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(54) Titre : TRAITEMENT DE SEMENCES ET/OU DE PLANTES AU MOYEN D'UNE CULTURE DE PSEUDOMONAS
(54) Title: TREATMENT OF SEEDS AND/OR PLANTS WITH PSEUDOMONAS CULTURE

(57) Abrégé/Abstract:
The invention relates to Pseudomonas strain termed Proradix, deposited under the Accession number PRORADIX - DSM 13134, for use in the treatment of seeds or plants.

Abstract

The invention relates to Pseudomonas strain termed *Proradix*, deposited under the Accession number PRORADIX - DSM 13134, for use in the treatment of seeds or plants.

Treatment of Seeds and/or Plants with Pseudomonas Culture

The present Invention relates to the treatment of seed and plants with bacteria of the genus Pseudomonas and to a new species of the genus Pseudomonas, which is particularly suitable for this purpose.

A method of strengthening and protecting plants in which microorganisms of the genus Pseudomonas are employed is already disclosed in DE 197 39 364 AI.

In the known method, at least one resistance inductor together with a useful microorganism is introduced into the plants' nutrient medium or into the seed of the plant, resistance inductor and microorganism having a complementary positive effect on the plant.

An example of a useful microorganism which may be mentioned is the strain Pseudomonas sp. Ps1 of the genus Pseudomonas fluorescens, which is deposited at the DSMZ.

This publication furthermore describes a method of how to isolate such a useful microorganism from soil and roots. In this method, a dilution series from soil and roots is plated out, incubated and tested for fluorescent colonies which are then used in a screening against harmful fungi. Furthermore, a strain found by the known method is studied for its combination ability with resistance inductors. In this manner, the strain Pseudomonas sp. Ps1 has been found.

However, experiments carried out by the applicant of the present application have revealed that the treatment described in the publication stated at the outset is as yet unsatisfactory, which can be attributed, inter alia, to the fact that expensive resistance inductors must be employed together with the microorganism described, and it has furthermore been revealed that the protective action is frequently unsatisfactory.

US 2,932,128 discloses a method for the treatment of seed in which the seed is moistened with a bacterial solution and then exposed to a vacuum in order to impregnate the seed.

In view of the above, it is an object of the present invention to provide novel solutions and methods and a novel microorganism for the treatment of seed and/or in plants.

In accordance with the present invention, there is provided a Pseudomonas strain termed Proradix, which was deposited under the provisions of the Budapest Treaty on 03.11.1999 at the DSMZ, 38124 Braunschweig under the deposit number Pseudomonas sp. PRORADIX - DSM 13134.

The Pseudomonas strain Proradix, which, according to studies by the DSMZ, probably constitutes another species within RNA group I of the Pseudomonadaceae, has been isolated by the inventor of the present application. A final attribution of Proradix was not possible, however, since only relatively little sequence similarity exists with validly described species of the genus Pseudomonas.

Field trials with the species Proradix have shown that it is a useful bacterium which is capable of colonizing the roots of useful plants, and there can exert its effect on the plant. It has been demonstrated in that connection that one application per vegetation period suffices and that additional resistance inductors can be dispensed with.

Proradix can be employed in a formulation as pickling liquor or as a powder and is suitable for the treatment of vegetable seed, in particular lamb's lettuce and carrots, of lawn seed and seed of woody species. Furthermore, it may be employed in green-manure seed for the biological soil conditioning and decontamination, and for the seed treatment and immersion treat-

ment of potatoes. In all these applications, Proradix has shown an improved quality compared to untreated control groups and in some cases also in comparison with chemical fungicides.

According to an aspect of the invention there is provided a method for the preparation of a solution for the treatment of plants and/or seed, comprising the steps:

a) providing an isolate of useful bacteria, preferably of the genus Pseudomonas,

b) providing a culture medium containing phosphorus compounds, nitrogen compounds and succinic acid,

c) inoculating the culture medium with the isolate, and

d) incubating the culture medium at approximately 26-32°C with gentle shaking for at least 50 hours.

According to a further aspect of the present invention there is provided pseudomonas strain termed Proradix, which was deposited on 03.11.1999 at the DSMZ, 38124 Braunschweig, under the provisions of the Budapest Treaty under the Accession number PRORADIX - DSM 13134.

The inventor has recognized that a solution thus prepared brings about a conditioning of the useful bacteria, which contributes to an improved protective action in the plants/seed treated with the bacteria. The inventor's current explanation of this conditioning effect is the induction of the formation of exopolysacoharides due to degradation products of the succinio acid. The exopolysaccharides are a protective substance forming a mucus which makes the bacteria particularly well storable on the seed/the plants.

In this context, the medium in which the bacteria are grown especially preferably contains K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$ and succinic acid, preferable contents being approximately 6.0 g of K_2HPO_4, approximately 3.0 g of KH_2PO_4, approximately 1.0 g of $(NH_4)_2SO_4$, approximately 0.2 g of $MgSO_4$ and approximately 4.0 g of succinic acid in approximately 1000 ml of deionized H_2O.

The inventor has found that exopolysaccharides are formed particularly rapidly in this medium, the cell density in a solution prepared by this method being less decisive for the seed treatment than the abovementioned conditioning.

On the other hand, the culture medium preferably contains glucose, preferred contents being approximately 6.0 g of K_2HPO_4, approximately 3.0 g of KH_2PO_4, approximately 1.5 g of $(NH_4)_2SO_4$, approximately 0.2 g of $MgSO_4$, approximately 2.0 g of glucose and approximately 4 g of succinic acid in approximately 1000 ml of deionized H_2O.

With this composition of the culture medium, a higher cell density can be achieved since the concentration of the carbon source present is higher. However, the inventor has recognized that exclusively glucose in the culture medium would rapidly lead to an acidic pH, which does not make possible further cultivation. This is why a mixture of glucose and succinic acid is chosen as carbon source so that the glucose is first metabolized, giving rise to a high cell density, but a low pH. After the glucose has been consumed, the succinic acid is metabolized, whereby the pH climbs, entailing survival, but slower growth, of the bacteria. This leads to the abovementioned for-

mation of exopolysaccharides and the simultaneous improved action of microorganisms grown in such a solution.

Naturally, it is particularly preferred in this context to employ an isolate of the species Proradix.

The invention furthermore relates to a solution prepared by the method described so far.

On the other hand, it is also possible to dry the solution in vacuo, which is advantageous particularly when the culture medium contains glucose, so that the resultant powder contains bacteria in high concentrations. This powder can then be dissolved in water for use in the field, it being possible to employ the water treated thus for spraying seed, potatoes and the like.

Against this background, the present invention also relates to a powder prepared thus.

Furthermore, the invention relates to a method for the treatment of seeds in which seed moistened with the novel solution is temporarily exposed to sub-atmospheric pressure.

This is because the inventor of the present application has recognized that bacteria, in particular bacteria conditioned in accordance with the novel method, preferably the species Proradix, are suitable for the vacuum treatment of seed and lead to outstanding protective results.

On the other hand, the invention also relates to a method in which the seed/the plants is/are sprayed with a solution of the novel powder in water. This simple method, too, results in an effective protection of the treated plants.

Finally, the invention also relates to the use of the Pseudomonas strain Proradix for the treatment of plants, and to plants and seed treated by the novel method or in accordance with the novel use.

Further advantages will be seen from the following description of examples for growing and using the novel method and the species Proradix.

Of course, the features which have been mentioned hereinabove and are yet to be demonstrated hereinbelow can be used not only in the combinations stated specifically, but also in other combinations or alone, while still being within the scope of the present invention.

Example 1: Isolation of the species Proradix

Soil samples and roots were suspended and plated in dilution series in a selective medium so that individual colonies where obtained. Fluorescent colonies were employed for an in vitro screening, in which over 500 isolates were tested for inhibiting the growth of phytopathogenic fungi, in particular the soil-dwelling fungus Rhizoctonia. Candidates of interest were selected and tested on plants and subsequently in field trials.

In these tests, the inventor of the present application has isolated the species Proradix (DSM 13134) of the genus Pseudomonas which, according to identification by the DSMZ, is another species within the RNA group I of the Pseudomonadaceae. A final attribution of Proradix was not possible, however, since only relatively little sequence similarity exists with validly described species of the genus Pseudomonas, but there is no doubt that the microorganism is a fluorescent representative of the Pseudomonas RNA group 1.

Example 2: Culturing the isolate

a) For the treatment of seeds, a Pseudomonas isolate, preferably the species Proradix, is inoculated into the following culture medium:

K_2HPO_4	6.0 g
KH_2PO_4	3.0 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4$	0.2 g
succinic acid	4.0 g

made up to 1000 ml with deionized H_2O.

The inoculated culture medium is incubated for 72 hours at 28°C/100 rpm; this gives a "liquid seed pickle".

b) To prepare a pulverulent formulation, a Pseudomonas isolate, preferably the species Proradix, is grown in the following culture medium:

K_2HPO_4	6.0 g
KH_2PO_4	3.0 g
$(NH_4)_2SO_4$	1.5 g
$MgSO_4$	0.2 g
glucose	2.0 g
succinic acid	4.0 g

made up to 1000 ml with deionized H_2O.

Depending on the concentration of the inoculum, the culture medium inoculated thus is incubated for 65-70 hours at 28-30°C/100 rpm.

The solution is subsequently made into a powder in a vacuum drying oven. Formulation auxiliaries: dry skim milk and gum arabic.

c) Both culture media induce the formation of exopolysaccharides, while this formation takes place very rapidly in the case of the culture medium described under a), the cell concentration is of no importance, since seed treatment does not require large amounts of bacteria.

However, a high cell concentration is indeed required in the case of the medium described under b), while the formation of exopolysaccharides is still desired. This is achieved by the mixture of glucose and succinic acid. First, the glucose is metabolized, which leads to a reduced pH, but to a high cell concentration. Once the glucose is consumed, the succinic acid is used as carbon

source, and its degradation products lead to a rise in the pH and to the formation of exopolysaccharides.

Example 3: Treatment of seed with Proradix

One possible treatment consists in dissolving the powder prepared in Example 2b) in water and using it to spray the seed or the young plants before planting.

Furthermore, it is also possible to spray the solution prepared with the culture medium described in Example 2a) directly onto seed/plants before planting.

If the treated seed is still to be stored before planting, it is first moistened with the solution described in Example 2a), and then introduced into a sealable chamber in which a temporary vacuum is generated.

In this manner, the bacteria penetrate the seed and can still efficiently exert their protective action, even after prolonged storage.

Example 4: Treatment of young lettuce plants of variety Garunda

Young plants of variety Garunda which are to be planted in one hectare of arable land are treated by pouring a solution of 50 ml Proradix (10^7 cfu/ml) per plant (Example 2b) over the young plants before they are planted up.

After harvesting, only 50% of the plants were not of marketable quality, while in the case of an untreated control 70% of the harvested plants were not of marketable quality.

In a comparative treatment with the chemical fungicides Switsch (NOVARTIS) (0.8 kg/ha) and Risolex (Spiess Urania) (3 l/ha), 58 and 48%, respectively, were not of marketable quality.

This result demonstrates that the lettuce is markedly less susceptible to black rot following treatment with Proradix, and the results are at least as good as in the case of treatment with chemical fungicides.

Example 5: Treatment of potatoes

Proradix is formulated as a powder as described in Example 2b), and the powder is dissolved in water as follows:

60 g of powder per 80 l of water
for 1 ha with seed potatoes
60 g of powder $\triangleq$ 4×10^{12} cfu

The potato tubers are sprayed automatically with this solution and then planted directly. As an alternative, the potato tubers are dusted directly with the powder.

Comparative experiments between untreated potatoes (control), potatoes treated with Proradix and potatoes treated with FZB24 (Bayer Leverkusen, base: Bacillus subtilis) gave approximately identical yields of marketable produce per hectare. However, the quality showed that treatment with Proradix afforded reli-

able protection against black speck (Rhizoctonia attack), approximately 45% of the harvested potatoes showing no symptoms and a further approximately 45% showing minor symptoms. In the control group, the corresponding figures are 10% and 50%, and 25% and 50% in the case of FZB24.

Again, superiority of Proradix to the conventional crop protection products is demonstrated.

Example 6: Treatment of carrots sold clean

Carrot seed is moistened with Proradix seed treatment of Example 2a) as described in Example 3 and vacuum- treated (seed infiltration), stored and then sown.

The harvested carrots were stored for 4 months at 4°C, then - as is customary in sales - stored for two weeks at room temperature and finally assessed.

The quality showed 50% without blemish for the variety Nerac (untreated control: 18%) and 27% of useless carrots (control: 58%). The figures for the variety Mocum were 36% (control: 14%) and 46% (control: 54%), respectively.

Thus, the treatment of carrots sold clean with Proradix leads to a markedly increased product quality.

In conclusion, it can therefore be said that for the treatment of plants and/or seed an isolate of useful bacteria, preferably of the genus Pseudomonas, preferably the species Proradix, is provided and incubated in a culture medium containing phospho-

rus compounds, nitrogen compounds and succinic acid. The solution can be used directly for spraying plants and/or seed, optionally followed by vacuum treatment. Furthermore, the solution can be vacuum-dried, the powder being dissolved in water prior to use.

Claims

1. Pseudomonas strain termed *Proradix*, which was deposited on 03.11.1999 at the DSMZ, 38124 Braunschweig, under the provisions of the Budapest Treaty under the Accession number PRORADIX - DSM 13134.

2. Method for the preparation of a solution for the treatment of plants and/or seed, comprising the following steps:

a) providing an isolate of Pseudomonas strain of claim 1;

b) providing a culture medium comprising phosphorus compounds, nitrogen compounds and succinic acid;

c) inoculating the culture medium with the isolate; and

d) incubating the culture medium at approximately 26 - 32°C with gentle shaking for at least 50 hours.

3. Method according to claim 2, wherein the culture medium comprises K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$ and succinic acid.

4. Method according to claim 2 or 3, wherein the culture medium further comprises glucose.

5. Method according to claim 3, wherein the culture medium comprises approximately 6.0g of K_2HPO_4, approximately 3.0g of KH_2PO_4, approximately 1.0g of $(NH_4)_2SO_4$, approximately 0.2g of $MgSO_4$, and approximately 4.0g of succinic acid in approximately 1000 ml of deionized H_2O.

6. Method according to claim 4, wherein the culture medium comprises approximately 6.0g of K_2HPO_4, approximately 3.0g of KH_2PO_4, approximately 1.5g of $(NH_4)_2SO_4$, approximately 0.2g of $MgSO_4$, approximately 2.0g of glucose and approximately 4.0g of succinic acid in approximately 1000 ml of deionized H_2O.

7. Method according to claim 6 wherein after incubation, the solution is vacuum-dried to prepare a powder.

8. A solution prepared by the method of any one of claims 2 to 6.

9. A powder prepared by the method of claim 7.

10. A method of treating seeds or plants, comprising the following steps:

a) moistening the seeds or plants with the solution of claim 8;

b) applying a vacuum to the seeds or plants moistened above; and

c) releasing the vacuum.

11. Method for treating seeds or plants, comprising the following steps:

a) dissolving the powder of claim 9 in water to obtain a solution; and

b) spraying or immersion treatment of the seed with the solution of step a) or pouring the solution of step a) over plants.

12. Use of the pseudomonas strain *Proradix* of claim 1 for the treatment of plants and/or seed.