AU8939491

(12) PATENT ABRIDGMENT (11) Document No. AU-B-39491/89
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 636112

(54) Title
METHOD FOR CULTIVATING MICROORGANISMS, PARTICULARLY OF THE FRANKIA GROUP, AND PREPARATION OF BACTERIAL INOCULUMS

International Patent Classification(s)
(51)[4] **C12N 011/04** **C12N 001/00** **C12N 001/04** **C12N 001/20**
C12N 011/10

(21) Application No. : **39491/89** (22) Application Date : **11.08.89**

(43) Publication Date : **30.11.89**

(44) Publication Date of Accepted Application : **22.04.93**

(62) Related to Division(s) : **67223/87**

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(56) Prior Art Documents
AU 551717 81153/82 C12N 11/10
AU 80764/82 C12N 11/04

(57) Claim

1. A method for the preparation of a microbial inoculum which comprises the following sequential steps:

1. entrapping microorganisms in a polymer matrix,

2. incubating said polymer matrix entrapped microorganisms in a medium which enables said microorganisms to grow until colonies are formed within said matrix, and

3. dehydrating said polymer matrix with entrapped colonies of microorganisms.

wherein said microorganisms are obtained from a two-phase process comprising the following sequential steps:

a) culturing microorganisms in a solid nutrient medium; and,

b) dividing into fragment said solid nutrient medium containing colonies of said cultured microorganisms.

P/00/011

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Form 10

PATENTS ACT 1952–1973

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE

Class:

Int. Cl:

Application Number:
Lodged:

Complete Specification—Lodged:
Accepted:
Published:

Priority:

Related Art:

TO BE COMPLETED BY APPLICANT

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Complete Specification for the invention entitled:

METHOD FOR CULTIVATING MICROORGANISMS, PARTICULARLY OF THE FRANKIA GROUP, AND PREPARATION OF BACTERIAL INOCULUMS

The following statement is a full description of this invention, including the best method of performing it known to me:—*

*Note: The description is to be typed in double spacing, pica type face, in an area not exceeding 250 mm in depth and 160 mm in width, on tough white paper of good quality and it is to be inserted inside this form.

11710/76-L

C. J. Thompson, Commonwealth Government Printer, Canberra

METHOD FOR CULTIVATING MICROORGANISMS, PARTICULARLY OF THE FRANKIA GROUP AND PREPARATION OF BACTERIAL INOCULUMS

This application is divided from copending Australian application No. 67223/87 in which is described and claimed a method of culturing microorganisms in a two-phase culture. The present invention relates to a method of preparing a microbial inoculum, which method may employ the microorganisms produced by the methods described in copending Australian patent application No. 67223/87, the disclosure of which is incorporated herein by way of reference.

The present invention relates to a method for culturing microorganisms and to a method for the production of an inoculum starting with the microorganisms obtained.

More particularly, the present invention relates to the production of inoculums required in horticulture and in forestry (for example, the production of inoculated Casuarina, alder, Hippophae and Elaeagnus seedlings in the nursery).

Amongst microorganisms which can be used as inoculum, actinomycetes belonging to the Frankia group (symbionts of nitrogen-fixing actinorhizal plants) and rhizobia must be mentioned.

Some microorganisms, particularly of the Frankia group, have the characteristic of producing a very low amount of biomass when they are cultured according to conventional methods. This very low biomass production is a serious disadvantage, both in the industrial context and in the context of fundamental research.

Therefore, the present invention provides a method for culturing microorganisms and for producing an inoculum, which makes it possible to produce much larger amounts of biomass than those obtained at present, and processes which enable the industrial requirements, for example of horticulturists, to be met.

In fact, considering their use, bacterial inoculums intended for use in horticulture or in forestry must

satisfy certain conditions:

first of all, they must consist of a support which is well defined and which ensures a good protection of the microorganisms concerned:

they must be easy to store, to dispatch and to use; and

finally, they must enable the microorganisms to be distributed widely at the infection sites of host plants, at the time of their application.

More particularly, the present invention relates to inoculums consisting of microorganisms entrapped in polymer matrices. Such method have already been described, particularly in the following French Patent applications: 77/10,254; 79/28,956; 81/04,474 and 83/02,847.

However, none of these methods enables the conditions listed above to be satisfied totally.

For this purpose, the invention described in copending Australian application No. 67223/87 provides a method for culturing microorganisms, which involves a two-phase culture, wherein:

a) the microorganisms are cultured in a solid nutrient medium ("stage a");

b) after culturing, this solid medium, colonized by microorganisms, is divided into fragments ("stage b"), and

c) a liquid nutrient medium is inoculated ("stage c") with the fragments obtained in "stage b" and the medium is incubated at 28-30°C. This stage corresponds to the two-phase culture.

After the growth of the microorganisms, the latter are recovered.

This method may be used, in particular, for the preparation of slow-growing actinomycetes, particularly those belonging to the Frankia group.

The method according to the invention described in copending Australian patent application No. 67223/87 makes use of the fact that some microorganisms, in particular of the Frankia group, grow much better

at a solid/liquid interface than in a liquid medium.

Thus, a liquid/solid two-phase culture with agar fragments, for example, enables not only the Frankia to adhere to their surface because of the thigmotropic nature of this microorganism (which nature is observed in some other microorganisms, as has already been pointed out), but also the Frankia to penetrate into these supports. The liquid medium not only contributes to the achievement of a solid/liquid interface which is favourable to microbia development, but also constitutes a nutrient reserve.

The solid or liquid nutrient media may have substantially the same compositions and may essentially consist of known media or of media derived from known media containing carbon, nitrogen, phosphorous and trace elements and other elements which ensure the growth of the microorganisms to be cultured.

The production of a solid medium may be carried out using agar, but other components may be employed in order to obtain a solid medium.

The "stage a" may be carried out in a container of the Petri dish type. After culture, the solid medium is divided into fragments by suitable means and then dispersed in a liquid nutrient medium. When the growth is complete, the microorganisms obtained are recovered by known techniques such as centrifugation.

Moreover, it has been observed that the addition of activated charcoal to the nutrient medium, especially when the addition is made to the solid medium ("stage a"), enables the amount of biomass to be increased significantly.

According to the present invention there is provided a method for the preparation of a microbial inoculum which comprises the following sequential steps:

1. entrapping microorganisms in a polymer matrix,
2. incubating said polymer matrix entrapped microorganisms in a medium which enables said microorganisms to grow until colonies are formed within said matrix, and
3. dehydrating said polymer matrix with entrapped colonies of microorganisms.

wherein said microorganisms are obtained from a two-phase process comprising the following sequential steps:

a) culturing microorganisms in a solid nutrient medium; and,

b) dividing into fragment said solid nutrient medium containing colonies of

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said cultured microorganisms entrapped in said polymer matrix.

The polymer matrices which may be employed are known to the person skilled in the art and appear particularly in the patent mentioned above. These may be, in particular, calcium alginate beads which are particularly well suited for the production of a microbial inoculum.

It is known that entrapped microorganisms, incubated in a medium which enables them to grow, are capable of continuing their growth within their polymer matrix. It has now been found that they are capable of forming microcolonies in this polymer matrix, which has many advantages. First of all, a high density inoculum is obtained, and the fine structure of the microcolonies also has the advantage of protecting the inoculum during its storage.

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For storage, these polymer matrices are preferably dehydrated without being divided. Thus, when calcium alginate beads are prepared, the latter are not divided into fragments but dehydrated as such.

In a preferred embodiment, the microorganisms entrapped in the polymer matrix are obtained by the method described above. The fragments, for example agar fragments, containing the microorganisms such as Frankia, are incorporated into the polymer matrix, for example calcium alginate.

Finally, when previously dehydrated microbial inoculum must be applied in situ, it is rehydrated with a buffer, for example a phosphate buffer, until a gel is obtained. This gel lends itself particularly well to the distribution of the microorganisms in the soil.

The method described above makes it possible to obtain an inoculum which may, after dehydration, be stored and transported without difficulty, produce a large amount of biomass and which can be distributed very easily in the form of a gel.

The following examples will enable the other advantages and features of the present invention to be demonstrated.

The culture media employed in these examples have the following compositions:

1. Qmod Medium (Lalonde and Calvert, 1979)

Components	Concentration
K_2HPO_4	0.3 g
NaH_2PO_4	0.2 g
$MgSO_4\ 7H_2O$	0.2 g
KCl	0.2 g
Yeast extract	0.5 g
Peptone	5.0 g
Ferric citrate (1% solution)	1 ml
Trace element solution*	1 ml
Lecithin	5 mg
Distilled water	1 l

pH adjusted to 6.8

* Trace element solution (g/l)

H_3BO_3 : 1.5; $MnSO_4 7H_2O$: 0.8; $ZnSO_4 7H_2O$: 0.6;
$CuSO_4 7H_2O$: 0.1;
$(NH_4)_6Mo_7O_{24} 4H_2O$: 0.2;
$CoSO_4 7H_2O$: 0.01.

2. Qmod Medium + activated charcoal (Diem and Dommergues, 1985)

Qmod medium above, to which activated charcoal has been added at a dose of 150 mg/l

3. BAP Medium (Murry et al., 1984)

Components	Concentration
K_2HPO_4	0.591 g
KH_2PO_4	0.952 g
NH_4Cl	0.267 g
$MgSO_4 7H_2O$	0.095 g
$CaCl_2 2H_2O$	0.010 g
FeNa-EDTA	0.010 g
Sodium pyruvate	1.1 g
Trace element solution*	1 ml
Vitamin solution**	1 ml
Distilled water	1 l

pH adjusted to 6.3

* Trace element solution (g/l)

H_3BO_3 : 2.86;
$MnCl_2 4H_2O$: 2.27; $ZnSO_4 7H_2O$: 0.22;
$CuSO_4 5H_2O$: 0.08;
$Na_2MoO_4 2H_2O$: 0.025; $CoSO_4 7H_2O$: 0.001.

** Vitamin solution (mg/l)

Thiamine HCl : 10; nicotinic acid: 50;
Pyridoxine HCl : 50; biotin : 225;
Folic acid : 10; Calcium pantothenate : 10;
Riboflavin : 10.

4. YEM Medium (yeast extract-mannitol) :

Components	Concentration
K_2HPO_4	0.5 g
$MgSO_4 7H_2O$	0.2 g
NaCl	0.1 g

Yeast extract (Difco)	1.0 g
Mannitol	10.0 g
Distilled water	1 l

5. Modified YEM Medium

Same composition as above, except that K_2HPO_4 is employed at a concentration of 0.1 g/l.

On the figures,

figure 1 represents the effect of adding activated charcoal to the culture medium on the growth of Frankia:

1a : Qmod medium on its own;

1b : same medium with activated charcoal (0.01%); the dark spots (arrows) appearing on the Petri dishes are Frankia colonies (the charcoal powder is not visible at this magnification).

figure 2 represents alginate beads colonized internally by Frankia:

2a : view of three beads (true diameter: approximately 5 mm);

2b : enlarged view of a bead in which Frankia colonies (true diameter of each colony : 100 200 µm) appearing as dark circular spots with diffuse borders; clusters of dark spots are observed around some colonies, these are sporangia formed during the growth of Frankia within the bead; the sporangia (arrows) are typical structures of Frankia.

EXAMPLE 1 - METHOD FOR CULTURING SOME SLOW-GROWING ACTINOMYCETES BELONGING TO THE FRANKIA GROUP, IN TWO STAGES AND IN A TWO-PHASE MEDIUM

The culturing is carried out in three stages:

"Stage a" : Frankia is cultured in Petri dish (10 cm) in an agar medium for 2 to 3 weeks. For this purpose, 10 ml of a 1.5% nutrient agar medium, molten at 40°C, is inoculated, within the mass, with 1 ml of a Frankia suspension obtained by homogenizing a Frankia culture.

After incubating for 2 - 3 weeks at 30°C, Frankia colonies develop in the agar (500 - 2000 colonies per dish). Each of these agar disks, invaded by Frankia colonies, is detached from the Petri dish and introduced, under sterile conditions, into a flask containing 50 ml of water and a bar magnet.

"Stage b" : The disks are thus finely divided into microgranules of agar which contain Frankia colonies or colony fragments.

"Stage c" : The suspension of fragments obtained as mentioned above is used to inoculate the liquid nutrient medium at a rate of 10 ml of Frankia fragment suspension per 50 ml of liquid nutrient medium. The incubation at 30°C lasts for 2 to 3 weeks. The composition of the nutrient medium (agar or liquid) varies depending on the strain of Frankia used. This may be either the Qmod medium or the BAP medium. This "stage c" forms the two-phase culture.

Determination of Frankia biomass obtained

As Frankia is a filamentous organism, it is not possible to estimate the biomass in terms of number of cells as in the case of bacteria. It is expressed either as weight of proteins (which is the solution adopted here), or as weight of dry matter.

After removing the nutrient medium by centrifugation, the centrifugation pellet is introduced into a test tube containing approximately 30 ml of distilled water and placed in a boiling water bath for 2 min in order to melt the agar in the microgranules. The entire contents of the tube are then poured into approximately 200 ml of boiling distilled water so as to dilute the molten agar in order to remove it, either by filtering through Millipore filter or by centrifugation. The culture is rinsed several times with distilled water and it is then harvested in order to carry out protein determination according to the method of Lowry et al (1951).

Comparison of the conventional method in liquid medium and the method according to the invention in a two-phase medium

In order to compare these two methods, it was

necessary to carry out the culturing in the liquid medium following the same sequence of operations as that recommended within the context of the present invention.

Table 1 summarizes the sequence of operations in the two cases.

TABLE 1

COMPARISON OF THE CONVENTIONAL LIQUID MEDIUM CULTURE METHOD AND THE TWO-PHASE CULTURE METHOD ACCORDING TO THE INVENTION WITH AGAR FRAGMENTS

Conventional liquid medium culture method	Two-Phase culture method with agar fragments
1 ml of *Frankia* cell suspension	1 ml of *Frankia* cell suspension
↓	↓
Culture in liquid medium for 2 to 3 weeks	Culture in agar medium for 2 to 3 weeks
↓	↓
Homogenization of the culture	Homogenization of the culture and production of agar fragments containing *Frankia* colonies
↓	↓
Continuation of the culture in liquid medium for 2 to 3 weeks	Transfer of fragments into liquid medium and culture for 2 to 3 weeks (two-phase culture)
↓	↓
Centrifugation, washing	Centrifugation, washing
↓	↓
Final product	Final product

Culture of the Frankia strain of Casuarina equisetifolia ORS 021001

The results given in Table 2 clearly show that the method according to the invention enables a much higher amount of biomass (x 20) to be obtained, starting with an identical inoculum and for the same incubation period.

Culture of three other Frankia strains

In order to check the reliability of the method according to the invention, this experiment was repeated at a different time of the year with three other Frankia strains, which are as follows:

ORS 022602 : Frankia of Allocasuarina stricta
ORS 060501 : Frankia of Colletia spinosa
ORS 140102 : Frankia of Hippophae rhamnoides

The results given in Table 3 confirm the improvement in Frankia biomass production by the culture method according to the invention compared with the conventional culture method. This improvement is of a smaller magnitude in this trial than in the previous trial.
This may be due to several reasons:

incubation period reduced to 4 weeks instead of 6 weeks, and

physiological state and the presence of variable amounts of the various Frankia structures (vegetative hyphae, sporangia) in the initial inoculum.

TABLE 2

COMPARISON OF THE GROWTH OF FRANKIA ORS 021001 CULTURED ACCORDING TO THE CONVENTIONAL LIQUID MEDIUM METHOD AND ACCORDING TO THE NEW TWO-PHASE CULTURE METHOD WITH AGAR MICROGRANULES

METHOD	FRANKIA BIOMASS (µg proteins/flask)	
	AT INOCULATION	AFTER 6 WEEKS OF INCUBATION (1)
Conventional method	2	55
Two-phase culture method	2	1 205

(1) 3 weeks in an agar medium and 3 weeks in a two-phase medium

Nutrient medium employed: Qmod

TABLE 3

COMPARISON OF GROWTH OF THREE STRAINS OF FRANKIA CULTURED ACCORDING TO THE CONVENTIONAL LIQUID MEDIUM METHOD AND ACCORDING TO THE NEW TWO-PHASE CULTURE METHOD WITH AGAR FRAGMENTS

METHOD AND STRAIN	FRANKIA BIOMASS (µg proteins/flask) AT INOCULATION	AFTER 4 WEEKS OF INCUBATION (1)
Strain ORS 022602		
Conventional method	2	26
Two-phase method	2	145
Strain ORS 060501		
Conventional method	3	58
Two-phase method	3	315
Strain ORS 140102		
Conventional method	6	41
Two-phase method	6	360

(1) 2 weeks in an agar medium and 2 weeks in a two-phase medium

Nutrient media employed: same medium as in Table 2, except for ORS 022602 which was cultured in a BAP medium

EXAMPLE 2 - EFFECT OF ADDING ACTIVATED CHARCOAL TO THE Qmod CULTURE MEDIUM ON THE NUMBER OF COLONIES OF FRANKLA STRAIN ORS 021001

The strain ORS 021001 is cultured in test tubes of size 18 x 180 mm, containing 10 ml of Qmod medium. The incubation is carried out at 30°C and the incubation periods are 3 weeks, 1 month and 2 months respectively. At the end of these incubation periods, each of the tubes contains 50 to 70 µg of Frankia proteins.

Each culture is decanted, 20 ml of fresh Qmod medium is added and the mixture is homogenized, under sterile conditions, using a bar magnet for 1 hour. Two dilutions (10^{-1} and 10^{-2}) of the suspensions obtained are made and two series of Petri dishes are inoculated by incorporating 0.5 ml of each dilute suspension into 20 ml of the Qmod agar medium with or without activated charcoal (Merck Item no. 2186) added at a dose of 150 mg per litre. The Petri dishes thus inoculated are incubated at 30°C for 3 weeks. The number of colonies formed are counted under a binocular microscope, the results being expressed on the basis of 1 ml of inoculum.

Table 4 clearly shows that the addition of charcoal to the medium increases the number of Frankia colonies observed, this increase being considerable in the case of the cultures incubated for two months.

This first result shows that activated charcoal promotes the germination of the spore type structures accumulated in the cultures incubated for two months (figure 1). On the other hand, microscopic observations show that newly formed hyphae are more branched in the Qmod medium with activated charcoal than in the Qmod medium on its own, and the latter result proves that activated charcoal promotes the growth of hyphae.

TABLE 4

EFFECT OF ADDING ACTIVATED CHARCOAL TO THE NUTRIENT MEDIUM (Qmod) ON THE NUMBER OF COLONIES FORMED FROM 1 ml OF FRANKIA ORS 021001 SUSPENSION

	NUMBER OF COLONIES FORMED	
FRANKIA CULTURE	Qmod MEDIUM	Qmod MEDIUM + CHARCOAL
3 week culture	1 000	3 400
1 month culture	8 200	266 000
2 month culture	4 200	940 000

EXAMPLE 3 - GROWTH OF FRANKIA IN ALGINATE BEADS

100 ml of Qmod medium are inoculated with 10 ml of a *Frankia* ORS 021001 cell suspension. After 4 weeks of incubation, a culture containing a *Frankia* biomass is obtained, the amount of biomass, as estimated by protein determination (Lowry et al., 1951), ranges up to 240 µg. The culture is decanted, it is transferred into the same quantity (100 ml) of fresh liquid Qmod medium, the mixture is homogenized with a bar magnet for 1 hour. The entrapment of *Frankia* in the alginate is then carried out. For this purpose, 100 ml of sterile liquid Qmod medium containing 4% alginate S170 (Satialgine S170, Société Bretonne de Produits Chimiques et Pharmaceutiques, 6 impasse Latécoère, Velizy 78140) are added. A total of 200 ml of suspension containing *Frankia* colony fragments in a Qmod medium containing 2% of alginate is thus obtained. This suspension is introduced drop by drop, under aseptic conditions, into a container containing a sterile aqueous $CaCl_2$ solution (1%) and stirred continuously with a bar magnet.

Under these conditions, the beads are formed after 20 to 30 minutes in the $CaCl_2$ solution. The beads are immediately rinsed 10 times with sterile distilled water.

The beads are transferred into flasks containing 50 ml of liquid Qmod medium (approximately 20 ml of beads per flask) and incubation is carried out at 30°C for 4 weeks. After this period, observations under the microscope show that many *Frankia* colonies (20 - 40 colonies/bead), totally free from any exogenous microbial contamination, developed within each bead. These colonies are of different sizes, but are perfectly identical with respect to morphology and radial growth pattern typical of *Frankia*. Many colonies produce sporangia, which are also typical (Figure 2).

EXAMPLE 4 - GROWTH OF RHIZOBIUM IN ALGINATE BEADS

Rhizobium strain A16 isolated from Albizia lebbeck is cultured in a conventional YEM medium. After 4 days of incubation, 1 ml of culture is withdrawn and introduced into 100 ml of modified YEM medium; this medium is low in phosphate and contains alginate S170 (Satialgine S170, Société Bretonne de Produits Chimiques et Pharmaceutiques, 6 impasse Latécoère, Velizy 78140). Beads are obtained by carrying out the operations in example 3. Two batches of these beads containing entrapped Rhizobium cells are prepared:

the first batch is immersed in a flask containing modified YEM liquid nutrient medium without alginate; and

the second batch is incubated under the same conditions, but in the absence of the nutrient medium.

After 48 hours, it is observed that the number of Rhizobium counted in the normal agar YEM medium increases from 175×10^3 per bead to 280×10^5 per bead in the first batch, whereas the number of Rhizobium remains static in the second batch. This result shows that as in the case of Frankia, the growth of Rhizobium within the alginate beads requires the incubation of these beads in a suitable liquid nutrient medium.

EXAMPLE 5 - REHYDRATION OF DEHYDRATED ALGINATE BEADS

The previously dehydrated alginate beads containing symbiotic microorganisms are immersed in a phosphate buffer solution (pH 6.8) with the following composition: KH_2PO_4 : 4.29 g; K_2HPO_4 : 4.34 g ; water q.s. 1 litre.

After 4 to 6 hours, the dehydrated beads regain the initial shape and consistency of fresh beads. It is very easy to obtain the inoculum in the form of a gel by crushing. This treatment with phosphate buffer is indispensable as it enables the microorganisms previously entrapped or developed after entrapment to be released and distributed.

EXAMPLE 6 - INOCULATION OF PLANTS

In order to monitor the quality of the inoculum obtained by the method according to the invention, the infectivity of a Frankia inoculum on Casuarina equisetifolia (Frankia strain ORS 021001) and that of a vesicular-arbuscular endomycorrhizal fungus (Glomus mosseae) inoculum are tested.

Frankia Strain ORS 021001

First of all, Casuarina equisetifolia plantlets are obtained by germinating surface-sterilized seeds (immersing in concentrated H_2SO_4 for 1 min, followed by rinsing in sterile distilled water). 4 week old plantlets are transplanted, at a rate of 1 plantlet per pot, into plastic pots (7 cm in diameter, 7 cm in height) containing sterile soil, moistened daily with sterile water. In order to inoculate the plantlets at the time of transplanting, the inoculum is prepared as follows:

120 mg of dehydrated beads are reswollen in the phosphate buffer mentioned above; and

after crushing, the gel obtained is mixed with sterile sand and distributed into 12 pots, which amounts to an addition of 10 mg of dehydrated beads to each inoculated pot.

Two batches of inoculum are employed: a first batch stored for two weeks only (inoculum no. 1), and a second batch stored at laboratory temperature for 1 year (inoculum no. 2). Concurrently with the inoculated pots, a series of uninoculated control pots were, of course, prepared. After 2 months of culture, the dry weight of the aerial parts of the plants (drying to constant weight), the number of nodulated plants and the number of nodules per plant were determined (Table 5).

TABLE 5

INOCULATION OF CASUARINA EQUISETIFOLIA PLANTLETS WITH ORS 021001 ENTRAPPED IN ALGINATE BEADS WHICH ARE DEHYDRATED AND LATER RESWOLLEN AT THE TIME OF USE IN A PHOSPHATE BUFFER

TREATMENT	DRY WEIGHT OF PLANTS (mg/plant)	NODULATED PLANTS/ TOTAL NUMBER OF PLANTS	NUMBER OF NODULES/PLANT
Control	80	0/12	0
Inoculum no. 1	220	12/12	3 - 8
Inoculum no. 2	170	12/12	3 - 5

Inoculum no. 1 Beads stored in the dehydrated form for 2 weeks at laboratory temperature

Inoculum no. 2 Beads stored in the dehydrated form for 1 year at laboratory temperature.

Glomus mosseae

Structures of Glomus mosseae (free hyphae or hyphae within the roots, and spores) are entrapped in alginate beads according to the method described by Diem et al. (1981) and Ganry et al. (1982). 7 day old Vigna unguiculata plantlets were transplanted into earthenware pots (15 cm in diameter, 20 cm in height) containing sterile soil. A first batch of 5 plants was inoculated with an inoculum consisting of a mixture of sand and gel prepared in the same way as described above, but using dehydrated beads containing the Glomus mosseae structures. These beads were stored for 1 year at laboratory temperature. The equivalent of 30 mg of dehydrated beads was added to each inoculated pot. A second batch of 5 plants consisted of uninoculated plants.

Table 6 shows that the inoculum of Glomus mosseae is perfectly infective at the dose employed and after storing for 1 year.

TABLE 6

INOCULATION OF VIGNA UNGUICULATA PLANTLETS WITH GLOMUS MOSSEAE ENTRAPPED IN ALGINATE BEADS WHICH WERE DEHYDRATED AND LATER RESWOLLEN AT THE TIME OF USE IN A PHOSPHATE BUFFER

TREATMENT	INFECTED PLANTS/ TOTAL NUMBER OF PLANTS	FREQUENCY OF INFECTION (%)
Control	0/5	0
Inoculum no. 1	5/5	86

Inoculum no. 1: Beads stored in the dehydrated form for 1 year

Frequency of infection: Number of infected pieces of root (3 mm)/total number examined

REFERENCES

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LOWRY O.H., N.J. ROSEBROUGH, A.L. FARR and R.J.RANDALL (1951) Protein measurements with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

MURRY M.A., M.S. FONTAINE and J.G. TORREY (1984) Growth kinetics and nitrogenase induction in *Frankia* sp. HFPArI3 grown in batch culture. Plant and Soil, 78: 61-78.

The claims defining the invention are as follows:

1. A method for the preparation of a microbial inoculum which comprises the following sequential steps:

1. entrapping microorganisms in a polymer matrix,

2. incubating said polymer matrix entrapped microorganisms in a medium which enables said microorganisms to grow until colonies are formed within said matrix, and

3. dehydrating said polymer matrix with entrapped colonies of microorganisms.

wherein said microorganisms are obtained from a two-phase process comprising the following sequential steps:

a) culturing microorganisms in a solid nutrient medium; and,

b) dividing into fragment said solid nutrient medium containing colonies of said cultured microorganisms, ~~entrapped in said polymer matrix.~~

2. A method according to claim 1 further comprising:

c) inoculating a liquid nutrient medium with said fragments containing colonies of cultured microorganisms,

d) maintaining said inoculated liquid nutrient medium under culture conditions, and

e) recovering said cultured microorganisms.

3. A method according to claim 1 or 2 wherein the polymer matrix employed is calcium alginate.

4. A method according to claim 1, 2 or 3 wherein said microorganisms are symbiotic with plants.

5. A method according to claim 4 wherein said microorganisms belong to the Frankia group or the Rhizobium group.

6. A method according to any one of the preceding claims wherein the polymer matrices are in the form of beads or granules.

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7. A method of preparing a microbial inoculum according to any one of claims 1 to 6 substantially as hereinbefore described with reference to any one or more of examples 4 to 6.

8. A microbial inoculum obtained by the method of any one of claims 1 to 7.

9. Application of the microbial inoculum as claimed in claim 8, wherein the dehydrated polymer matrices are rehydrated with a buffer until a gel is obtained, before use.

10. A microbial inoculum according to claim 8 substantially as hereinbefore described.

DATED this 29th day of January, 1993.

INSTITUT FRANCAIS DE RECHERCHE SCIENTIFIQUE POUR LE DEVELOPPEMENT EN COOPERATION (ORSTOM)

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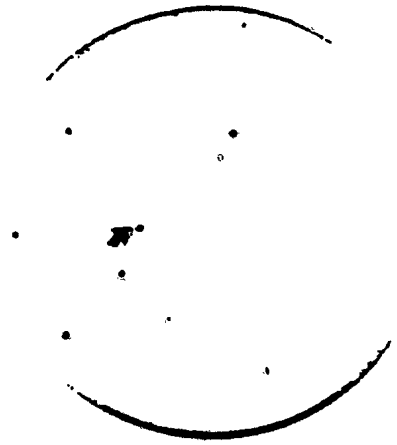
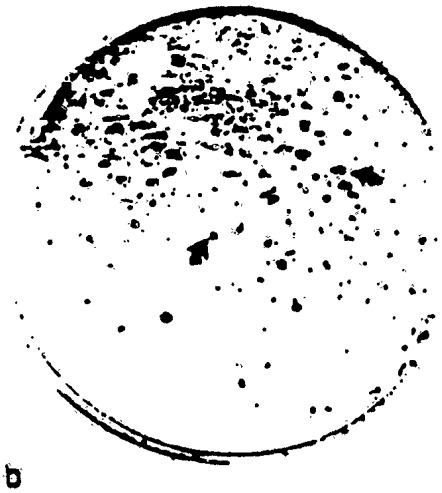
FIG_1



1a



1b

FIG_2

2a

2b