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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 June 2019 (20.06.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/116203 A1

(51) International Patent Classification:
*C07K 7/06* (2006.01) *A01N 37/46* (2006.01)

(21) International Application Number:
PCT/IB2018/059834

(22) International Filing Date:
10 December 2018 (10.12.2018)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:
102017000142231 11 December 2017 (11.12.2017) IT

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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).

**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))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

(54) Title: PEPTIDES WITH FUNGICIDAL ACTIVITY, THEIR COMPOSITIONS AND RELATED USES IN AGRONOMIC FIELD

(57) Abstract: The present invention relates to new peptides with antimicrobial and fungicidal activity, the related phytopharmaceutical compositions and in particular their use for the control of *Plasmopara viticola* in viticulture.

WO 2019/116203 A1

"Peptides with fungicidal activity, their compositions and related uses in agronomic field"

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The present invention relates to new peptides with antimicrobial and fungicidal activity, the related phytopharmaceutical compositions and in particular their use for the control of *Plasmopara viticola* in viticulture.

Plant diseases caused by pathogenic agents such as viruses, bacteria and fungi are responsible for agricultural production losses quantifiable around 16% at the global level and can also influence the quality and safety of foods [1]. In particular, fungicides constitute most of the phytosanitary products used in agriculture and viticulture is one of the leading sectors in terms of use of fungicides. It is estimated that, in Europe, 68,000 tonnes/year of fungicides are used to control grapevine diseases, accounting for 65% of the sum of the fungicides used in agriculture (Eurostat report, 2007).

The Oomycete *Plasmopara viticola*, obligate biotroph, is the causal agent of peronospora, one of the most severe grapevine diseases in the world [2].

Among the most common symptoms of the diseases are stem necrosis, grape withering and leaf discoloration. Severe attack generally determine the early fall of the leaves (phyloptosis). In the absence of treatments and in the presence of favourable meteorological conditions, peronospora can destroy up to 75% of the harvest in a single season and can weaken the new buds, causing loss of vigour and reduced production even in subsequent years, with consequent severe economic losses [3].

Currently, grapevine peronospora is controlled by frequent applications of fungicides, such as copper in the form of soluble salts (sulphate, Bordeaux mixture, etc.) or synthetic active ingredients.

However, the efficiency of these treatments, and in general of treatments with phytosanitary products, is very low; it is estimated that less than 0.1% of the active ingredient applied to crops is actually able to impact the designated pathogen [4]. The remaining part accumulates in the soil and from there, by washout of the contaminated grounds, can reach and pollute surface water and underground

aquifers, becoming a hazard for fresh water organisms and human beings. Fungicides accumulated in soils can damage arthropods, earthworms, fungi, bacteria, protozoa and in general all the organisms that contribute to the function and to the structure of the soils. The chronic exposure to phytosanitary products of useful insects, like bees, and of wild birds can cause reductions in their reproductive capacity, with consequences at the level of species and ecosystem, while exposure to high concentrations can even cause the death of single individuals. Pets can also be influenced by exposure to phytosanitary products. Some active ingredients have poor degradability and hence they remain in the environment for a long time. For example, organochlorine insecticides, such as DDT, were identified in the surface water of the United States 20 years after their use had been forbidden. Moreover, the phytosanitary products that enter the food chain can experience the phenomenon of biomagnification. This term means the tendency of some chemical substances to become ever more concentrated the farther up one rises in the trophic chains. As a consequence, the concentrations accumulated in the tissues of organisms can be many times higher relative to the surrounding environment.

This is a partial list of the negative effects that agricultural technologies and practices can have on the ecosystem and that justify the need to develop new antimicrobial compounds that can be used in agriculture for control of vegetable diseases, characterized by law toxicity and a reduced environmental impact (Commission Regulation (EC) No 473/2002 of 15 March 2002).

The authors of the present invention have now identified a family of peptides characterized by a primary sequence of eight amino acids able to interact with the catalytic domain of cellulose synthase and to inhibit the activity of the enzyme. In particular, the inventors have verified that the peptides according to the invention are able to contrast the growth of *Plasmopara viticola* on grapevine, with improved properties of activity, specificity, biodegradability, toxicity and with relatively low production costs thanks to its small dimensions [5].

The peptides of the invention can advantageously be employed in the eradication and prevention of peronospora in different crops, in particular in grapevines, and they represent a valid alternative to copper-based preparations because:

a) they are highly effective, as the minimum inhibitory concentration (MIC), *in vitro*, is around 20-50 μM;

b) they are not cytotoxic for human cells, as demonstrated by the assay for the determination of cell viability (MTT assay);

c) they are specific for *Plasmopara viticola*, while they are innocuous on non-target organisms, such as the soil bacteria *Agrobacterium tumefaciens* and *Bacillus amyloliquefaciens* and the Ascomycete *Erysiphe necator* (or *Uncinula necator*), causal agent of grapevine oidium;

d) they have a low cost of production, due to the small dimensions (eight amino acid residues) [5].

Moreover, the peptides according to the invention are sustainable fungicides that can sustain a reduction in use, or even the replacement, of traditional copper-based fungicides, as required by Commission Regulation (EC) no. 473/2002, thus promoting the transition towards green and sustainable agriculture.

Therefore, the present invention relates to a peptide characterized by a length sequence of 8 amino acids having the following sequence (I):

$$(X)_n - R - (Y)_m - R - (Z_i)_p \qquad (I)$$

wherein m is an integer number between 1-6;

n and p are each one an integer number between 0-5;

with m+n+p =6;

R is Arg;

wherein X, Y, Z are L-aminoacids selected from the group consisting of Leu (L), Thr (T), Ala (A), Cys (C), Gln (Q),

with the provision that the aminoacid Leu (L) is present twice and the aminoacids Thr (T), Ala (A), Cys (C), Gln (Q), are present once in the sequence of formula (I).

According to a preferred embodiment of the invention the peptide of formula (I) is characterized by an amino acid sequence such that:

n= 0;

m= 5;

p = 1;

$Y_1 = L$; $Y_2 = T$; $Y_3 = A$; $Y_4 = Q$; $Y_5 = C$

$Z_1 = L$

or it is characterized by the amino acid sequence RLTAQCRL (SEQ ID NO:1).

According to an alternative preferred embodiment of the invention the peptide of formula (I) is characterized by an amino acid sequence such that:

n= 2

m= 1

p = 3

$X_1 = L$; $X_2 = C$

$Y_1 = L$

$Z_1 = A$; $Z_2 = T$; $Z_3 = Q$

or it is characterised by the amino acid sequence LCRLRATQ (SEQ ID NO:2).

The peptides according to the invention can be prepared by Fmoc solid-phase peptide synthesis or by recombinant expression. In particular, the nucleotide sequence for the expression of the octapeptide having SEQ ID No:1 is as follows:

5'-CGTCTGACGGCGCAGTGTCGTCTT-3' (SEQ ID NO:6).

Instead for the peptide having SEQ ID NO:2 it is possible to use the nucleotide sequence 5'-CTG TGT CGT CTT CGT GCG ACG CAG-3' (SEQ ID NO:7).

The present invention also contemplates the peptidomimetic variants of said peptides, i.e. molecules that, while maintaining the structure and the bioactivity of the original molecule, exhibit greater stability (given by better resistance to proteolysis and/or to both physical and chemical degradation). These variants derive from the incorporation of amino acid residues not present in nature (D-amino acids) instead of L-amino acids and/or non-proteinogenic amino acids.

By way of example, peptide fluorination is an effective strategy to improve the stability of the peptides of the invention with respect to enzymatic, chemical and thermal denaturation. Fluoroalkyl groups increase local hydrophobicity and facilitate membrane traversing.

For practical uses in agriculture it is often preferable to use fungicide compositions containing the appropriately formulated active ingredients.

Therefore, further objects of the present invention are a phytopharmaceutical composition comprising at least one peptide according to the invention, a solid or liquid solvent and/or diluent, possibly adjuvants and/or co-formulants of various nature.

According to a preferred embodiment of the present invention, the aforesaid phytopharmaceutical composition can comprise one or more further active ingredients such as fungicides other than the peptide, selected from the group consisting of phytoregulators, antibiotics, herbicides, insecticides, fertilizers and/or mixtures thereof.

The aforesaid phytopharmaceutical compositions can be in solid form (such as granules, granules dispersible in water, dry powders etc.) of in liquid form (for example solutions, suspensions, emulsifiable concentrates, emulsions, microemulsions etc.): the selection of the type of composition will depend on the specific use.

The total concentration of the active peptide in the aforesaid compositions can vary within a broad range; in general, it varies from 1% to 90% by weight relative to the total weight of the composition, preferably from 5% to 50% by weight relative to the total weight of the composition.

The application of these phytopharmaceutical compositions can take place on each part of the plant, for example on leaves, stems, branches and roots, or on the seeds themselves before sowing, or on the soil in which the plant grows.

According to a preferred embodiment of the present invention, the compositions are in spray formulation.

A further object of the present invention, therefore, is the use of the peptides according to the invention or of the fungicidal compositions comprising at least one peptide of the invention for control of phytopathogenic fungi (Oomycetes class) in agricultural crops.

Thanks to their capability for specific interaction with the catalytic domain of the cellulose synthase enzyme the peptides according to the invention are able to

perform a preventive fungicidal action and exhibit very low or zero toxicity on the treated crops.

Examples of phytopathogenic fungi that can be effectively treated and combated with the peptides according to the invention belong to the Oomycetes class and are selected from the group consisting of *Plasmopara viticola, Peronospora spp, Phytophtora spp. (e.g. Phytophthora nicotianae, Phytophthora infestans, Phytophthora ramorum, Phytophthora sojae*), *Pseudoperonospora cubensis* and *Bremia lactucae.*

The main crops that can be protected with the compounds according to the present invention comprise fruit-bearing plants (e.g. grapevine), citrus trees (e.g. orange, lemon, tangerine, grapefruit), leguminous plants (e.g. bean, pea, alfalfa, clover, soy), vegetables (e.g. lettuce, onion, tomato, potatoes, eggplant, pepper), cucurbits (e.g. pumpkin, zucchini, cucumber, cantaloupe, watermelon), tobacco, coffee, tea, cocoa, sugar beet, sugar cane or cotton.

According to a particularly preferred embodiment of the present invention, the peptides described above have been found to be considerably effective in the control of *Plasmopara viticola* on grapevine.

The peptides of the invention can also be used in the control of *Phytophtora infestans* on tomato and potato; of *Bremia lactucae* on lettuce; of *Phytophthora parasitica* on pepper, eggplant, onion, citrus trees or *Phytophthora nicotianae* on tobacco or cotton; of *Phytophthora sojae* on leguminous plants; of *Pseudoperonospora cubensis* on cucurbits.

A further object of the present invention is a method for controlling phytopathogenic fungi in agricultural crops, which consists of applying on any part of the plants to be protected or on the soil effective, non-phytotoxic doses of compositions comprising the peptide according to the invention.

A further object of the present invention is then a method for controlling phytopathogenic fungi in agricultural crops, which consist of applying effective doses of the peptides according to the invention, used as such or formulated in fungicidal compositions as described above. The effective dose in the aforesaid compositions can vary within a broad range; in general, it varies from 1% to 90%

by weight relative to the total weight of the composition, preferably from 5% to 50% by weight relative to the total weight of the composition.

The application of these compositions can take place on each part of the plant, for example on leaves, stems, branches and roots, or on the seeds themselves before sowing, or on the soil in which the plant grows.

The quantity of compound to be applied to obtain the desired effect can vary according to different factors such as, for example, the compounds used, the crop to be preserved, the type of pathogen, the degree of infection, the climatic conditions, the method of application, the adopted formulation.

Doses of compounds between 10 g and 5 kg per hectare of agriculture crop generally provide sufficient control.

The present invention will now be described, for non-limiting illustrative purposes, according to a preferred embodiment thereof, with particular reference to the attached figures, wherein:

- Figure 1 shows the amino acid sequence of cellulose synthase 2 of *P. viticola* (PvCesA2). The catalytic domain used in the yeast two-hybrid assay is underlined.
- Figure 2 shows the fungicidal activity of the peptide NoPv1 co-inoculated together with *P. viticola* on grapevine leaves; dpi, days post infection.
- Figure 3 shows the images of grapevine foliar disks inoculated with *P. viticola* in the presence or absence of the peptide NoPv1. (a-c) As control, the sporangia of *P. viticola* were resuspended in water and used to infect the grapevine foliar disks (5 drops for each foliar disk). In these conditions, the sporulation of *P. viticola* is observable starting from 5 days from inoculation. (b-d) The same sporangia of *P. viticola* were mixed with the peptide NoPv1 (200 μM) and then used to infect the grapevine foliar disks. In this case, the presence of NoPv1 was able to inhibit completely the growth of *P. viticola* without causing any damage to foliar tissues. The images refer to 7 days post infection.
- Figure 4 shows the fungicidal activity of the peptide NoPv1 administered before infection with *P. viticola* (pre-inoculation).
- Figure 5 shows the comparison between the fungicidal activity of the peptide NoPv1 with two fungicides currently on the market: Kocide 2000®, based on

Copper and Pergado®. The fungicides were administered with the Potter spray Tower before the inoculation of *P. viticola*. The data refer to 5 and 7 days post infection.

- Figure 6 shows the fungicidal activity of the peptide NoPv1 on greenhouse-cultivated grapevine plants. The peptide was administered to the leaves by spray a day before infection with *P. viticola* and the severity of the infection was assessed 7 days from inoculation. The data clearly indicate that NoPv1 is also effective in greenhouse conditions.

- Figure 7 shows the bactericidal/bacteriostatic activity of NoPv1 verified on *Agrobacterium tumefaciens* and *Bacillus amyloliquefaciens*. NoPv1 was used to verify its impact on the growth of soil bacteria such as *Agrobacterium tumefaciens* (a) and *Bacillus amyloliquefaciens* (b). Bacterial growth was analysed for 5 hours in the presence of NoPv1 (100 μM and 200 Mm) and in its absence (Control). The optical density measurements at 600 nm were carried out every hour. The charts clearly show that NoPv1 have no inhibitory effect on bacterial growth.

- Figure 8 shows the biological activity of NoPv1 on *Phytophthora infestans* and *Erysiphe necator*. (a) Alignment of the amino acid sequence of the PvCesA2 domain, used in the yeast two-hybrid assay, with the homologue of *P. infestans* (PiCES2). (b) The ability of NoPv1 of inhibiting the mycelial growth of *P. infestans* at 18 °C was verified *in vitro* through the addition of NoPv1 to the culture medium and the measurement of the diameter of the colony of the oomycete at 4, 5, 7 and 11 days post inoculation. The images refer to 7 days post inoculation. (c) Young grapevine leaves were treated with NoPv1 (200 and 400 μM) and inoculated with oidium spores. The high level of infection, observed 14 days post infection, indicates that NoPv1 does not alter the growth of *Erysiphe necator*, even at high concentrations. The images refer to 14 dpi.

- Figure 9 shows the MTT assay to assess the potential cytotoxicity of NoPv1. Immortalised human cells (HKC8), cultivated in the medium DMEM-F12 at different densities (1000, 3000 and 6000 per 100 μl), were grown in the presence of 400 μM NoPv1 and in its absence (Control) for 24 and 48 hours. The results obtain

demonstrate the absence of any effect of NoPv1 on the growth and on the vitality of the cells in the culture.

- Figure 10 shows the fungicidal activity of the "scramble" peptide of NoPv1 (NoPv1sc) on *P. viticola.* (a) the activity of the peptide NoPv1sc solubilised in water and inoculated together with *P. viticola* on grapevine foliar disks, in the form of droplets, was analysed 5 and 7 dpi. (b) Photos of grapevine foliar disks with *P. viticola* in the presence of NoPv1sc (20, 50 and 100 μM) and in its absence (Control). (c) Fungicidal activity of the peptide NoPv1sc, solubilised in water and administered with the Potter spray tower before infection with *P. viticola*. The data refer to 5 and 7 dpi.

- Figure 11 shows the fungicidal activity of mutated versions of the peptide NoPv1 analysed on co-inoculated grapevine foliar disks. The activity of the peptides NoPv1 R1A, where the Arg in position 1 was replaced with Ala, NoPv1 R7A, where the Arg in position 7 was replaced with Ala, and NoPv1 R1A-R7A, where both Args were replaced with Ala, was analysed at 5 and 7 dpi. The charts highlight the importance of the two Args for the fungicidal activity of NoPv1.

The better to illustrate the invention, the following examples are provided below to illustrate the invention without limiting it.

EXAMPLE 1: *Identification of the peptide according to the invention NoPv1 (SEQ ID NO:1)*

The peptide NoPv1 having amino acid sequence RLTAQCRL (SEQ ID NO: 1) was selected by a yeast two-hybrid assay in which small peptides had to be identified, able to interact with the catalytic domain of the cellulose synthase 2 of *P. viticola*, PvCesA2 (Figure 1), to inhibit the activity of the enzyme.

It has been demonstrated in *Phytophthora infestans*, a pathogenic Oomycete close to *Plasmopara viticola* from the phylogenetic viewpoint, that the cellulose synthases play a fundamental role in the establishment of the infection [6]. The peptide library was generated following the protocol shown in [7], which provides the use of a modified yeast expression vector, in which the gene of thioredoxin A of *Escherichia coli* (TrxA), encoding for the scaffold protein, was fused in frame with the transcriptional activation domain (Activation Domain, AD) of the yeast

transcription factor GAL4 (TrxA-GAL4AD). The fragment of random nucleotide sequences of 24 base pairs (bp), encoding for the 8 amino acid long peptide library, was then inserted in the vector containing the fusion protein TrxA-GAL4AD, more precisely it was positioned in the catalytic centre of the protein TrxA. The insertion of random peptide sequences in this position makes the enzyme TrxA inactive, eliminates the potential interactions with its specific natural interactors and exposes on the surface the random sequence of 8 amino acids. The library thus built was used to transform a yeast strain (AH109) bearing the vector pGBKT7 in which the catalytic domain of the gene PvCesA2 was fused with the DNA Binding Domain (BD) of the yeast transcription factor GAL4 (PvCesA2-GAL4BD). The peptides able to interact with the catalytic domain of PvCesA2 were selected on the basis of the capability of the positive yeast clones to grow on a selective medium. The plasmid DNA was then purified and sequenced. The sequence of the fragment of 24 nucleotides allowed to deduct the primary structure of the peptide NoPv1.

Properties of the peptide NoPv1

The peptide NoPv 1 is a peptide consisting of eight L-amino acids, $NH_2$-Arg – Leu – Thr – Ala – Gln – Cys – Arg – Leu-COOH (SEQ ID NO:1) with a molecular weight of 960.16 Da, an isoelectric point at pH 10.43, a net charge of 1.9 at pH 7, and good solubility in water. It was hypothesised that peptides with a net positive charge, between +2 and +9, are excellent antimicrobial peptides because their charge promotes the initial electrostatic interaction with the negatively charged phospholipid membranes of bacteria, fungi and other micro-organisms ([8][9][10][11]). However, the positive charge of NoPv1 is not sufficient to explain its specificity for *P. viticola* and the absence of toxicity on human cells.

To verify the importance of the primary sequence of the peptide NoPv1 for its biological activity, a scramble peptide was generated, with the name of NoPv1sc ($NH_2$-Leu – Cys – Arg – Leu – Arg – Ala – Thr – Gln-COOH, SEQ ID NO:2).

The peptide NoPv1sc demonstrated its ability, both in co-inoculation assays and in pre-inoculation assays, to inhibit the infection from *P. viticola* in a similar manner to NoPv1, suggesting that both the primary sequence of the peptide, and its

biochemical properties are important and both contribute to determine its biological activity (Figure 10).

Lastly, a test was conducted of the importance for the biological activity of NoPv1 of the two arginine residues, which provide the peptide with the positive charge, by the generation of three new peptides in which one or both arginine residues were replaced by alanine resides (Ala-scan) (Figure 11).

All the peptides thus generated, NoPv1 R1A ($NH_2$-Ala – Leu – Thr – Ala – Gln – Cys – Arg – Leu-COOH, SEQ ID NO: 3), NoPv1 R7A ($NH_2$-Arg – Leu – Thr – Ala – Gln – Cys – Ala – Leu-COOH, SEQ ID NO:4), and NoPv1 R1A-R7A ($NH_2$-Ala – Leu – Thr – Ala – Gln – Cys – Ala – Leu-COOH, SEQ ID NO:5) proved to be unable to prevent the infection of *Plasmopara viticola* at the levels of NoPv1, underlining the fundamental role played by the positive charge in the biological activity of the peptide NoPv1.

EXAMPLE 2: *Synthesis of the peptide and purification*

The peptides were prepared by Fmoc solid-phase peptide synthesis [12].

The peptides were prepared using the Wang resin with a loading of 0.4 mmol $g^{-1}$ and on 0.2 mM scale.

0.2 M of solutions of 9-Fluorenylmethyloxycarbonyl (Fmoc) amino acids were used in N,N-dimethylformamide (DMF) or 1-Methyl-2-pyrrolidinone (NMP). As coupling reagent, a mixture of 1-hydroxybenzotriazole/O-benzotriazol-N,N,N0,N0-tetramethyluronium hexafluoro-phosphate (HOBT/HBTU) 0.45M in DMF and as diisopropylethylamine (DIPEA) 2 M in NMP.

The coupling reaction was conducted for 5 min at 40 W with a maximum temperature of 75°C. The deprotection is carried out with 2 cycles of 5 min and 10 min respectively (75°C, 40 W).

For the deprotection reaction of the Fmoc group, a 20% piperidine solution in DMF was used.

The detachment of the resin was carried out using the reagent K (trifluoroacetic acid/phenol/thioanisole/triisopropylsilane/water, 82.5:5:5:5:2.5 v/v) for 3 hours. The peptide was precipitated from cold ethyl ether and purified by RP-HPLC with a 5-

70% gradient of the solvent B (solvent A:water/acetonitrile/TFA 95/5/0.1; solvent B: water/acetonitrile/TFA 5/95/0.1) in 20 min with a flow of 20 ml/min. The peptides were lyophilised and preserved at 0°C.

EXAMPLE 3: *Study on the biological activity of the peptide NoPv1*

To assess the effects of the peptide NoPv1 on the infection by *P. viticola*, different types of experiments were carried out both *in vitro*, in controlled conditions in a laboratory by inoculation on foliar disks obtained from susceptible plants of *Vitis vinifera* cultivar (cv) Pinot noir, and *in vivo* on greenhouse-grown plants.

Co-inoculation assays

The fungicidal activity of the NoPv1, dissolved in water at different concentrations (20, 50, 100, 150, 200, 400 μM) and inoculated together with *P. viticola* on grapevine leaves in the form of small drops, was assessed at 5 and 7 days from inoculation (dpi), in accordance with the protocol described in [13] and [14].

Each foliar disk was inoculated positioning on it 5 drops (of 10 μl each) of a suspension of sporangia of *P. viticola* (at the concentration of $1 \times 10^5$ sporangia/ml) as shown in Figure 2.

For each concentration to be tested, 5 replications were carried out, each consisting of 5 foliar disks. The disks, placed in Petri dishes with a plastic film, were incubated in the dark in a growth chamber at the temperature of 22 ± 1 °C for one night to allow the penetration of *P. viticola* inside the foliar tissues. The following day, the drops were dried with filtering paper and the disks were incubated again in the growth chamber, at the same temperature and a photoperiod of 16 hours of light and 8 ours of darkness, for a period of seven days. The severity of the disease was assessed at five and seven days after inoculation as the percentage of surface area of the foliar disk covered by sporulation. This percentage was calculated as the sum of the severity of the disease in each of the five drops positioned on the disk. To each drop was assigned a value variable from 0% (absence of sporulation at the drop) to 20% (abundant sporulation).

NoPv1 showed a weak antimicrobial activity at the concentration of 20 μM, but it was able to block almost completely the infection of *P. viticola* at 100 μM (Figures 2 and 3).

Pre-inoculation assays:

The effects of the peptide NoPv1 against *P. viticola* were assessed also using the Potter spray Tower (Burkard Scientific, UK). The Petri dishes containing the foliar disks were sprayed with 1.67 ml of a 400 μM solution of NoPv1 (corresponding to a standard dosage of 10 hl/ha in a vineyard with *Pergola Trentina* cultivation system) at the pressure of 55 kPa.

The peptide NoPv1 was applied at different times (from seven days to two hours) before the inoculation of the pathogen. After each application, the foliar disks were left to dry in a chemical fume hood and the preserved in the growth chamber. The foliar disks were then sprayed with a fresh suspension of sporangia of *P. viticola* (0.6 ml of suspension for each Petri dish, containing 5 foliar disks, at a concentration of $1 \times 10^5$ sporangia/ml) and incubated for one night in the dark in a growth chamber at the temperature of 22 ± 1°C. The following day, the dishes were made to dry in a laminar flow hood and then maintained in the growth chamber of a period of seven days. At 7 dpi, the percentage of surface area of the foliar disk covered by sporulation was assessed visually.

The peptide NoPv1 showed a significant capability of inhibiting the *Plasmopara viticola*, even when applied seven days before infection indicating that NoPv1 can be used as a fungicide with preventive action. The peptide (400 μM) was supplied to foliar disks by means of the Potter spray Tower at different times before the infection with *Plasmopara viticola* (g, days; O, hours). The corresponding controls (Co), without peptide NoPv1, are shown in the chart. The chart also shows the co-inoculation assay (0, O). The data show that NoPv1 is effective even when it is administered before the infection, hence in pre-inoculation. The values of severity of the infection shown in the chart refer to 7 days from the inoculation of *Plasmopara viticola* (7 dpi) (see Figure 4).

Even more interesting is the fact that NoPv1 showed a very similar effectiveness to copper sulphate, currently the best fungicidal compound used in agriculture, in

preventing the attack of peronospora and it also showed a far higher effectiveness than Pergado SC, because the strains of *P. viticola* that attack vineyards in Trentino have become resistant to this fungicide (see Figure 5).

Assays on plants in greenhouse

The ability of NoPv1 to prevent the *Plasmopara viticola* infection was also analysed in greenhouse conditions, in this case the solution of NoPv1 was sprayed on the plants to be tested one day before inoculation with *Plasmopara viticola* (Figure 6). In detail, the plants of *V. vinifera* cv *Pinot Noir* were cultivated in a greenhouse (20°C, 70% ± 10% RH), so that each plant had two buds bearing 10-15 leaves each. The solution of the peptide NoPv1, at the concentration of 400 of 800 μM, was applied on the lower page of all leaves by a spray. For each concentration to be tested, five plants were used. To each plant were applied 15-20 ml of solution of the peptide NoPv1 on the basis of the number of leaves present. As a control, five plants were sprayed with water (untreated control). For the inoculation of *Plasmopara viticola*, the plants were sprayed with a fresh suspension of sporangia ($1.8 \times 10^5$ sporangia / ml) and incubated in the dark in a greenhouse for one night. The percentage of foliar surface area covered by sporulation was assessed visually at 7 dpi. In this case, too, NoPv1 confirmed its protective action, since it demonstrated its ability to reduce the *P. viticola* infection in greenhouse conditions.

EXAMPLE 4: *Properties of the peptide NoPv1*

Specificity of NoPv1

The micro-organisms present in the ground represent an essential component of the soil ecosystem, as well as of the agricultural system. They perform a fundamental role in the biogeochemical cycles of the nutrients and each of them, indicated as PGPR bacteria (plant growth-promoting rhizobacteria), are able to establish more or less close relations with the superior plants, promoting their growth [15]. Among PGPR bacteria we find micro-organisms belonging to the genera *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas* and *Serratia*.

Therefore, it was verified whether the peptide NoPv1 could somehow influence the growth of these bacteria. For this purpose, the peptide NoPv1 was added to a concentration of 200 μM in liquid cultures of *Agrobacterium tumefaciens* and *Bacillus amyloliquefaciens*. In both cases, the addition of the peptide NoPv1 to the culture medium caused no alteration in bacterial growth (Figure 7), suggesting that NoPv1 can be used in vineyards without the occurrence of undesired bacteriostatic or bactericidal effects on the soil ecosystem. The biological activity of NoPv1 was also tested on the Oomycete fungus *P. infestans*, closely correlated to *P. viticola*, and on the Ascomycete *Erysiphe necator*, causal agent of grapevine oidium (Figure 8).

The catalytic domain of the gene PvCesA2 of *P. viticola* used in the yeast two-hybrid assay is extremely similar to the corresponding gene of *P. infestans* (PiCES2), having an identity equal to 97% (Figure 8a); consequently, NoPv1 proved to be able to influence the growth of *P. infestans in vitro* (Figure 8b) when added to the growth medium. On the contrary, NoPv1 does not block the growth of *Erysiphe necator* (Figure 8c), an Ascomycete that has a cell wall constituted mainly by chitin, and not by cellulose as in Oomycetes. These experimental results strongly support the hypothesis that the activity of NoPv1 is specific and hence that the peptide acts inhibiting the activity of the cellulose synthase.

On the basis of these results it is also possible to hypothesise that the peptide NoPv1 can be active against any organism having an enzyme for the cellulose synthase that is very similar to PvCesA2. Analyses conducted with the TBLASTIN software allowed us to identify numerous organisms, all Oomycetes and plant pathogens, whose cellulose synthases show an amino acid sequence homology above 90% with PvCesA2.

The list of oomycetes and of the host vegetable species whose cellulose synthase has an amino acid sequence homology above 90% with PvCesA2 is shown in Table 1 below.

The homology analysis was conducted using the TBLASTN software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1

| Pathogenic agent | Identity | Host plants |
|---|---|---|
| *Bremia lactucae* | 99% (referred to the gene PvCesA3) | Specific pathogen of the Compositae. Causal agent of the lettuce peronospora, the most fearful adversity that can affect this crop |
| *Phytophthora parasitica* o *Phytophthora nicotianae* | 97% | There are over 300 host species, including numerous ornamental plants and several crops (pepper, eggplant, tomato, onion, citrus fruits, tobacco, cotton) |
| *Phytophthora infestans* | 97% | Causal agent of the peronospora of potato and tomato |
| *Phytophthora ramorum* | 95% | Pathogen with marked polyphagy, high virulence and high spreading rate. Currently, two strains have been isolated, an American one and a European one, identified on several host plants. **American strain:** Pinaceae, Taxodiaceae, Fagaceae, Ericaceae, Caprifoliaceae, Aceraceae, Rosaceae, Hippocastanaceae, Lauraceae, Rhamnaceae, Amamelidaceae. **European strain:** Taxaceae, Fagaceae (*Fagus selvatica*, *Quercus ilex*, *Castanea sativa*), Ericaceae (*Rhododendron* spp., *Vaccinium* spp., *Arbutus unedo*), Caprifoliaceae (*Viburnum* spp.), Theaceae |
| *Phytophthora sojae* | 95% | In addition to soy (*Glycine max*) this oomycete can also attack other crops, including tomatoes and various leguminous plants (alfalfa, clover, pea, bean) |
| *Pseudoperonospora cubensis* | 91% | Causal agent of cucurbit peronospora, among the main foliar diseases of pumpkin, zucchini, cantaloupe, watermelon, cucumber and cucurbits in general, both in protected culture and in full field |

Potential cytotoxicity of the peptide NoPv1

The peptide NoPv1 never showed phytotoxicity symptoms, even when used in relatively high concentrations.

No damage was observed either on the foliar disks, or on the young or adult leaves of the greenhouse plants treated with the peptide.

The potential cytotoxicity of the peptide NoPv1 on human cells was tested *in vitro* by the cell viability test, or MTT assay.

It is a standard colorimetric assay that allows to measure the activity of the succinate dehydrogenase mitochondrial enzyme, active only in living cells, and able to reduce MTT (bromide of 3-[4,5-dimethylthiazol-2-il]-2,5-diphenyltetrazole, yellow coloured) to formazan, a blue/purple coloured substance, whose formation can be followed measuring absorption at 570 nm. Therefore, formation and absorbance levels at 570 nm are directly proportional to the quantity of vital cells present in the sample. Cultures of immortalised human cells (HKC8) at different densities (1000, 3000 and 6000 per 100 µl) were grown in DMEM-F12 medium in the presence of 400 µM NoPv1 and their vitality was measured after 24 and 48 hours. No significant differences were observed between control and treated, indicating that NoPv1 is not toxic for human cells (see Figure 9).

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# CLAIMS

1. Peptide characterized by a length sequence of 8 amino acids having the following sequence (I):

$$(X)_n - R - (Y)_m - R - (Z_i)_p \qquad (I)$$

wherein m is an integer number between 1-6;

n and p are each one an integer number between 0-5;

with m+n+p =6;

R is Arg

wherein X, Y, Z are L-aminoacids selected from the group consisting of Leu (L), Thr (T), Ala (A), Cys (C), Gln (Q),

with the provision that the aminoacid Leu (L) is present twice and the aminoacids Thr (T), Ala (A), Cys (C), Gln (Q), are present once in the sequence of formula (I).

2. Peptide according to claim 1, wherein:

n= 0;

m= 5;

p = 1;

$Y_1 = L$; $Y_2 = T$; $Y_3 = A$; $Y_4 = Q$; $Y_5 = C$

$Z_1 = L$

3. Peptide according to claim 1, wherein:

n= 2

m= 1

p = 3

$X_1 = L$; $X_2 = C$

$Y_1 = L$

$Z_1 = A$; $Z_2 = T$; $Z_3 = Q$

4. Phytopharmaceutical composition comprising at least one peptide according to any one of the claims 1-3 as active principle, and optionally a solid or liquid solvent and /or diluent, at least an adjuvant and/or coformulating agent.

5. Phytopharmaceutical composition according to claim 4, comprising one or more further active ingredients such as a fungicide other than the peptide according

to any one of the claims 1-3, selected from the groups consisting of phytoregulators, antibiotics, herbicides, insecticides, fertilizers and/or mixtures thereof.

6. Use of the peptide according to any one of the claims 1-3 or a phytopharmaceutical composition according to anyone of the claims 4-5, as antimicrobial or fungicide agent in agronomic field.

7. Use of the peptide according to claim 6, for treating or preventing the infections mediated by Oomycetes.

8. Use of the peptide according to claim 7, wherein said Oomycetes are selected from the group consisting of *Plasmopara viticola, Peronospora spp, Phytophtora spp.*, *Pseudoperonospora cubensis* and *Bremia lactucae.*

9. Use of the peptide according to claim 7, for treating or preventing peronospora caused by *Plasmopara viticola* on vines.

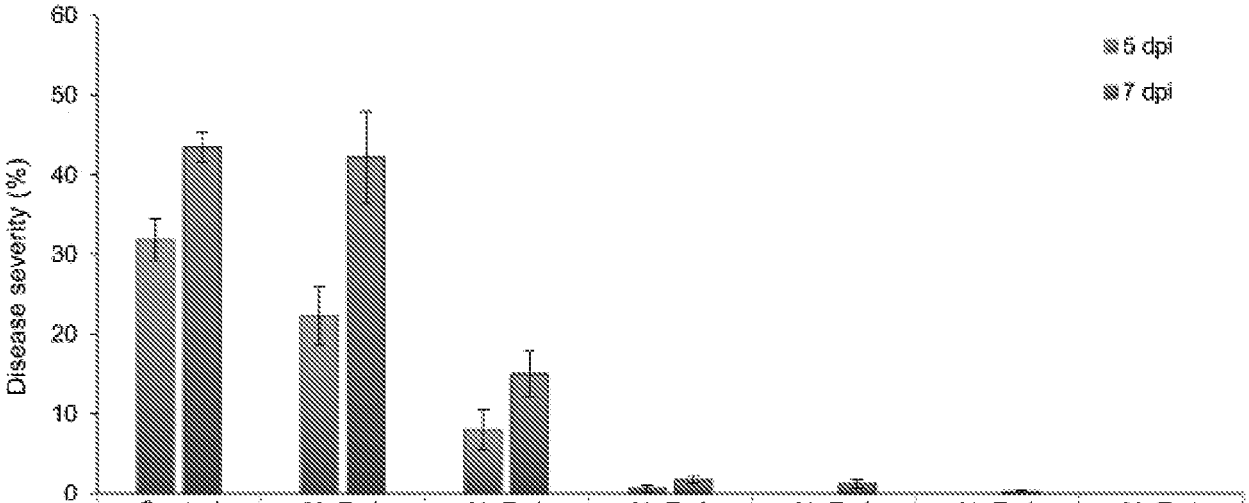
10. Method for controlling phytopathogenic fungi in agricultural crops, which consists in applying, on any part of the plants to be protected or on the ground, effective and non-phytotoxic doses of a peptide according to any of the claims 1-3, used as such or formulated in phytopharmaceutical composition according to one or more of claims 4-5.

11. Method for controlling phytopathogenic fungi according to claim 10, wherein said phytopathogenic fungi belong to Oomycetes class and are selected from the group consisting of *Plasmopara viticola, Peronospora spp, Phytophtora spp.*, *Pseudoperonospora cubensis* and *Bremia lactucae.*

12. Method for controlling phytopathogenic fungi according to any one of the claims 10-11, wherein said agricultural crops comprising fruit bearing crops, preferably vine, citrus, legume, horticultural crops, cucurbitaceous crops, tobacco, coffee, the, cacao, sugar beet or cotton.

MFGNDKQLLIKHEDYELHGTPATGANDGGAGFYTQEAHLVHQQGHADPRGPALPPMNVSDAVGLGGQRDNIIS
VHGYMHKQGKRTIKGPIHKSWKRRYFALEKAKIYYFHSHLECRQYFTTRNADLVVGAIELKDALQLRPCARLD
LPHKGFEVHTKRRVWVLCPETDEEYRMWFQGVERAIVANGAGNIIERKLPNVRKYLMKGNQTYRFFYFLFVIA
GIVELLALVFWFAIGLEPCDAARLEVDCNTITLTSLDELRCSSEPFSGYFTPPTWYLQVADVENVICFRDPPI
PQWISYFAMILAELLTFALGVLYYLGMWKPVRRGAHYFDEFEPPVPDELWPKVDILLCHYSEPAEEAIDTLMA
CMNVQYPPHLLQIWVCDDGYCKTKWTKGNPIPTVELNKGILETAGDLRQEVAQFMYDRVCDPNEDMEVYAWRK
LHSSANLPSPSRCKVVNRADCAVGSFRDDYRYPGLPHVTFIGRVKPETHYSKAGNINNCLYNEGANGRYLIIL
DTDMQPHPKFILATLPFFFDDEDRQDKAKYICCGVGCNAVAKLCCASCQIAGVPEEQISYCSKDCFENAMHVQ
SAVHRRQVNGTMSDTNASKIDMRCMNCDAKLPKSGVCRKCGNKGADGEDVSSLHTYSDDVRDNAVAFVQTPQY
FRDCIQLQIGDPMGHRNATFYDAIQTGQDGYDCASFAGTNAIFRREALDSIGGIQYGSLTEDCYTGQVLCSMG
WKAQYFRKDFEGEPSERIRLAEGLIPDSVAGSLAQRKRWAKGNFQIALMNKKTQYFDPEWKMPEVQVPTYHKS
NKFMRRVFYFNSTLYPLGSITAILFYYITIYFLFSGYAPIYMAGERLVYALVPKLLVQGLLSALSNRTVENSD
VIRSQEVWFAYAFTNCTAVLEAFWWKITGREPKWFNTGGAKRGSIAELPNVIIFFGTVVGILWAVVRFLAGYN
SIQTSHGASLLFASLMMGLFLAVKLAPSVRMSIQEYFGWSYESLMDQGNVVGSISIAFGLIFVTLWVWIEQPT
SNPF

**FIG. 1**




| | Control | NoPv1 20 µM | NoPv1 50 µM | NoPv1 100 µM | NoPv1 150 µM | NoPv1 200 µM | NoPv1 400 µM |
|---|---|---|---|---|---|---|---|
| 5 dpi | 31,84 | 22,32 | 7,96 | 0,60 | 0,00 | 0,00 | 0,00 |
| 7 dpi | 43,44 | 42,20 | 15,00 | 1,68 | 1,20 | 0,20 | 0,00 |

**FIG. 2**

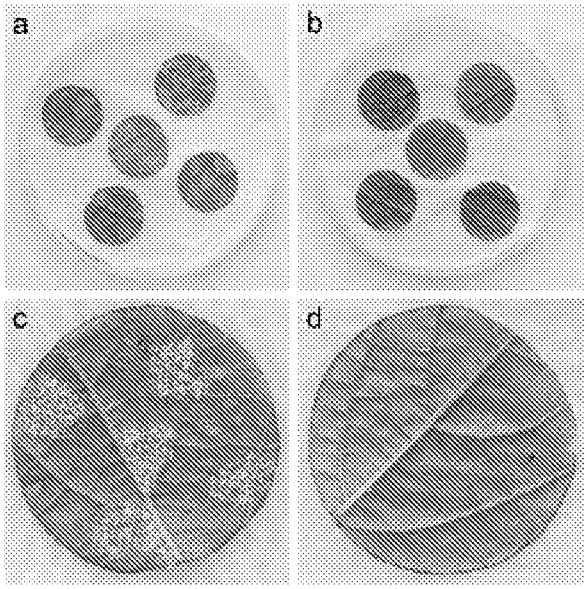
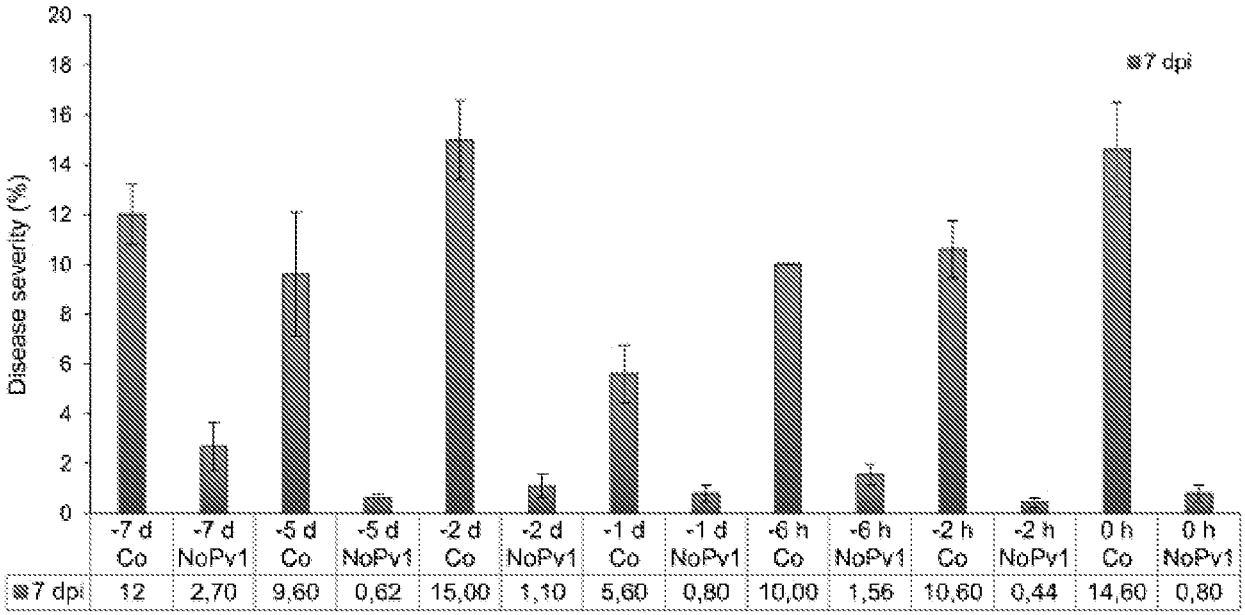
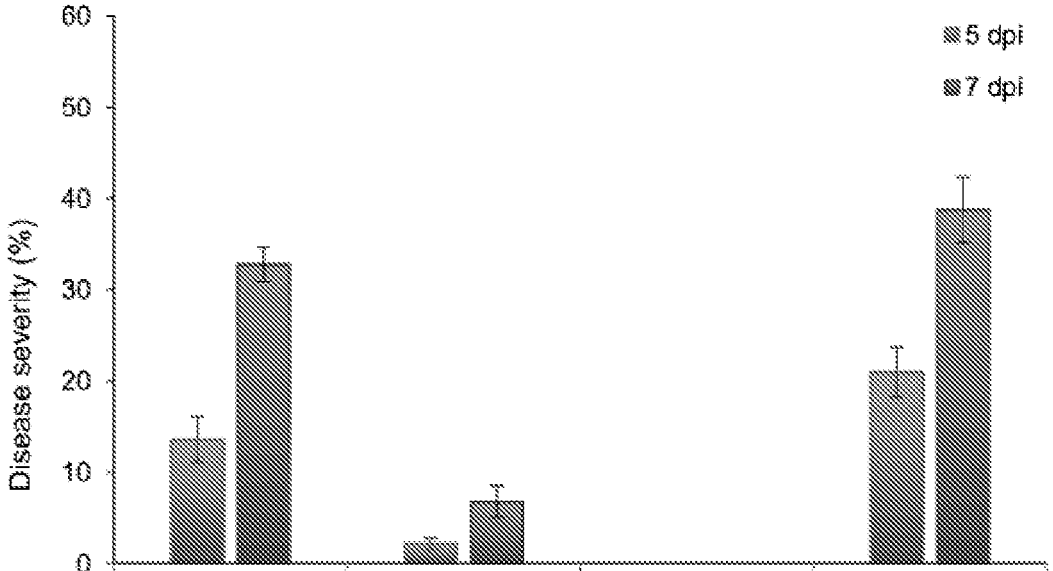



FIG. 3



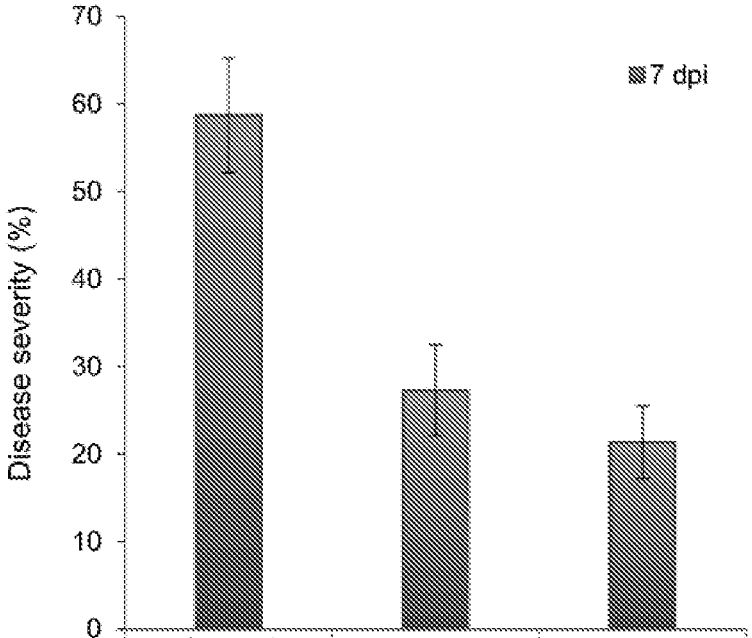

| | -7 d Co | -7 d NoPv1 | -5 d Co | -5 d NoPv1 | -2 d Co | -2 d NoPv1 | -1 d Co | -1 d NoPv1 | -6 h Co | -6 h NoPv1 | -2 h Co | -2 h NoPv1 | 0 h Co | 0 h NoPv1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ⊠7 dpi | 12 | 2,70 | 9,60 | 0,62 | 15,00 | 1,10 | 5,60 | 0,80 | 10,00 | 1,56 | 10,60 | 0,44 | 14,60 | 0,80 |

FIG. 4




| | Control | NoPv1 200 µM | Kocide (Copper) | Pergado |
|---|---|---|---|---|
| 5 dpi | 13,6 | 2,20 | 0,00 | 21,00 |
| 7 dpi | 32,80 | 6,80 | 0,00 | 38,80 |

FIG. 5




| | Control | NoPv1 400 µM | NoPv1 800 µM |
|---|---|---|---|
| 7 dpi | 58,71 | 27,28 | 21,32 |

FIG. 6

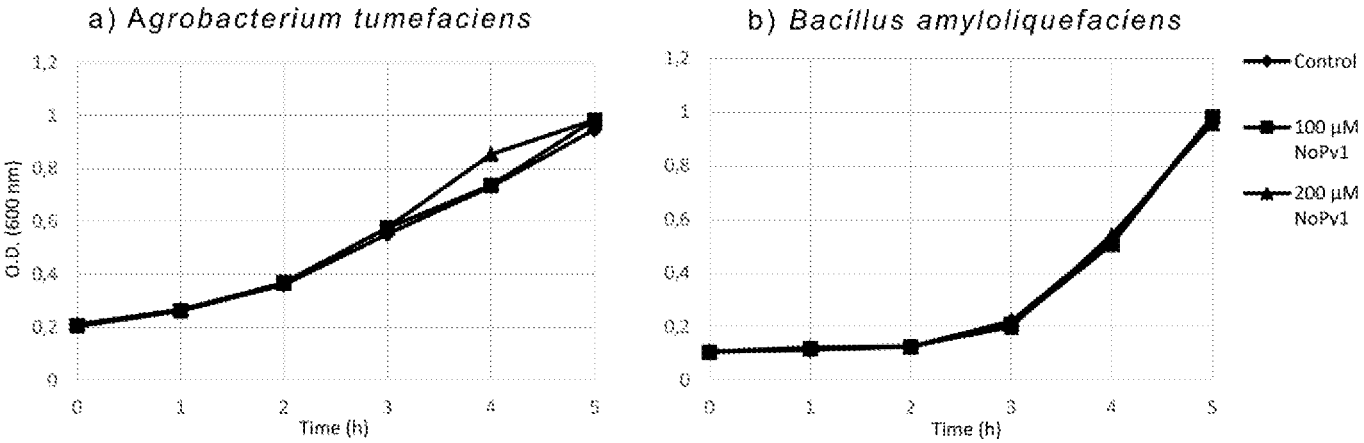
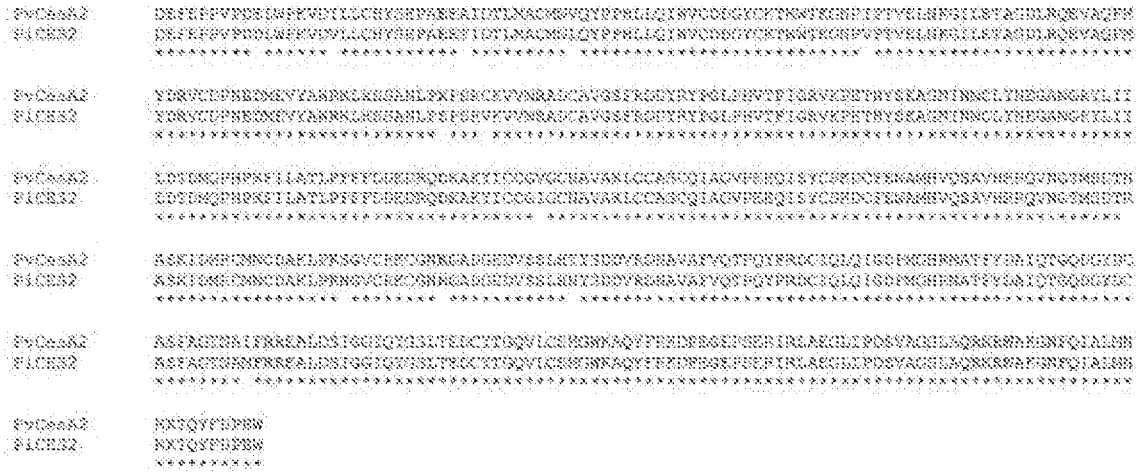
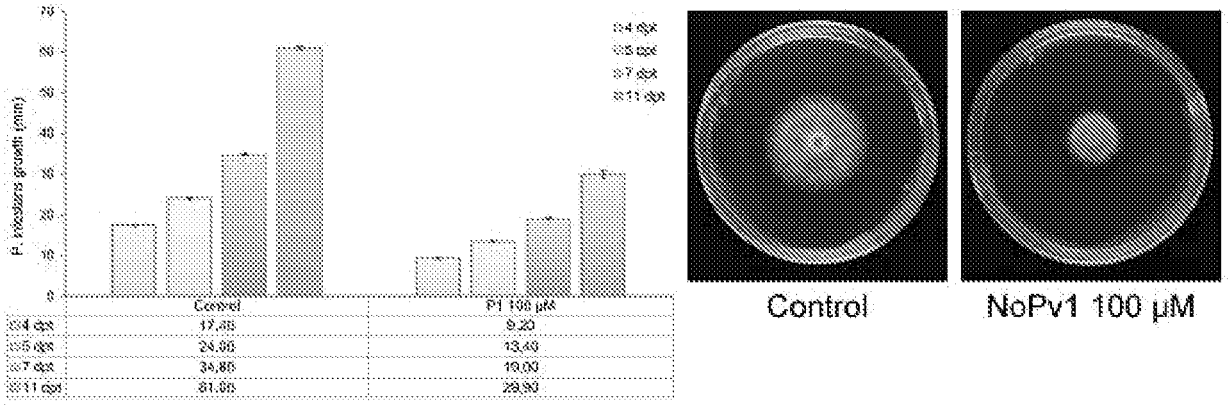

a) Agrobacterium tumefaciens
O.D. (600 nm)
1,2
1
0,8
0,6
0,4
0,2
0
0
1
2
3
4
5
Time (h)
b) Bacillus amyloliquefaciens
1,2
1
0,8
0,6
0,4
0,2
0
0
1
2
3
4
5
Time (h)
Control
100 µM NoPv1
200 µM NoPv1

FIG. 7

a

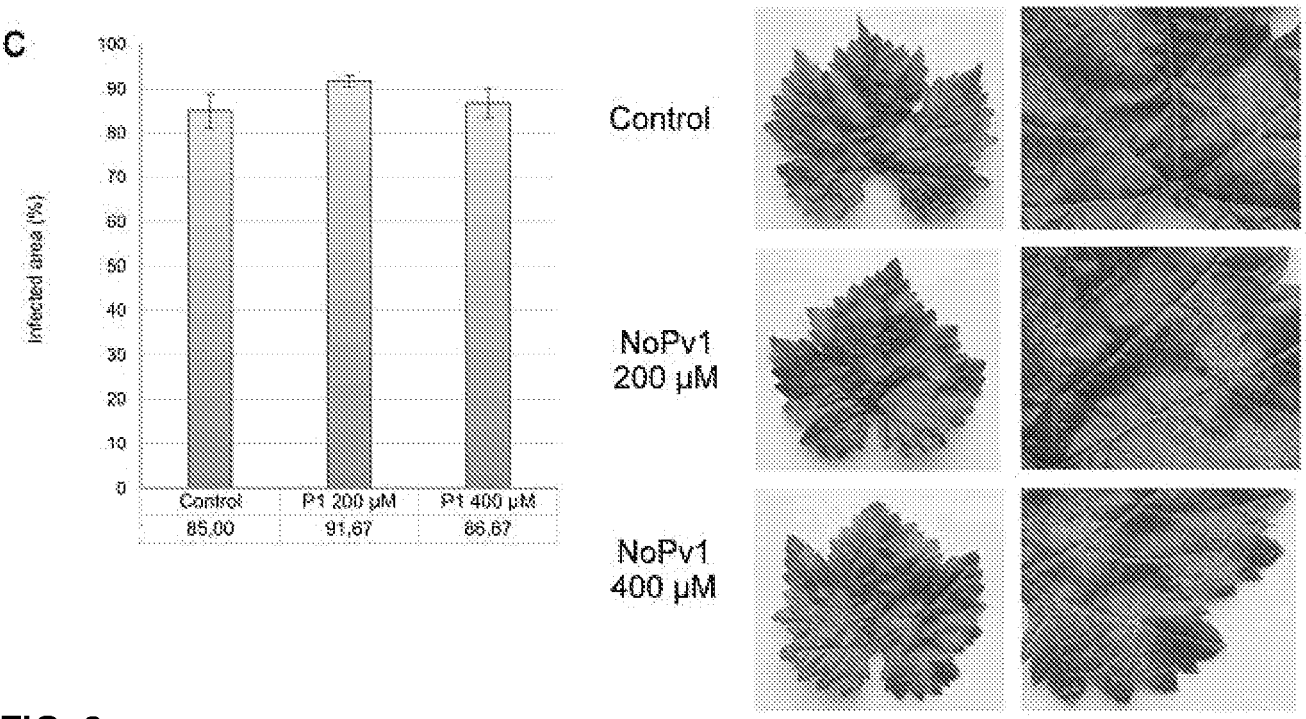


b




| | Control | P1 100 µM |
|---|---|---|
| 4 dpi | 17,48 | 9,20 |
| 5 dpi | 24,88 | 13,48 |
| 7 dpi | 34,88 | 19,00 |
| 11 dpi | 81,88 | 29,90 |

c




| Control | P1 200 µM | P1 400 µM |
|---|---|---|
| 85,00 | 91,67 | 86,67 |

FIG. 8

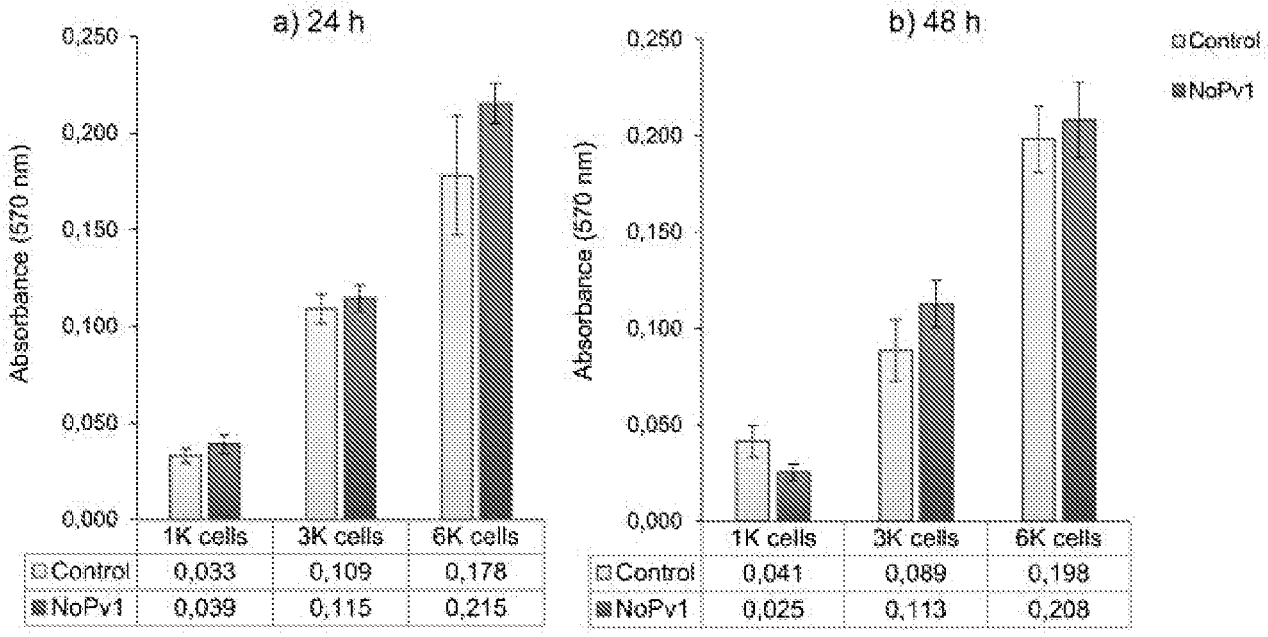
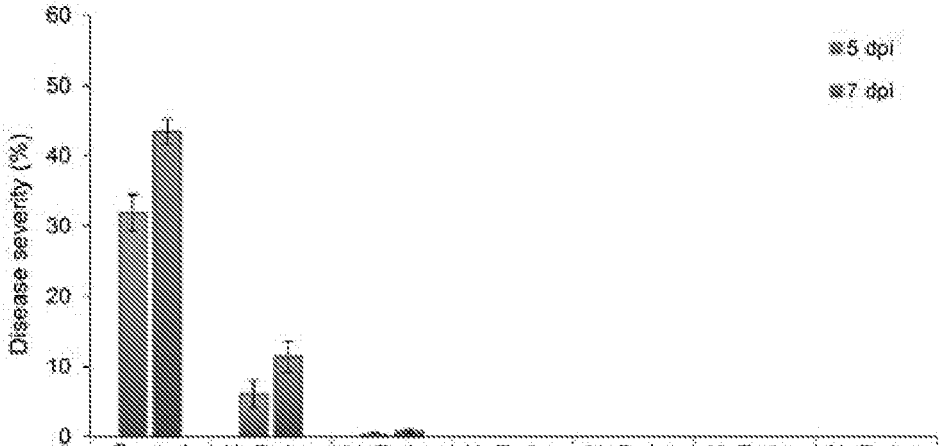

a) 24 h
b) 48 h
Absorbance (570 nm)
0,250
0,200
0,150
0,100
0,050
0,000
Control
NoPv1
1K cells
3K cells
6K cells
Control 0,033 0,109 0,178
NoPv1 0,039 0,115 0,215
Control 0,041 0,089 0,198
NoPv1 0,025 0,113 0,208

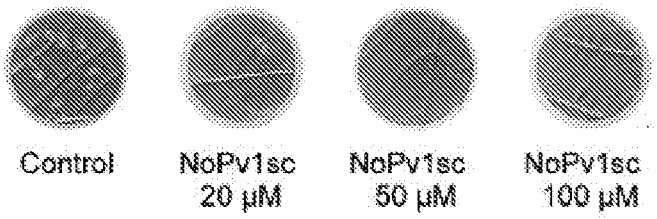
FIG. 9

a

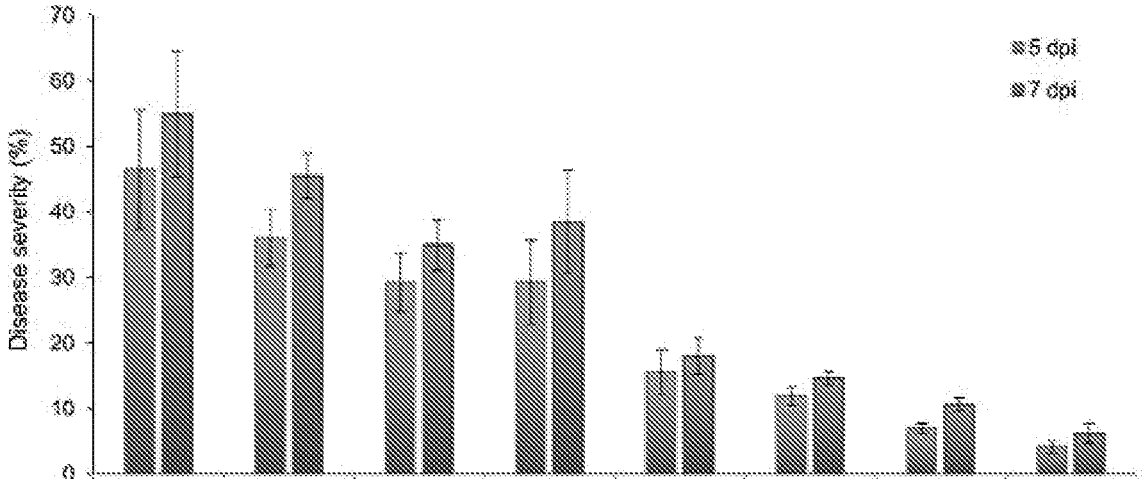



| | Control | NoPv1sc 20 µM | NoPv1sc 50 µM | NoPv1sc 100 µM | NoPv1sc 150 µM | NoPv1sc 200 µM | NoPv1sc 400 µM |
|---|---|---|---|---|---|---|---|
| 5 dpi | 31,84 | 6,08 | 0,28 | 0,00 | 0,00 | 0,00 | 0,00 |
| 7 dpi | 43,44 | 11,40 | 0,72 | 0,00 | 0,00 | 0,00 | 0,00 |

b




c




| | Control | NoPv1sc 50 µM | NoPv1sc 75 µM | NoPv1sc 100 µM | NoPv1sc 125 µM | NoPv1sc 150 µM | NoPv1sc 200 µM | NoPv1sc 400 µM |
|---|---|---|---|---|---|---|---|---|
| 5 dpi | 46,45 | 35,98 | 29,24 | 29,26 | 15,58 | 11,91 | 7,03 | 4,15 |
| 7 dpi | 55 | 45,49 | 34,96 | 38,43 | 18,02 | 14,63 | 10,63 | 6,21 |

FIG. 10

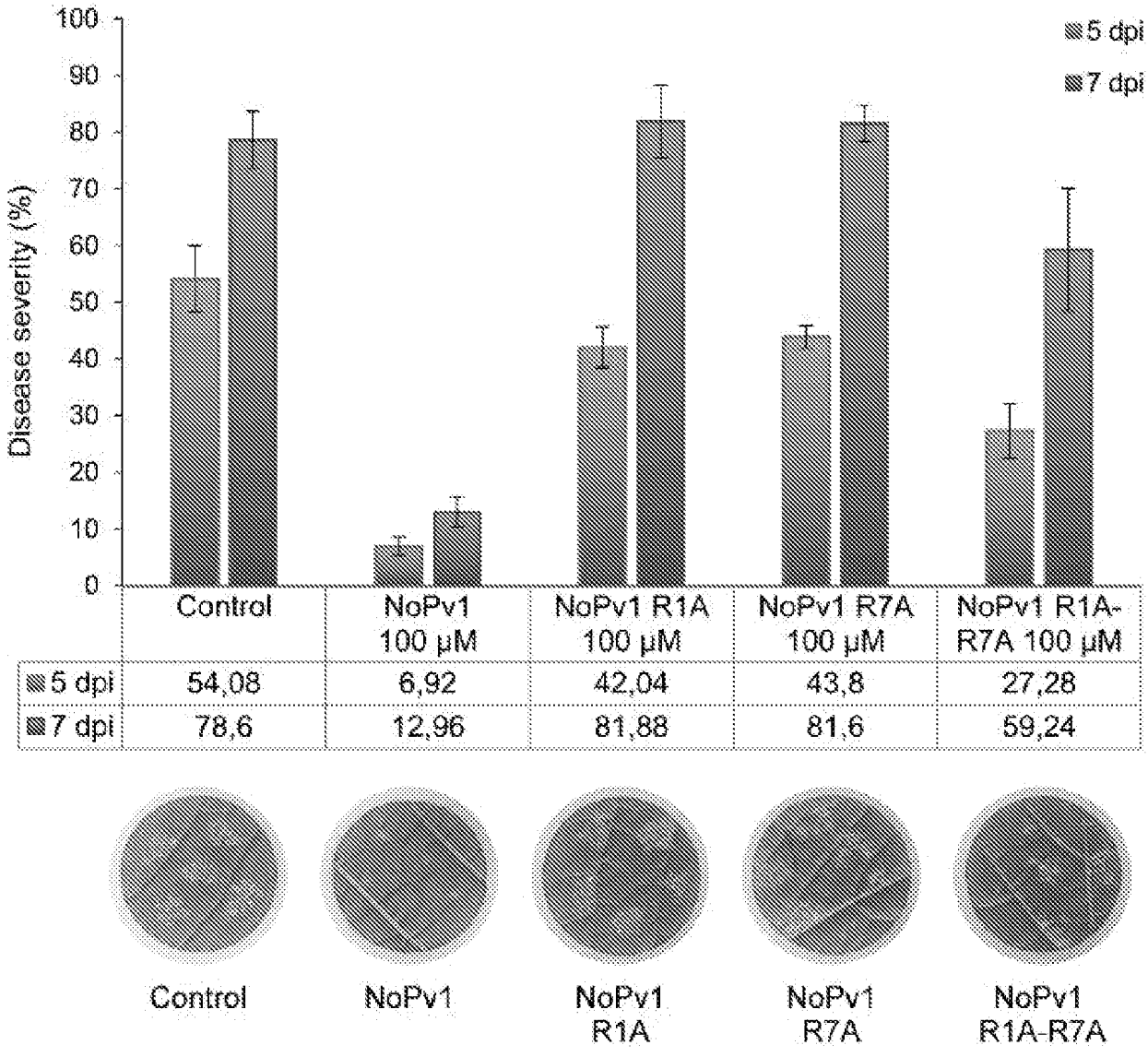

Disease severity (%)
100
90
80
70
60
50
40
30
20
10
0
5 dpi
7 dpi
Control
NoPv1 100 µM
NoPv1 R1A 100 µM
NoPv1 R7A 100 µM
NoPv1 R1A-R7A 100 µM
5 dpi 54,08 6,92 42,04 43,8 27,28
7 dpi 78,6 12,96 81,88 81,6 59,24
Control
NoPv1
NoPv1 R1A
NoPv1 R7A
NoPv1 R1A-R7A

FIG. 11

# INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2018/059834

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/06 A01N37/46
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, BIOSIS, WPI Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 2009/143133 A2 (VIRGINIA TECH IP)<br>26 November 2009 (2009-11-26)<br>the whole document<br>----- | 1-12 |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 6 May 2019 | 29/05/2019 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Wiame, Ilse |

1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2018/059834

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2009143133 A2 | 26-11-2009 | CA 2724569 A1 | 26-11-2009 |
| | | EP 2291188 A2 | 09-03-2011 |
| | | US 2010093601 A1 | 15-04-2010 |
| | | WO 2009143133 A2 | 26-11-2009 |