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(54) Title: A METHOD OF GROWING AND PRESERVING FUNGI AND BACTERIA

(57) Abstract

A microorganism-containing inoculum for use in agriculture, horticulture and/or silviculture comprises a porous inorganic substrate such as perlite having a microorganism-containing nutrient culture medium associated therewith.

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"A METHOD OF GROWING AND PRESERVING FUNGI AND BACTERIA".

This invention relates to a method for growing and preserving microorganisms such as fungi and bacteria, and in one particular aspect it relates to a method for production of an inoculum containing microorganisms for use in agriculture, horticulture and/or silviculture for infecting soil and/or plants with the microorganisms.

It is known that certain microorganisms, including but not restricted to types of bacteria and fungi, live in symbiotic association with plants, and that such an association can benefit the plant in a number of ways, such as promoting growth and protecting the plant against pathogens. It is often advantageous in the commercial production of plants to infect the plants with certain types of fungi or bacteria in order to exploit this beneficial symbiotic association of the plant and fungi or bacteria, thereby enabling more productive and more profitable agriculture, horticulture and sylviculture. The ability to deliberately infect soil and/or the roots of plants with particular types of fungi or bacteria is necessary before a beneficial infection of the plants with fungi and bacteria can be exploited.

This present invention provides a method which enables the growth of large volumes of microbial biomass

in a manner which allows for easy and convenient production of inoculum with which to infect soil and plants. The method is inexpensive and is practical to use in very large scale applications, such as for broadacre wheat cultivation, and has a large number of advantages over previously described methods to infect plants with microorganisms. The method is also suitable for large scale growth of microorganisms for the purpose of producing secondary metabolites.

In accordance with the present invention, there is provided a method for culturing a microorganism characterised in that said microorganism is grown in a nutrient culture medium in the presence of a porous, inorganic substrate.

In another aspect, this invention provides a method for producing a microorganism-containing inoculum, which comprises the steps of culturing the microorganism by the method broadly described above, and subsequently drying the resultant culture biomass to produce said microorganism-containing inoculum.

This aspect of the invention also extends to a microorganism-containing inoculum which comprises a porous, inorganic substrate having a microorganism-containing culture medium associated therewith.

The method of this invention uses a highly porous inorganic substrate as a medium upon and within which to grow microorganisms such as fungi or bacteria. In a preferred embodiment of this method, the porous substrate is sterilized by poisonous gas, by heat, by radiation or by steam or any other method. A sterile nutrient solution is then added to the porous substrate in sufficient quantity to saturate the porous substrate. A small amount of a pure culture of the desired fungi or

bacteria is then added to the nutrient-saturated porous substrate and the mixture is left at the optimum growth temperature for the fungi or bacteria for sufficient time to allow growth of the fungi or bacteria throughout the substrate.

The porous substrate containing the fungus or bacteria may then be slowly dried in a stream of air. When dried, the material can be used as an inoculum by being added to soil whereupon in contact with water, the fungi or bacteria contained within the porous substrate rapidly regenerates and grows out from the porous substrate into the soil. When the dried material is mixed with seed and the mixture sown in suitable soil, the fungi or bacteria which grows from the porous material is temporally and physically well placed to infect the plant which germinates from the seed.

In one particularly preferred aspect of the invention, the inoculum is used as a means for large scale inoculation of soil and plants such as wheat with the sterile red fungus (SRF- See Dewan and Sivasithamparam, 1988, post).

Porous substrates which are suitable for the growth of fungal or bacterial biomass in accordance with this invention include but are not restricted to perlite, vermiculite, diatomite and terra cotta clay. Perlite which is a type of obsidian rock is particularly suitable, as it is readily available and has a very low bulk density due to the presence of many small air pockets within its structure. Because of the presence of many intraparticular spaces, perlite can adsorb more than twice its own weight of water. Perlite has a very large surface area for its mass, thus dries out rapidly and easily.

The advantage of the use of an inoculum in accordance with this invention over other inoculum delivery methods for fungal microorganisms is that other methods use carrier material such as sterilised straw or seed which contains organic material. It is known that organic material causes deleterious changes in the rhizosphere which inhibit plant growth. The present method uses only a very small amount of organic material. If the amount of organic material in the nutrient growth medium is sufficiently low, then the fungus or other microorganism which grows from the dried, infected perlite grows away from the perlite where it can infect adjacent plant roots. The residual perlite left in the soil improves soil structure and water holding capacity. The rate of growth of aerobic microorganisms is very rapid on the preferred perlite substrate as described due to the low bulk density of perlite and the ease of gas exchange.

The present invention thus provides a useful method to prepare, use and preserve bacteria and fungi for both small scale and large scale applications. It is very useful as an immobilization matrix for the continuous culture of bacteria and fungi for the production of useful metabolites. It is a useful and convenient method to store microbes for prolonged periods without having to freeze them, or lyophilise them. It is a useful way to infect large fields with particular desirable microorganisms.

Perlite is particularly preferred as a carrier for inoculum in accordance with this invention as it contains no organic matter and can be completely sterilised during the manufacturing process. Furthermore, perlite has a very low ion exchange capacity, therefore there is no tendency for pH and salt concentrations in nutrient media to change upon contact with the perlite, hence it is easy

to maintain optimal conditions for the growth of microorganisms.

Perlite is highly porous, hence it can absorb large amounts of nutrient medium and allows good gas exchange to promote rapid growth of microorganisms. It also has a large surface area to mass ratio, hence after microbial growth has progressed to the desired point, water can be removed rapidly by placing the material in an air current. Because of rapid evaporation, the perlite and biomass stay relatively cool due to the heat of vaporization of water. This means that a hot air current dries the perlite even faster without causing heat damage to microorganisms.

When a SRF-containing, perlite based, inoculum preparation is dried to less than 10% water, the SRF contained therein is extremely stable in storage at room temperature and remains viable for greater than one year. Further, when the SRF/perlite is rehydrated, SRF regenerates and mycelial outgrowth occurs more rapidly than any other preserved form of SRF (e.g. lyophilised or frozen glycerol stocks). A sufficiently large amount of viable SRF is contained within the perlite matrix such that the volume of SRF/perlite required to effectively treat plants on a broadacre scale is very much less than many commonly applied agricultural treatments. Volumes of 2 to 6 Kg per hectare are usually adequate to promote SRF root colonisation in all plants. This makes the technique cheap and practical for large scale applications. Larger volumes may of course be used if desired in particular circumstances.

Perlite can be conveniently manufactured in particle sizes ranging from less than 10 micron to greater than 2 cm. The ideal particle sizes for producing SRF inoculum for broadacre applications is

between 0.5 to 5 mm. Such particles of perlite have good flow characteristics are readily compatible with standard seeding equipment. Perlite is relatively cheap and the precursor ore, obsidian, is plentiful throughout the world occurring at sites of either recent or ancient volcanic activity. The cost of the delivery vehicle therefore becomes a trivial part of the cost of treating fields. Perlite is considered a very safe material and no occupational health and safety hazards have been reported to be associated with any of its uses. It is thus unlikely to cause any health or safety problems to agriculturalists using perlite inoculum. Finally, any residues of perlite which remain in soil after cropping will enhance the agricultural utility of the soil by improving water holding capacity and aeration. Such benefits will continue to accrue over years of using the perlite delivery system.

Further features of the present invention will be apparent from the following Examples which illustrate this invention.

EXAMPLE 1

Perlite was obtained from the Australian Perlite Company Pty.Ltd. (Class P500). To a 50 g sample of perlite in a 400 ml conical flask was added 100 ml of potato broth (50 g shredded potato boiled in 500 ml of water for 5 minutes, then potato removed by straining through cheesecloth and liquid volume made up to 1 L with water). The flask was sealed with aluminium foil and autoclaved at 121°C for 15 minutes. When the flask was cool, a piece of agar containing the sterile red fungus (SRF) described by Dewan and Sivasithamparam (Dewan, M.M. and Sivasithamparam, K. (1988). Trans.Br.Mycol.Soc. 91 (4): 687-717) was placed in the flask which was sealed.

The flask was kept at 20°C and shaken gently once per day for five days. A 500 g sample of perlite was then added to 1 L of the potato broth and autoclaved in a 6 litre flask. The contents of the small flask were added to the large flask which was kept at 20°C for five days, shaking the flask every day. The culture of fungus in perlite was then dried under a gentle stream of air for 24 hours. After this time it weighed 750 grams.

When the dried perlite containing the sterile red fungus was placed on a petri dish containing potato/dextrose/agar, after 24 hours SRF mycelia were clearly visible over the perlite and had grown into the agar in a 1 cm circle around the perlite. When the dried perlite containing the sterile red fungus was placed on a petri dish containing water agar, after 24 hours there was very little SRF mycelia on the perlite, but SRF had grown into the agar in a 2 cm pattern around the perlite.

When the dried perlite containing sterile red fungus was placed on top of 250 g of sand in a plastic cup alongside wheat seeds and covered with a further 50 g of sand then 40 ml of water, after seven days the roots of the wheat plantlets were found to be covered with sterile red fungus and were 40% longer than roots from wheat plants treated exactly the same way except for the omission of the perlite containing sterile red fungus.

It can be determined from this example that it is possible to infect wheat in the field with sterile red fungus by sowing perlite prepared as described in the example with wheat seeds using standard agricultural equipment. The volume of perlite required is less than the volume of wheat and due to the low bulk density of perlite, the weight of perlite required to completely infect wheat with sterile red fungus is usually between 2 and 6 Kg per hectare. Because of the low cost and ready

availability of perlite, it is thus practical to infect whole wheat fields with sterile red fungus by using the method of this invention.

EXAMPLE 2

(i) Preparation of nutrient broths.

Potato dextrose broth: fresh potato (150 g) was shredded and boiled for 5 minutes in 600 ml of water purified by reverse osmosis (RO water). The broth was strained through cheesecloth to remove coarse potato pieces. D-glucose (50 g) was added to the potato broth and the volume adjusted to 1 litre. The solution was then sterilized in an autoclave.

Potato sucrose broth: fresh potato (150 g) was shredded and boiled for 5 minutes in 600 ml of RO water. The broth was strained throughout cheesecloth to remove coarse potato pieces. Commercial grade sucrose (50 g) was added to the potato broth and the volume adjusted to 1 litre. The solution was sterilized in an autoclave.

(ii) Preparation of small scale inocula.

Perlite (20 g lots, P500 grade supplied by Western Perlite) was autoclaved at 120°C for 30 minutes in 250 ml flasks. Potato sucrose or potato dextrose broth was added to each flask at ratios of between 1:1 and 2:1 (broth:perlite v/w). Each flask was inoculated with a disk of potato dextrose agar (PDA) containing growing margins of an SRF culture. Each flask was mixed twice daily, and incubated at 20°C for 8-10 days.

(iii) Preparatic of larger scale inocula.

Perlite (3 g lots) was autoclaved at 120°C for 30 minutes in autoclavable plastic bags supplied by Disposable Plastics, South Australia. Nutrient broth was added to each bag at a ratio of between 1:1 and 2:1 (broth:perlite v/w). Each bag was inoculated with 30 g

of perlite previously colonized by sterile red fungus. The bags were heat sealed, mixed regularly and stored at 20°C for 8-10 days.

(iv) Preservation of Sterile Red Fungus in Perlite.

Perlite inoculated with SRF was weighed in a petri dish and dried overnight at 37°C, 50°C, 55°C or 65°C. All samples were reduced in weight by approximately 50%. Samples of the dried perlite were plated onto PDA.

(v) Results.

A broth to perlite ratio closer to 2:1 resulted in more SRF production, but a greater amount of polysaccharide gel was produced by the fungus, reducing the friability of the resultant dried SRF/perlite. All samples of dried SRF/perlite regenerated viable SRF mycelia when incubated on PDA plates. Those dried at 37°C and 50°C regenerated most rapidly. There was no bacterial or fungal contamination present. This indicates that perlite inoculated with SRF and dried can be stored successfully with few contamination or regeneration problems.

EXAMPLE 3

This example relates to field trials carried out on wheat with a sterile red fungus-containing inoculum prepared in accordance with the present invention in order to verify that perlite is an effective SRF delivery system in the field and that the fungus delivered by this inoculum is effective in protecting wheat against the "take-all" fungus Ggt (*Gaeumannomyces graminis* var. tritici) and in promoting growth.

1. Inoculum Preparation.

Three types of inocula were used for field trials, one based on rye-grass, one using perlite with a low

nutrient growth medium and one using perlite with a high nutrient medium.

Rye-grass was prepared by a simple scale-up of the standard laboratory practice, i.e. from 50 g batches to 5 Kg batches, using 20 litre plastic drums instead of glass beakers. Drums were sterilized with hypochlorite and water-soaked Wimmera rye-grass was sterilized in small batches in an autoclave. 20 Kg of inoculum was prepared and tested by plating onto water agar. When greater than 99% of tested seeds were infected with SRF, the seed was dried slowly over three days in a laminar flow hood, then weighed out into heat sealed plastic bags ready for each trial plot.

Perlite was prepared in 1 Kg batches by adding perlite and growth media into plastic autoclavable bags which very conveniently fitted into the autoclave. Low nutrient media was a simple potato broth (50 g of thin sliced potato per litre of water) whereas high nutrient media contained additional glucose (50 g per litre) as well as potato. Cultures were initiated by the addition of 100 g of previously prepared SRF/perlite to bags prior to sealing. Bags were mixed daily and when greater than 99% of particles were infected with SRF, the material was dried in a laminar flow hood for about 24 hours. Individual doses for each field plot were then weighed and sealed into plastic bags.

2. **Experimental Design.**

Plots were sown at the Mt.Barker Field Station, some 20 km west of Mt.Barker, Western Australia, on June 8, 1989 where there is a moderate to high risk of infection by take-all fungus. 100 separate plots of 30 m x 1.8 m were used. Of these plots 20 were controls which received seed (50 Kg per hectare) and fertilizer (150 Kg per hectare Agras No.1). A relatively large number of

control plots were sown as it was anticipated that there would be a fairly large variance between individual plots which was a function of slight differences in soil and independent of the treatments applied.

The eighty remaining plots were divided into 10 treatments with eight replicates of each treatment according to the following:

R1 = 20 Kg/Ha sterilised rye-grass (no SRF) as rye-grass controls.
R2 = 20 Kg/Ha SRF infected rye-grass.
R3 = 40 Kg/Ha SRF infected rye-grass.

P1 = 6 Kg/Ha sterilized perlite (no SRF) as perlite controls.

N1 = 3 Kg/Ha low nutrient SRF infected perlite.
N2 = 6 Kg/Ha " " " " "
N3 = 9 Kg/Ha " " " " "

G1 = 3 Kg/Ha high nutrient SRF infected perlite.
G2 = 6 Kg/Ha " " " " "
G3 = 9 Kg/Ha " " " " "

Treatment R1 and P1 were included to eliminate the possibility that any effects measured were due to the presence of carrier rather than SRF.

The different treatments were allocated to the specific plots in a totally random fashion using a random coding sequence. All sites were sown using cone seeders. This apparatus is a miniature version of a conventional seed drill (sowing eight rows of seed and fertilizer) except that it uses a very precise metering system to ensure that rows are sown uniformly independent of the gradient of the land. It is also designed such that

there is no need to clean the machinery out between each treatment in order to prevent cross-contamination between treatments.

3. **Summary of Results.**

(a) Rainfall: (mm recorded at station, and historic average). June 82(98); July 120(107); August 68(92); September 34(81); October 88(72); November 40(43); December 11(29).

As is typical of the area known for its reliable rain, the research station received very close to the average 350 mm for the growing season, with rain every month.

(b) Sowing and emergence: The plot was sown relatively late for the area which resulted in germination during a very cold time of the year. This probably had a significant effect in reducing the total number of wheat plants in each plot.

(c) Presence of fungi: The presence of SRF was confirmed in roots of rye and perlite treated plots in winter and spring, but none was detectable in summer. The exact proportion of plants infected with SRF could not be determined due to the imprecise nature of root analysis. Ggt was possibly isolated from a control plot, but there was no evidence of "take-all" disease pathology in control or test plots, as judged by rotted roots or darkening of the stem collar.

(d) Growth and yield enhancement: Sampling on August 28, 1989 demonstrated a clear statistically significant growth enhancement when SRF was present. In later sampling this enhancement appeared to be lost, but this loss was most likely due to the presence of weeds having a larger effect on growth than SRF treatment. The final yield data indicate that even in the presence of a severe weed infectation, SRF improved yield by around 10%.

CLAIMS:

1. A method for culturing a microorganism characterised in that said microorganism is grown in a nutrient culture medium in the presence of a porous, inorganic substrate.

2. A method according to claim 1 wherein the inorganic substrate is saturated with the nutrient culture medium prior to growth of the microorganism therein.

3. A method according to claim 1, or claim 2, wherein the inorganic substrate is selected from perlite, vermiculite, diatomite and terracotta clay.

4. A method according to claim 3, wherein the inorganic substrate is perlite.

5. A method according to claim 4, wherein the size range of the perlite is between 0.5 and 5 mm.

6. A method according to any one of claims 1 to 5 wherein the microorganism is the sterile red fungus (SRF).

7. A method for producing a microorganism-containing inoculum, which comprises the steps of culturing a microorganism by the method of any one of claims 1 to 5, and subsequently drying the resultant culture biomass to produce said microorganism-containing inoculum.

8. A microorganism-containing inoculum produced by the method of claim 7.

9. A microorganism-containing inoculum which comprises a porous inorganic substrate having a microorganism-containing nutrient culture medium associated therewith.

10. An inoculum according to claim 9 wherein the inorganic substrate is selected from perlite, vermiculite, diatomite and terra cotta clay.

11. An inoculum according to claim 10 wherein the inorganic substrate is perlite.

12. An inoculum according to claim 11, wherein the size range of the perlite particles is between 0.5 and 5 mm.

13. An inoculum according to any one of claims 9 to 12 wherein the microorganism is the sterile red fungus (SRF).

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 90/00252

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C12N 11/14 // A01N 63/00, 63/04, (C12N 11/14, C12R 1:645)

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	C12N 11/14

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU : IPC as above
Chemical Abstracts (1982-1990)

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	GB,A, 2113711 (HITACHI SHIPBUILDING AND ENGINERRING CO LTD) 10 August 1983 (10.08.83)	(1-3)
X	EP,A, 0314439 (NATIVE PLANTS INCORPORATED) 3 May 1989 (03.05.89)	(1-3, 7-10)
X	EP,A, 0226394 (AGRACETUS) 24 June 1987 (24.06.87)	(1-3, 7-10)
X	US,A, 3580811 (HIDY) 25 May 1971 (25.05.71)	(1-3)
X	Derwent Abstract Accession No. 85-067520/11, Class D16, SU,A, 1109432 (NOUCHPOLY) 23 August 1984 (23.08.84)	(1-4)

(continued)

* Special categories of cited documents: 10

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

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"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

"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

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 August 1990 (13.08.90)	27 August 1990
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	R.E. GRANT

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	AU,A, 21898/88 (ALCAN INTERNATIONAL LIMITED) 9 March 1989 (09.03.89)	(1-2)
X	DE,A, 3335643 (CHISSOASAHI FERTILIZER CO LTD) 5 April 1984 (05.04.84)	(1-4, 7-8)
X	BE,A, 897340 (GENEX CORPORATION) 14 November 1983 (14.11.83)	(1-3)
A	GB,A, 2185908 (MANVILLE CORPORATIOIN AND RANCH) 5 August 1987 (05.08.87). See page 3 line 124 - page 4 line 5 and claims	(1, 3, 4)
A	Patent Abstracts of Japan, C-599, page 111, JP,A, 01-37232 (KOHJIN CO LTD) 7 February 1989 (07.02.89)	(1)
A	Derwent Abstract Accession No. 87-077726/11, class L02, SU, A, 1244180 (PONOMAREV YUE) 15 July 1986 (15.07.86)	(1-4)

V. [] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[] Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1. [] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[] The additional search fees were accompanied by applicant's protest.

[] No protest accompanied the payment of additional search fees.