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Növények és vetőmag kezelése peronosporomycetes fertőzés ellen/alatt

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

A fordítást a szabadalmas az 1995. évi XXXIII. törvény 84/H. §-a szerint nyújtotta be. A fordítás tartalmi helyességét a Szellemi Tulajdon Nemzeti Hivatala nem vizsgálta.

The present invention relates to the treatment of plants and/or seeds against/in case of an affection by a pathogen of the class of Peronosporomycetes, namely *Phytophthora* sp.

Peronosporomycetes, formerly designated as Oomycota or Oomycetes, belong to the pathogens causing several redoubtable plant diseases. *Phytophthora infestans*, one of the most important species, can e.g. induce the leaf or late blight in the tomato or the leaf and tuber blight in the potato. In potatoes the affection results in significant losses of yields in particular in damp summers but in parts also in spring, where the pathogen finds optimum conditions for the proliferation, which usually can exceed 20 % of the average yield. In outdoor tomatoes the disease can even result in a complete loss the harvest.

The first symptoms of these diseases become visible on the leaves and stipes: starting from the edges of the leaves dark brown spots appear. They increase in size rapidly during humid weather or strong dew formation and form on the bottom of the leaves a white fungoid turf which reminds of mold. At the ends of the fungoid filaments spores container do develop, so-called sporangia. The spores are released at wet conditions and actively penetrate the plant tissue by the aid of a germ tube. At temperatures beyond 10 °C the sporangia also directly germinate.

Especially in ecological but also in conventional potatoe agriculture the primary affection, the so-called stipe affection, poses a significant problem since it can rapidly result in an early outbreak of a secondary affection. The pathogen reaches the tubers even during the growing period or at the harvest via wounds, natural intracellular openings, so-called lenticels, and germinal elements. Affected tubers comprise greyish blue discolored spots and brown tuber tissue without a sharp delimitation to healthy tissue. They are uneatable. The pathogen also overwinters in the tubers. Even one infected rotten tuber can be sufficient to induce an epidemic in a potato population.

Extensive research work performed at the Bavarian State Institute for Agriculture (Bayerische Landesanstalt für Landwirtschaft) in the last years shows, that at high soil moisture an infection occurs via the soil. Under such conditions spores form on the diseased plant tuber which are distributed by the soil water. By this manner neighbouring plants or stipes of the own plant can already become infected in the soil which likewise

grow up sick. This results in the typical *Phytophthora* infestive nests. The plants show already very early a stipe affection.

Other authors also come to the conclusion that after abundant rainfalls and high soil moisture *Phytophthora infestans* either grow from the infected plant tuber to the top in the stipe or sporulate on the surface of the mother tuber; see Radtke et al. (2000), Adler (2001), Zellner (2004).

So far controlling the leaf and tuber/late blights has not been solved in a satisfying manner. This is mainly because of e.g. *Phytophthora* sp. show resistances to active agents belonging to the group of phenylamides and carbamates as well as the active agent dimethomorph.

For a protection against an affection by *Phytophthora* sp. the potato plants or the seeds, i.e. the seed potatoes, therefore are often treated with copper solutions. However it has turned out that with copper sprayings the primary or stipe affection, in particular in the organic but also in the conventional potato growing, is hard to control. In addition, the use of copper results in significant environmental pollutions so that the European Community has aspirations to minimize the use of copper.

Pythium sp. is another important species belonging to the Peronosporomycetes which in several seedlings causes the so-called damping off disease, referred to in the English language as "damping off". Also the treatment of this plant disease has not been solved in a satisfactory manner.

Against this background the object underlying the invention is to provide an alternative to the currently used preparations for the treatment of plants and/or seeds against an affection by the pathogen of the class of Peronosporomycetes, namely *Phytophthora* sp., by means of which the use of copper can be preferably avoided or at least reduced.

This object is met by the use of the bacterium *Pseudomonas* sp. Proradix (DSMZ 13134).

The *Pseudomonas* strain Proradix which was deposited on 3 November 1999 under the deposition number DSM 13134 at the DSMZ, 38124 Braunschweig, Germany, according to the Budapest Treaty, is a new species within the RNA group I of the Pseudomonadaceae. A final allocation of Proradix has not taken place so far since only a

relatively low sequence similarity exists to species of the genus *Pseudomonas* which are described up to now.

The effectiveness of the *Pseudomonas* strain Proradix against an affection of plants and/or seeds by a pathogen of the class of Peronosporomycetes, such as *Phytophthora sp.*, was surprising and not to be expected.

The deposited *Pseudomonas* strain Proradix is extensively described in the WO 01/40441 to which herewith reference is made. There an effect of Proradix against black rot in salad and late blight in potato tubers is described. Both plant diseases are caused by *Rhizoctonia solani*.

Rhizoctonia solani is part of the real fungi, therefore belongs to the fungus kingdom (Fungi). *Rhizoctonia* is the asexual form of *Thanatephorus cucumeris* and is a kind of fungus that belongs to the group of basidia fungi and can be found worldwide. The basidia fungi include well-known edible mushrooms such as the porcino and the button mushroom.

It is further said in literature that the *Pseudomonas* strain Proradix can be used against an affection of plants or seeds by the genus *Fusarium sp.* *Fusarium* is a living being also belonging to the kingdom of real fungi, which can cause a variety of plant diseases which are referred to fusarioses.

Based on the experiments described in the WO 01/40441 and the remaining prior art a skilled person may therefore assume that the *Pseudomonas* strain Proradix can also be used for the treatment of plants and seeds against an affection by other fungi.

It was however completely surprising that Proradix has a positive effect on plants or seeds being affected by *Phytophthora sp.* These, however, do not belong to the real fungi (Fungi) but to the Chromalveolata.

The Chromalveolata are completely differently organized than the real fungi. In contrast to the fungi the Chromalveolata have plastids which originate from a secondary endosymbiosis with a representative of the achaeplastida. That means in the course of its evolutionary development they have incorporated a protozoan with plastids from endosymbionts and reduced the primary host to such a degree that only the plastid was left.

The Peronosporomycetes are therefore much closer related. With brown algae, gold algae, and diatoms than with the real fungi; see Adl et al. (2005). The Peronosporomycetes live in the water, in water animals, in the soil or in terrestrial plants as saprophytes or parasites.

Against this background one could have assumed that a substance which is effective against fungi is ineffective against an affection of plants or seeds by Peronosporomycetes.

Particularly surprising is the good effect of Proradix against an affection of plants or seeds by a pathogen of the class of Peronosporomycetes for the reason that the inventors have so far undisclosed results of a scientific examination which supposedly prove that Proradix should have no positive effect against *Pythium aphanidermatum*, a representative of the Peronosporomycetes; Hamelink and Paternotte (2008), "Efficacy of Proradix against *Pythium aphanidermatum* in cucumber". The inventors, however, were able to reproduce the positive effect of Proradix against this very pathogen in many cases and suspect that the opposite results may be caused by a methodical mistake of the experimentors.

The invention is fully solved with the provision of the *Pseudomonas* strain Proradix.

With the *Pseudomonas* strain Proradix for the first time an active agent is provided which is a real alternative to copper. The inventors could demonstrate by experiments in potato tubers and seeds of cucumbers in an impressive manner that in comparison to untreated plants the affection of the stipes in plants treated with the *Pseudomonas* strain Proradix as the only active agent (monotherapy) could be significantly reduced. As a consequence, by means of Proradix the leaf and tuber/late blight as well as the "damping off disease" can be treated or prevented in an effective manner. These positive effects of Proradix can be compared with those which can be achieved by the treatment with copper.

As the inventors were able to realize the *Pseudomonas* strain Proradix can significantly reduce an affection of not yet infected plants or tubers by *Phytophthora* sp. and in this way protect the harvest yields by a growing at the root.

It is therefore a particular advantage that the *Pseudomonas* strain Proradix can be prophylactically used in non-affected plants/seeds.

In fact, in the prior art so far no other bacterium is described which provides comparable positive results against an affection of plants or seeds by Peronosporomycetes.

The pathogen is *Phytophthora* sp.

This measure has the advantage that the most important phytopathogenic species within the Peronosporomycetes is treated in a targeted manner. As the inventors were able to demonstrate the *Pseudomonas* strain Proradix shows especially for this species an optimum effect.

It is preferred if the use of the bacterium *Pseudomonas* sp. Proradix according to the invention is directed against a primary affection or stipe affection by *Phytophthora* sp., preferably *Phytophthora infestans*.

This measure has the advantage that a large problem, which especially appear in organic potato production, is treated, which can poorly be controlled with copper sprayings and can rapidly result in an early outbreak of a secondary affection.

In a preferred variation according to the invention potato plants (*Solanum tuberosum*) and/or tubers hereof or the seeds of the cucumber (*Cucumis sativus*) are treated.

This measure has the advantage that two of the most important cultivated plants with large economic significance, which are very often affected by Peronosporomycetes or *Phytophthora infestans*, respectively, can be effectively protected or treated. As the inventors could demonstrate especially here Proradix provides particularly good results.

The *Pseudomonas* strain Proradix can be used in the context of a monotherapy as the only active agent but also in combination with other active agents. This may result in synergistic effects.

The *Pseudomonas* strain Proradix according to the invention can be provided in any formulation, such as a solution but also as a powder. Formulations of these kinds are known to the skilled person. Examples of formulations are given in the WO 01/40441 to which reference is explicitly made. Also suitable are formulations which are provided by the company Sourcon-Padena GmbH & Co. KG, Tübingen, Germany.

Also disclosed is a method for the production of a composition for the treatment of plants and/or seeds against an affection by a pathogen of the class of

Peronosporomycetes, comprising the following steps: (a) provision of the bacterium *Pseudomonas sp. Proradix* (DSMZ 13134) in a phytotherapeutically effective amount, (b) formulation of the bacterium.

The invention also relates to a method for the production of a composition for the treatment of plants and/or seeds against an affection by a pathogen of the class of Peronosporomycetes, namely *Phytophthora sp.*, comprising the following steps: (a) provision of plants and/or seeds, (b) incubation of the plants/seeds with a solution containing the bacterium *Pseudomonas sp. Proradix* (DSMZ 13134), wherein the solution is preferably administered by mordanting, spraying or immersion of the plants/seeds.

The features, advantages and characteristics of the use according to the invention apply likewise to the methods according to the invention.

The invention is now explained in more detail by means of embodiments resulting in further characteristics, features and advantages. The embodiments are for illustrative purposes and do not limit the scope of the invention.

Example 1: Scientific classification of the Peronosporomycetes *Phytophthora infestans* and *Pythium ultimum* or *Pythium aphanidermatum*

Phytophthora infestans is scientifically classified as follows:

Classification:	Living being
Domain:	Eucaryota
Phylum:	Chromalveolata
Subphylum:	Stramenopile
Class:	Peronosporomycetes
Order:	Peronosporales
Family:	Peronosporaceae
Genus:	<i>Phytophthora</i>
Species:	<i>Phytophthora infestans</i>

Pythium ultimum and *Pythium aphanidermatum* are scientifically classified as follows:

Classification:	Living being
Domain:	Eucaryota

Phylum:	Chromalveolata
Class:	Peronosporomycetes
Order:	Pythiales
Family:	Pythiaceae
Genus:	<i>Pythium</i>
Species:	<i>Pythium ultimum</i> or <i>Pythium aphanidermatum</i>

Phytophthora infestans, *Pythium ultimum* and *Pythium aphanidermatum* therefore belong to the kingdom of Chromalveolata, however not to the kingdom of real fungi (Fungi).

[0045] The basidiomycete *Rhizoctonia solani*, which is described in the prior art as being treatable with Proradix, is, however, scientifically classified as follows:

Classification:	Living being
Domain:	Eucaryota
Phylum:	Real fungi (Fungi)
Strain:	Basidiomycota
Class:	Basidiomycetes
Order:	Polysporales
Family:	Corticaceae
Genus:	<i>Rhizoctonia</i>
Species:	<i>Rhizoctonia solani</i>

Rhizoctonia solani belongs therefore to the kingdom of real fungi and to the class of basidiomycetes to which also the edible mushrooms such as the button mushroom or porcino belong.

In prior art furthermore an effect of Proradix against *Fusarium* is described. *Fusarium* is scientifically classified as follows:

Classification:	Living being
Domain:	Eucaryota
Phylum:	Real fungi (Fungi)
Strain:	Ascomycota
Class:	Sordariomycetes
Order:	Hypocreales

Family: Nectriaceae
Genus: *Fusarium*

Also *Fusarium* belongs therefore to the real fungi.

The inventors were therefore able to demonstrate in a completely surprising manner that an active agent which is so far only described with respect to real fungi can also be used against non-fungi, namely against pathogens of the class of Peronosporomycetes.

Example 2: Isolation of Proradix.

The isolation of the bacterium *Pseudomonas* sp. Proradix is described in detail in the WO 01/40441, there example 1. Reference is explicitly made thereto and the disclosure there is made the disclosure of the present invention.

Example 3: Treatment of potatoes with Proradix.

Potato tubers (*Solanum tuberosum*) of the variety "Hansa" were artificially infected by *Phytophthora infestans* by means of a dosing-syringe. Healthy tubers of the variety "Fasan" were mordanted with a Proradix solution (Proradix®WG, Sourcon-Padena GmbH & Co. KG, Tübingen, Germany) according to the manufacturer's instructions. The concentration of the aqueous liquid mordant was approximately 60 g Proradix bacteria per hectare or approximately 4×10^{12} CFU per 2,500 kg potato tubers.

As a reference, in parallel healthy tubers of the variety "Fasan" were mordanted with an aqueous copper solution (Cuprozin®, Spiess-Urania Chemicals GmbH, Hamburg, Germany) according to the manufacturer's instructions. The concentration was approximately 460.6 g copper hydroxide per liter, corresponding to approximately 300 g/l of pure copper. About 0.4 l of this solution were applied per hectare or per approximately 2,500 kg of potato tubers.

The planting of both varieties was made by hand on a testing area in Sanitz of the company AGRO Service Nord GmbH, Wietzenhof, Germany. For this purpose the planting locations were exactly marked each 30 cm. The subsoil was compacted so that the rain water was withheld for a while and not directly drained off, since a sporulation of *Phytophthora infestans* only happens at wet conditions. Then, at first the healthy tubers of the variety "Fasan" were planted. Afterwards, the infected tubers of the variety "Hansa"

were directly placed on the tubers of the variety "Fasan" to be examined and slightly earthed up. This creates the preconditions for a potential affection of the healthy non-infected tubers of the variety "Fasan". The germinated plants of the variety "Hansa" were removed. By doing so, the evaluation only of the germinated mordanted plants of the variety Fasan could be realized in a targeted manner.

After a cultivation of approximately two months the affection of the variety Fasan by *Phytophthora infestans* was evaluated in the respective parcels once a week until the harvest after additional approximately two months.

This experiment was executed two times, one time in the year 2008 and one time in the year 2009. In the following table I the result is summarized:

Table I

Treatment	Stipe affection	
	First approach (year 2008)	Second approach (year 2009)
Untreated tubers	5 %	91.8 %
Tubers treated with Proradix	1.5 %	30 %
Tubers treated with copper	0.7 %	16.3 %

These results demonstrate in an impressive manner that a treatment of potato tubers with the bacterium *Pseudomonas sp.* Proradix results in a significant decrease of the primary or stipe affection by the Peronosporomycet *Phytophthora infestans*, which is comparable to such achieved by a copper treatment.

Example 4: Treatment of cucumber seeds with Proradix (not part of the invention)

5 g of Proradix (Proradix®WG, Sourcon-Padena GmbH & Co. KG, Tübingen, Germany) were dissolved in 50 ml water for 30 to 60 min. In this solution about 60 seeds of the cucumber (*Cucumis sativus*) were incubated for additional 30 to 60 min, 24 h pre-

germinated and then s-seeded in soil which was contaminated with *Pythium ultimum*. In a parallel approach as a control the same amounts of seeds were treated in water without Proradix in a similar manner. The cultivation was effected over 2 weeks. In the following the number of germinated plants not affected by the damping off disease was evaluated. This experiment was in view of the data of Hamelink and Paternotte (loc. cit.) performed in a total of three times. The result is shown in the following table II.

Table II

Treatment	Germination rate First approach (year 2008)	Germination rate Second approach (year 2009)	Germination rate Third approach (year 2009)
Untreated seeds	51 %	66 %	58 %
Seeds treated with Proradix	73 %	85 %	71 %

This experiment demonstrates in an impressive manner that Proradix is also in the position to protect cucumber seedlings from the damping off disease caused by *Pythium ultimum*.

The inventors have conducted the same experiment under identical conditions on a land with soil with *Pythium aphanidermatum* and achieved comparable results. They were not able to confirm the conclusions of Hamelink and Paternotte (loc. cit.). According to the findings of the inventors Proradix shows positive effects against all species of the genus *Pythium*.

Conclusion:

The inventors were surprisingly able to demonstrate that the *Pseudomonas* strain Proradix, which is so far only described for the treatment of plant diseases caused by fungi, can be successfully used for the treatment of plants and/or seeds which are affected by a pathogen of the class of Peronosporomycetes, thus by a pathogen that does not belong to the fungi. This could be exemplarily demonstrated with the genera *Phytophthora* and *Pythium* and the potato and cucumber. The *Pseudomonas* strain Proradix can be

provided as a solution. The solution can be directly used for the treatment of the plants and/or seeds by the way of spraying, mordanting or immersion. The solution can also be vacuum-dried, and the obtained powder is dissolved in water before use.

SZABADALMI IGÉNYPONTOK

1. *Pseudomonas sp.* Proradix (DSMZ 13134) baktérium alkalmazása növények és/vagy vetőmag kezelésére *Phytophthora sp.* fertőzés ellen/alatt.
2. Az 1. igénypont szerinti alkalmazás, azzal jellemezve, hogy a kórokozó *Phytophthora infestans*.
3. Az előző igénypontok bármelyike szerinti alkalmazás, azzal jellemezve, hogy a növény burgonya (*Solanum tuberosum*) és/vagy ennek gumója.
4. Az 1-3. igénypontok bármelyike szerinti alkalmazás, azzal jellemezve, hogy a kezelés levélfoltosság és gumómételey ellen történik.
5. Az 1-4. igénypontok bármelyike szerinti alkalmazás, azzal jellemezve, hogy a vetőmag uborka (*Cucumis sativus*) vetőmagja.
6. Az 1-5. igénypontok bármelyike szerinti alkalmazás, azzal jellemezve, hogy a növények és/vagy a vetőmag kezelése a palántadőlés betegség („damping off”) ellen történik.
7. Eljárás növények és/vagy vetőmag kezelésére *Phytophthora sp.* fertőzés ellen/alatt, az alábbi lépésekkel:
 - a) előkészítjük a növényeket és/vagy a vetőmagot,
 - b) a növényeket/vetőmagot *Pseudomonas sp.* Proradix (DSMZ 13134) baktériumot tartalmazó oldattal inkubáljuk.
8. A 7. igénypont szerinti eljárás, azzal jellemezve, hogy a (b) lépést az oldatot a növények/vetőmag csávázásával, permetezésével vagy merítéssel kezeléssel visszük fel.