Primer: SEQ ID No.3	<u>CATATGAACACGGAAAACCAG</u> GAT	NdeI
Reverse Primer: SEQ ID No.4	<u>AAGCTTCGCGCTCGGGGATTCC</u> ATGCT	<u>HindIII</u>

Forward Primer- SEQ ID No.5	<u>GAATTC</u> ATGCCGGCGCGCTGCGC GGC	<u>EcoRI</u>
Reverse Primer SEQ ID No.6	<u>AAGCTT</u> GCTCGGGGATTCCATGC TCGC	<u>HindIII</u>
Forward Primer SEQ ID No.7	<u>AAGCTT</u> AACACGGAAAACCAGG AT	<u>HindIII</u>
Reverse Primer SEQ ID No.8	<u>GAATTC</u> CGCGCTCGGGGATTCCA TGCT	<u>EcoRI</u>

*The primers were designed using PrimerQuest Tool of IDT (Integrated DNA technologies, Inc, USA) and synthesized from Eurofins Genomics India Pvt. Ltd., Bangalore, India). For cloning purpose, suitable restriction sites (marked as underlined) were incorporated at 5' end of selected primers to make them synthetic primers in nature.

Example 3: Assessing antifungal activity of the purified protein

Antifungal activity of protein having SEQ ID No.2 was assayed by treating *Rhizoctonia solani* strain BRS1 sclerotia with different concentrations (5, 10 and 15µg/ml) of purified protein. As control, the sclerotia were treated with three different solutions 10 mM Phosphate buffer saline (PBS) pH 7.4, 50mM wash (one component used in protein elution) and heat inactivated Bg_9562 protein (by incubating in boiling water for 40 min). After treatment, sclerotia were placed on the PDA (Himedia, India) plates and incubated at 28°C for further growth. Result (as shown in Figure 3a) summarizes that 15µg/ml of the protein was efficient in preventing the growth of *R. solani*, while proper fungal growth was observed in case of different controls. Fungal growth inhibition upon eBg_9562 protein treatment is summarized in Figure 3b. The eBg_9562 treated sclerotia failed to grow while the control sclerotia showed proper growth. Further, upon different time intervals (such as 24h, 48h and 72h), sclerotial growth in terms of area of the mycelial lawn on PDA plates for both Bg_9562 as well as eBg_9562 protein treated along with various control treated samples were measured and data is summarized in Figure 3c. Overall, the data clearly demonstrate that in comparison to Bg_9562, the eBg_9562 is more efficient in preventing the fungal growth. Further, Table 3 represents the tabulated data showing the comparison fungal

growth inhibition upon various treatments (*as referred in Figure 3c*).

TABLE 3: Fungal growth inhibition upon various treatments

Sample	Observed growth area (cm ²)		
	24h	48h	72h
Buffer control	0.28	1.53	4.52
Heat inactivated. Bg_9562	0.28	1.57	4.60
50mM wash buffer	0.25	1.66	4.13
Bg_9562	0.00	0.48	0.69
eBg_9562	0.00	0.00	0.00

Table 3 depicts that after 72 hours of growth upon treatment with evolved Bg_9562, the fungi shows nil growth against fungi treated with wild type Bg_9562 protein that shows the growth of 0.69 cm² after 72 hours of treatment. Notably various control treatments do not inhibit the growth of fungi. Therefore, the present invention provides novel genes and proteins that are capable of broad spectrum anti-fungal activities. The novel genes and proteins are obtained from *Burkholderia gladioli* strain NGJ1 and expressed in pET28a expression vector. The eBg_9562 is effective and provides nil anti-fungal activity even after 72 hours of its treatment under various conditions (*as depicted by Figure 3c*). The modification in the protein has led to enhanced protein production as confirmed by protein estimation data through Bradford *in Figure 3d*. The figure depicts that the protein concentration of eBg_9562 is nearly 20mg/liter compared to the protein concentration of wild Bg_9562 that produces less than 10mg/liter of protein.

Furthermore, *R. solani* sclerotia were initially grown in PDB media for 48h to have germinating mycelia and then subjected to treatment with 15µg/ml of Bg_9652 protein and 10mM PBS buffer (*as a control*). Upon further incubation at 28°C, the treated and control mycelia were subjected to MTT assay as per the protocol described (Meshulam et al, 1995). Result of MTT assay suggested that the protein treatment could induce cell death as there was no formation of colored compound (*as shown in Figure 4a*). While due to active metabolism, formation of colored compound was detected in case of control mycelia (*as shown in Figure 4a*).

Example 4: Establishing the role of the antifungal protein through reverse genetics approach

In order to demonstrate that the SEQ ID No. 2 protein is indeed involved in antifungal and mycophagous behaviors of *B. gladioli* strain NGJ1, we adopted reverse genetics approach. For this the pGD1 plasmid was obtained by cloning 209 bp of the wild type *Bg_9562* gene using a Forward primer having SEQ ID No. 5 and reverse primer having SEQ ID No. 6 as disclosed in Table 1 into pK18 mob vector (Schäfer A et al, 1994). The pGD1 was mobilized into *B. gladioli* strain NGJ1 by using published protocol (Schäfer A et al, 1990) and the selection of $\Delta 9562$ insertion mutants (NGJ100 and NGJ101) were performed on kanamycin (50 μ g/ μ l) containing PDA plates. The $\Delta 9562$ insertion mutant bacterium was confirmed through PCR and sequencing (*as shown in Figure 5a & 5b*).

Further for complementation, full lengths of the gene was amplified from NGJ1 genome by using forward primer having SEQ ID No. 7 and reverse primer having SEQ ID No. 8 as disclosed in Table 2 and cloned into pHM1 vector. The pHM1- 9562 plasmid was electroporated into NGJ100 and NGJ101 strain and positive Kan^R and Spec^R colonies were selected on KBA plates. The NGJ100 and NGJ101 containing pHM1-9562 complementing plasmid (NGJ102 and NGJ103) was further confirmed through PCR and sequencing.

After, both *Bg_9562* mutants (NGJ100 and NGJ101) and complementing (NGJ102 and NGJ103), *B. gladioli* strains were tested for the anti-fungal and mycophagous activity of *R. solani*. The mutant bacterium failed to demonstrate the antifungal and mycophagous activity (*as shown in (Figure 6a)*). Fungal sclerotia were treated with the bacterial cultures of *Bg_9562* mutants (NGJ100 and NGJ101), complements (NGJ102 and NGJ103) and wild type NGJ1 (WT) individually for 4 hours at 28°C followed by subsequent incubation on PDA plates for 7 days (*as shown in (Figure 6b)*).

Example 5: The protein demonstrate broad spectrum antifungal activities

The effect of purified proteins was also tested on various fungi including several agricultural important fungal pathogens (*as illustrated in Table 1*) and the experimental data as evident by Figures 3 and Table 3 of the specification.

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Claims:

1. A protein with a broad-spectrum antifungal activity, comprising an amino acid sequence of SEQ ID No. 2 wherein said amino acid sequence is encoded by the nucleotide sequence of SEQ ID No. 1.
5
2. The protein of claim 1, wherein the amino acid sequence and the nucleotide sequence are non-naturally occurring and genetically engineered from the *Burkholderia gladioli* Bg_9562 gene.
10
3. The protein of claim 1 or 2, wherein the protein consists of the amino acid sequence of SEQ ID No. 2 and has a molecular weight of at least ~13 kDa, a pI of about 4.65 and a pH optimum at about 7.4.
- 15 4. The protein of any one of claims 1 to 3, expressed inside the cells of bacteria, yeast, insects, plants, humans or animals using recombinant DNA technology.
5. The protein of any one of claims 1 to 4, for use in treatment of fungal diseases.
- 20 6. The protein of any one of claims 1 to 4, for use in inhibiting growth of fungi and inducing cell death in fungal mycelia.
7. The protein of any one of claims 1 to 4, for use in controlling fungal disease in crops.
- 25 8. The protein of claim 7, wherein said fungal disease is sheath blight disease of rice caused by *Rhizoctonia solani*.
9. A method to prepare a protein having antifungal activity and comprising an amino acid sequence of SEQ ID No. 2, wherein the method comprises the steps of:
30 (i) identifying the protein that possesses antifungal activity and mycophagous activity

from *Burkholderia gladioli* strain NGJ1,

- (ii) analyzing the amino acid sequence of the identified protein in step (i) to find critical residues associated with antifungal activity as well as mass production, and artificially designing a gene sequence that is SEQ. ID No. 1 by incorporating mutations at 11 positions and synthesizing the designed gene through gene synthesis,
- (iii) over expressing the protein with desired changes of step (ii) in an expression vector to get recombinant protein,
- (iv) purifying the over expressed protein of step (iii) by chromatography,
- (v) assessing the antifungal activity of the purified protein of step (iv), and
- (vi) establishing the role of antifungal protein by a reverse genetics approach to produce the protein from Bg_9562.

10. A composition for use as an antifungal comprising the protein of any one of claims 1 to 4, an oil selected from the group consisting of almond oil, rapeseed oil and sesame oil; thickeners selected from the group consisting of beeswax, cocoa butter and shea butter; emulsifiers selected from the group consisting of alcohols; and water.

11. The composition for use of claim 10 wherein the alcohol is cetyl alcohol.

12. The composition for use as claimed in claim 10 or 11 for use in controlling fungal disease wherein the fungus is selected from the group consisting of *Rhizoctonia solani*, *Magnaporthe oryzae*, *Saccharomyces cerevisiae* and *Candida albicans*.

13. A process for preparing an antifungal film, wherein the process comprises steps:
(i) producing a protein comprising an amino acid sequence of SEQ ID No. 2 in bulk quantities by fermentation in a bioreactor;
(ii) encapsulating the fermented product obtained from step (i) in chitosan or non-chitosan based nanoparticles;

(iii) using the encapsulated product obtained from step (ii) to develop water base or oil base cream/ointment; and

(iv) converting the product obtained from step (iii) into the antifungal film, wherein the antifungal film is made from starch/oils.

5

14. A method for controlling fungal disease of plants or crops, comprising: applying a polypeptide comprising the sequence of SEQ ID NO: 2, or the composition for use as an antifungal of claim 10 or 11, to an infected plant, infected plant part, or infected crop.

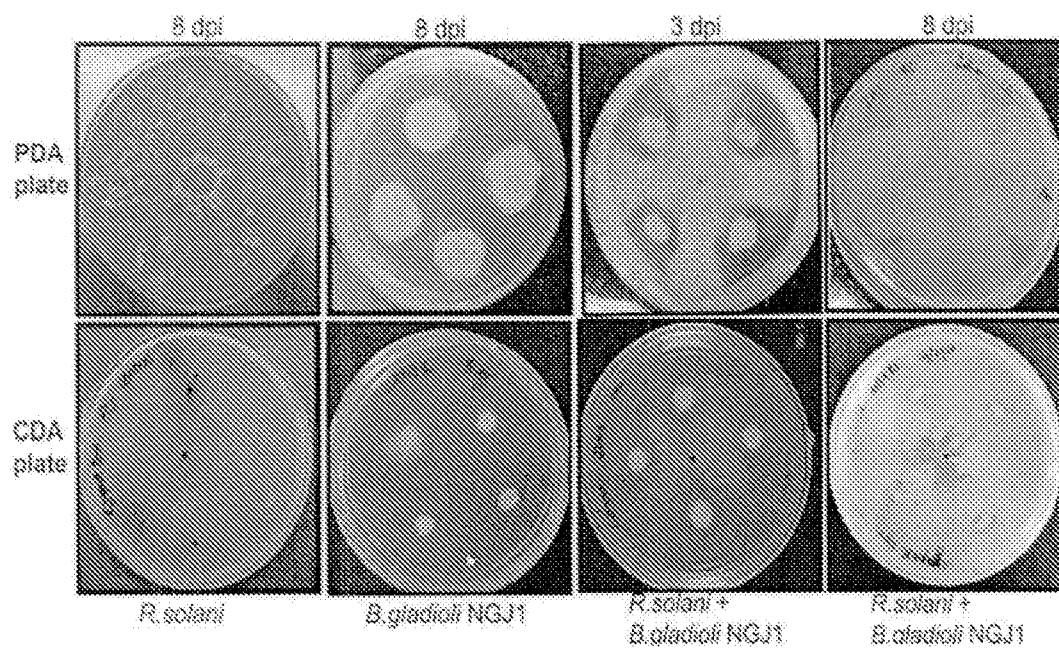


FIG 1.

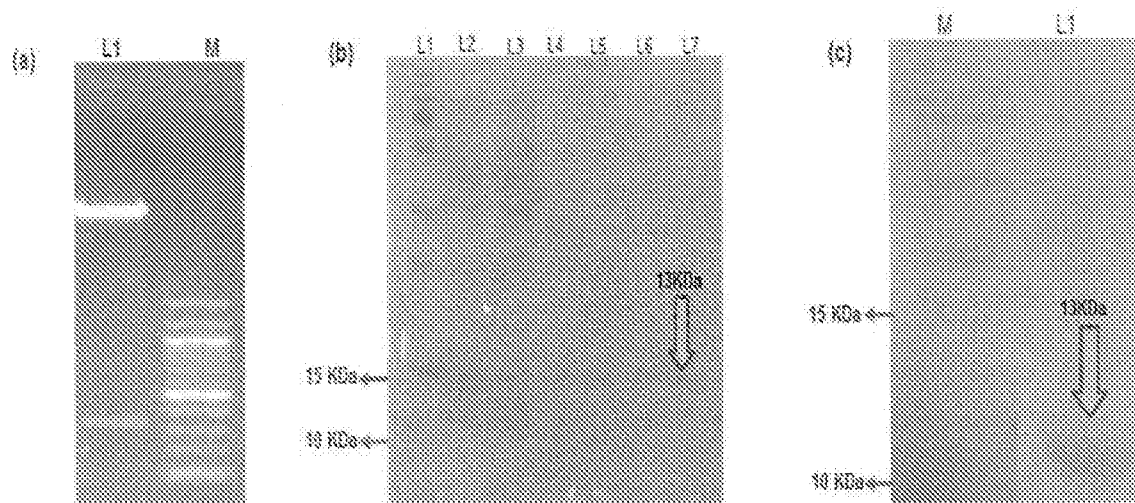
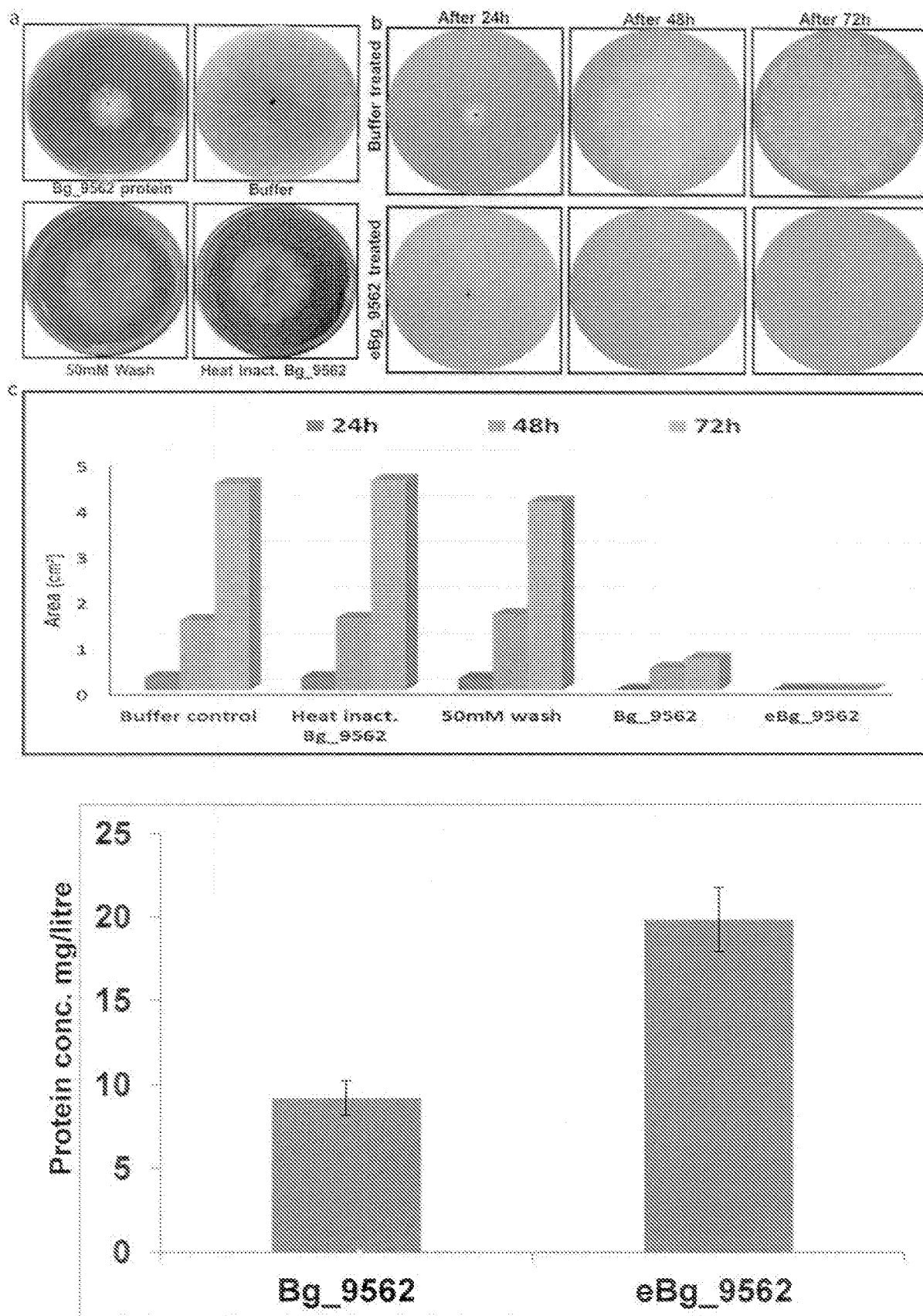


FIG 2.

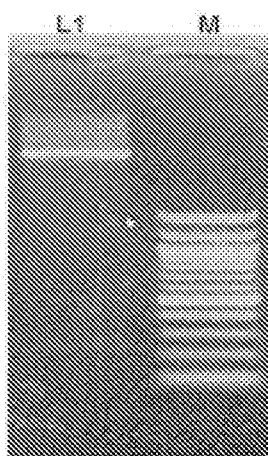


(a)



FIG 4.

(a)



(b)

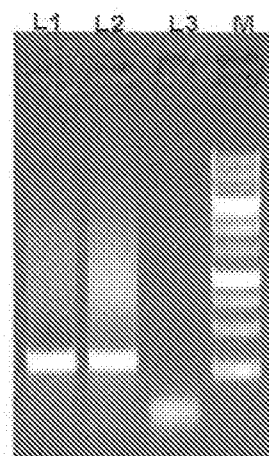


FIG 5.

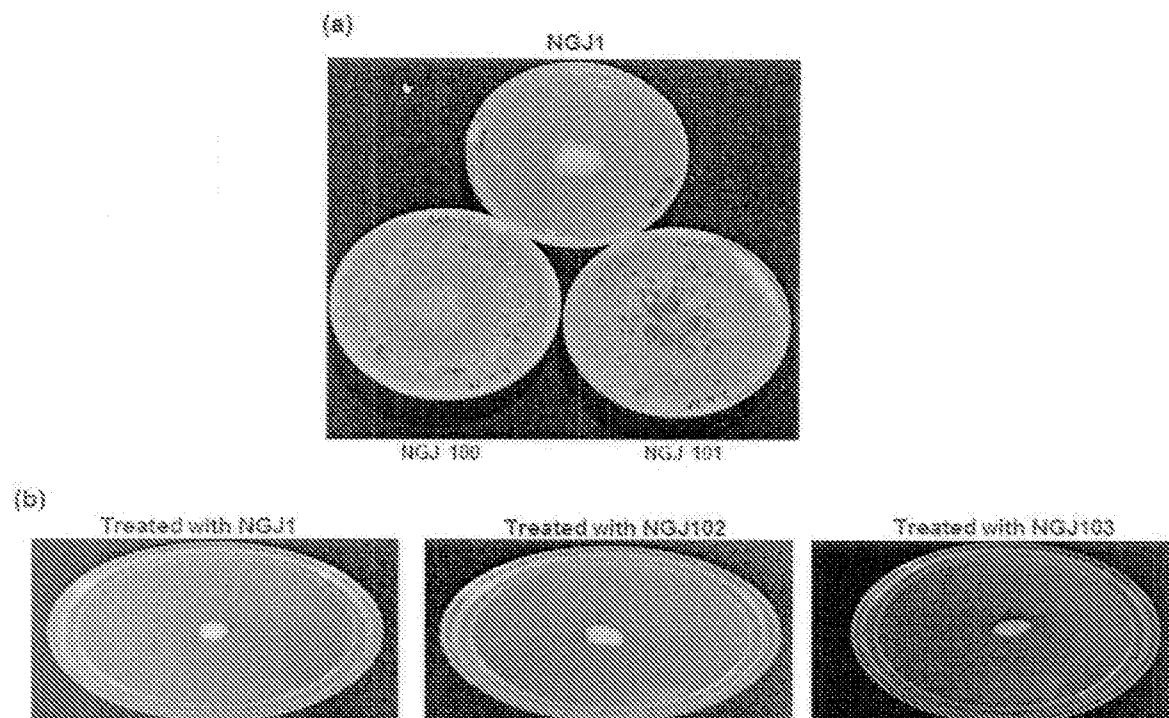
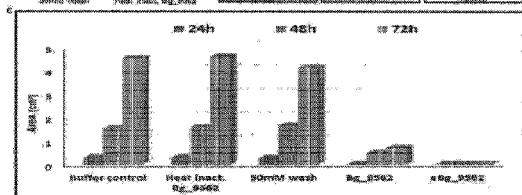
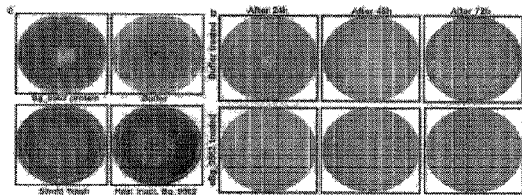


FIG 6.



e

