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(54) Title: LIQUID MYCORRHIZAL INOCULANT

(57) Abrégé/Abstract:
The invention relates to the field of agriculture, soil biotechnology and, specifically, the production and application of arbuscular mycorrhizal fungi, a micro-organism which improves efficiency in nutrient uptake by plants, moisture level, crop production and protection against certain root diseases, etc. The invention consists of a liquid product which is applied to arbuscular mycorrhizal fungi, using an aqueous medium which can achieve stabilisation of the mycorrhizal propagules, thereby enabling the formation of soil aggregates, and a demonstrated viability in liquid medium of between 12 and 18 months and which can be used in irrigation and fertigation systems. The inventive inoculant is applied directly to fields or greenhouses under protected cultivation and can also be used to overcome spore dormancy mechanisms and to obtain an increased concentration level which enables and facilitates handling and transport. The inventive product can be applied in order to manage mycorrhizal fungi in crops under large-scale irrigation system with improved productivity and environmental impacts.

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(54) **Title:** LIQUID MYCORRHIZAL INOCULANT

(54) **Título:** INOCULANTE MICORRIZÓGENO LÍQUIDO

(57) **Abstract:** The invention relates to the field of agriculture, soil biotechnology and, specifically, the production and application of arbuscular mycorrhizal fungi, a micro-organism which improves efficiency in nutrient uptake by plants, moisture level, crop production and protection against certain root diseases, etc. The invention consists of a liquid product which is applied to arbuscular mycorrhizal fungi, using an aqueous medium which can achieve stabilisation of the mycorrhizal propagules, thereby enabling the formation of soil aggregates, and a demonstrated viability in liquid medium of between 12 and 18 months and which can be used in irrigation and fertigation systems. The inventive inoculant is applied directly to fields or greenhouses under protected cultivation and can also be used to overcome spore dormancy mechanisms and to obtain an increased concentration level which enables and facilitates handling and transport. The inventive product can be applied in order to manage mycorrhizal fungi in crops under large-scale irrigation system with improved productivity and environmental impacts.

(57) **Resumen:** La solución técnica propuesta se relaciona con la rama de la agricultura, la biotecnología del suelo y específicamente con la producción y aplicación de los hongos micorrizógenos arbusculares, microorganismo que mejora la eficiencia en la toma de nutrientes por las plantas, nivel hídrico, producción vegetal, protección contra alguna de las enfermedades de la raíz, etc. Consiste en un producto líquido para la aplicación de los hongos micorrizógenos arbusculares, partiendo de la utilización de un medio acuoso que permite lograr una estabilización de los propágulos micorrízicos que posibilita la formación de agregados de suelos y una viabilidad comprobada en medio líquido entre 12 y 18 meses que se puede utilizar en los sistemas de riego y fertirriego, aplicándose en forma directa en campos o invernaderos de cultivo protegido, además de permitir la superación de los mecanismos de dormancia de las esporas y de lograr un elevado nivel de concentración que permite y facilita su anejo y transportación. Con la aplicación de este producto, se pueden manejar los hongos micorrizógenos en cultivos bajo sistemas de riego en gran escala, con una mejora productiva y ambiental.

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LIQUID MYCORRHIZAL INOCULANT

DESCRIPTION.

Technical field

The present technical solution is related with agricultural sciences, particularly with organic biofertilizer production, and more specifically, for obtaining one liquid mycorrhizal inoculant that can be applied to soil and plants both directly and through irrigation systems.

Previous art

Multiple functions have been attributed to mycorrhizal associations, among them are the improvement of the absorbing root system surface through a remarkable increase of this system, soil toxin tolerance increment, solubilization of certain nutrient elements (N, P, Ca, Mg, Mn, Cu, Zn, B, etc.), resistance to adverse conditions, such as: drought, salinity, absorption selectivity, improvement of soil physical properties derived from the formation of stable aggregates and certain protection against root pathogens. The application of this association to agriculture implies a strong environmental impact, because it favors a better balance among the different microorganism populations present in the mycorrhizosphere and rhizosphere of mycorrhizal fungus-plant association and in general a higher protection against the biotic and abiotic stress effects.

The obtaining and later application of inoculants based on arbuscular mycorrhizal fungi in plants have particular characteristics, because they do not grow in axenic media alone, but complete their cycle of life in the host plant roots, so that once concluded the vegetable life cycle (for the production of these inoculants 3 to 4 month-old short cycle plants are used), the root system colonized by the fungus is extracted, as well as the fungal propagules (extramatrical mycelium and spores) developed during the association, as well as the substratum where they have been carried out, constituting all these components a mycorrhizal inoculant on a solid base (Fernandez, F. Chapter 3. Bases Científico Técnicas para el manejo de los sistemas micorrizados eficientemente. In: Rivera, Fernández, eds. INCA, Havana. 2003).

In the current production of this type of inoculant, the following substrata are used (Feldman, F. and Idozak, E. Inoculum production of Vesicular-arbuscular Mycorrhizal

Fungi for use in tropical nurseries. In: Methods in Microbiology. Volume 24 ISBN 0-12-521524-X. Eds. Academic Press Limited. 1992; Fernandez, F *et al*, Author's Certificate 22641. OCPI. 1999):

- Soil
- Soil + Organic Matter
- Organic matter
- Sand
- Clays
- Clays + Sand
- Clays + Organic Matter
- Sand + Organic Matter + Soil
- Nutrient solution in liquid flow chamber
- Vermiculite
- Pearlet
- Moss peat
- Expanded montmorillonite clay
- Combinations of all these subtracts

Mycorrhizal products developed from the previously mentioned substrata, are directly applied to dressed seed with 10%, according to the total weight of the seed to be put straight to the field (Fernandez, F *et al.* Author's Certificate 22641. OCPI. 1999) or the direct application of inoculants at the rate of 1 to 2 T. h^{-1}. In the case of seedbed and nursery crops, the application rate should not exceed 1 $kg.m^2$, and 10 g/seed, always putting the product below the seed to be sown respectively (Sieverding, E. Vesicular. Arbuscular Mycorrhiza in Tropical Agrosystem. Deutsche Gesellsschaft fur tecniische Zusammenarbeit (GTZ) GMBH, Federal Republic of Germany. 1991. 371 p).

The application of these inoculants by irrigation systems or fertigation is mainly due to the absence of a liquid inoculant that meets the appropriate characteristics, allowing it to support the abrupt changes of the hydrostatic pressure generated in the irrigation tubes.

The production of arbuscular mycorrhizal inoculants has been developed on the basis of applying solid, semisolid to flocculant substrata and on a smaller scale the use of these microorganisms in liquid media.

Regarding solid media, there are some examples of patents that have used several multiplication substrata, such as: US4945059, JP7123979, JP4187081, DE10161443, and DE10221762. These inoculants are unsuitable, since they generally manage big volumes of substrata, spore germination is affected by long dormancy periods, propagule concentration in this product is natural, that is, it cannot be concentrated with at the desire size.

In the case of semisolid inoculants, the following patents have been published: EP0726305, EP0596217, EP0072213, US5120344, EP0495108, EP0475433, US5436218, US3437625, US3557562, EP0023347, US2856380, US3857991, and GB2381264. These products are based on t substrata used like gels, copolymers, clays, in flocculant media. As in the previous case, important volumes are managed in the agricultural application which are not able to overcome the dormancy periods in the spores from mycorrhizal fungi.

The liquid inoculants reported along with patents, are basically related with ectomycorrhiza (CN1420167, US2 004208852) and we have only found a liquid reported by Japanese people (JP4141023), based on the use of a stabilizer (citric acid or its salt) to protect spores.

In general, well-known mycorrhizal products do not have a uniform spore germination and dormancy mechanisms of these fungal species cannot be yet improved, because the substrata on which these inoculants are presented do not confer stressing situations to the propagules that accelerate the germination processes of the spores once inoculated under field conditions.

On the other hand, mycorrhizal inoculants manufactured so far, which have a natural limit of mycorrhizal propagules, have not been reported, because the obtaining procedure does not bear the concentration of a higher quantity of specific propagules. The well-known mycorrhizal product with a higher concentration at present has a spore relation: mycelium of 50 spores.g^{-1} substratum: 65 mg. mycelium^{-1} substratum

(Fernández, F. Chapter 3. Bases Científico Técnicas para el manejo de los sistemas micorrizados eficientemente. In: Rivera, Fernández, eds. INCA, Havana. 2003).

The specific glycoprotein of these fungi, called Glomaline, accelerates the formation processes of stable micro aggregates in the soil, improving their physical structure, increasing the oxygen levels, and enabling a better colonization of the organism. As Glomaline is only excreted in a natural way during the symbiosis, or under the action of a certain medium of excretion, it does not appear excreted to the solid support in the well-known mycorrhizal inoculants, and the levels where it is found in the soil, depend on the symbiotic status with the organism, and they cannot rise beyond what this symbiosis allows to.

Description of the Invention

The main objective of this invention consists of obtaining of a liquid product that permits a massive handling of mycorrhizal fungi, with higher quality and efficiency, so that it can be straight applied in appropriate and profitable doses especially by located irrigation system or another's ones.

For obtaining this inoculant, the following steps were developed:

1. Seeding host plants of *Braquiaria decumbens*, *Sorghum vulgare* or another micotrophic obligatory species of host plants which grow on a reproductive substratum: soil, kaolinitic or montmorillonic clays, pearlet, zepeolite, vermiculite and organic matter (applied according to symbiotic needs).

2. These plants are directly inoculated by placing, 10 grams of pure strain of mycorrhizal fungi below the seeds, for their production the following species have been individually used: *Glomus fasciculatum*, *Glomus clarum*, *Glomus spurcum*, *Glomus mosseae*, *Glomus intrarradices*, *Gigaspora margarita*, and other species of Glomales and Diversisporales, but other species of mycorrhizal fungi can also be use.

3. At the end of plant life cycle, its top part is removed, and a consistent solid substratum is obtained in a mixture of mycorrhizal propagules (colonized roots, fungal mycelium and resistance spores) and the initial support.

4. Later on, mycorrhizal propagules are extracted by humid decanted and sifted method through several sieves that go from a rancid of 400 to 40 *u*m, according to the

mycorrhizal fungi species in question; the solid fraction gathered in the finest sieve, is centrifuged and then the liquid fraction is decanted with fungal propagules.

5. When all the mycorrhizal components have been separated, these are superficially disinfected with a solution of Chloramine T (2%) during 5 minutes; they are washed three times with sterile distilled water and placed in sulfate of streptomycin during 24 hours. Once ended they are packed in a watery mixture containing Sorbitol (between 5 and 10 %), Mannitol (2 %),Tween™ 40, (5 %), Tween 80 (2 %), Polyethylenglycol 4000, to apply at a range between 100 and 500 $mg.L^{-1}$ and agar from 0.8 to 0.1 %. In this case, the product can. be manufactured up to permissible physical limits of propagule $unit.mL^{-1}$ of culture medium.

6. The watery solution with propagules would be composed by the following elements:

a)Active components:

-Mycorrhizal propagules: Resistance spores, arbuscular extramatrical mycelium, Glomaline and smaller than 40 um roots, colonized with the fungus in question.

b) Other components:

"Sorbitol, at a range between 5 and 10 %,

"Mannitol, at a range between 2 and 5 %,

"Tween 40, at a range between 5 and 7 %,

"Tween 80 at a range between 2 and 7 %,

"Polyethyleneglycol applied at a range between 100 and 500 $mg.L^{-1}$

"Agar, at a range between 0.8 and 0.1 %.

Once these procedures concluded, a product is ready to be applied in the field or through located irrigation system, guaranteeing that mycorrhizal propagules, especially spores, are be protected of the effects of the superficial osmotic pressure and that once they are applied in water, they are able to break spore dormancy mechanisms and reach a strong colonizing capacity, developing a positive crop answer.

The proposed invention consists of an inoculant that, according its form of production, can be highly infective, because very high concentrations can be reached, without more limitations than the physical ones that allow this aqueous dissolution, or the conveniences for its application, achieving concentrations of the order of 1 or 2 million spores. L^{-1} and 1 and 2 grams of mycelium $.L^{-1}$. In a preferred embodiment, the polyethyleneglycol has molecular weights between 4,000 and 10,000 and a solubility in the inoculant between 100 and 500 $mg.L^{-1}$.

Also, because of the fact that mycorrhizal propagules are in an aqueous medium, Glomaline secretion is stimulated, which is present at high concentrations in the composition of this inoculant, conferring it special characteristics, speeding up this way, with its direct application, the processes of forming stable microaggregate in the soil, improving its physical structure immediately, and increasing oxygen levels, which enables a better colonization of this microorganism, guaranteeing an efficient mycorrhizal symbiosis development.

This inoculant promotes the direct application of the product in the soil, decreasing its volume when increasing its concentration, enabling this way its handling and transportation, and eliminating work.

It is mounted in a concentrated liquid support, with 8-months viability, which can be completely applied through fertigation systems, achieving a homogeneous distribution, checked by experiments at different scales. This fact has guaranteed a successful inoculation of all plants under the irrigation system, which is observed by significant yield increments (see technical report).

References

- Dell Amico. et al, Journal of Agricultural Science.. 2002
- Feldmann, F and Idczak, E. Inoculum production of Vesicular –arbuscular Mycorrhizal Fungi for use in tropical nurseries. In: Methods in Microbiology. Volume 24 ISBN 0-12-521524-x. Eds. Academic Press Limited. 1992
- Fernández, F. Capitulo 3. Bases Científico Técnicas para el manejo de los sistemas micorrizados eficientemente. En: Rivera, Fernández, eds. INCA, La Habana. 2003.
- Fernández, F et al,. Patente 22641. OCPI. 1999
- Sieverding, E. Vesicular Arbuscular Mycorrhiza in Tropical Agrosystem. Deutsche Gesellsschaft fur techniische Zusammenarbeit (GTZ) GMBH, federal Republic of Germany.1991. 371 p.

CLAIMS:

1. A liquid mycorrhizal inoculant which comprises:

a. Mycorrhizal propagule which consists of resistance spores, arbuscular external mycelium, Glomaline and smaller than 40 µm roots, colonized with the fungus in question,

b. between 5 and 10% sorbitol,

c. between 2 and 5% mannitol,

d. between 5 and 7% Tween 40,

e. between 2 and 7% Tween 80,

f. between 100 and 500 $mg.L^{-1}$ polyethyleneglycol, and

g. 0.1% agar.

2. The liquid mycorrhizal inoculant according to claim 1, wherein the inoculant is applied in water through irrigation or fertigation systems.

3. The liquid inoculant mycorrhizal according to claim 1, wherein the inoculant comprises 1 to 2 million $spores.L^{-1}$ and 1 to 2 grams of $mycelium.L^{-1}$.

4. The liquid inoculant mycorrhizal according to claim 1, wherein the polyethyleneglycol has molecular weights between 4,000 and 10,000 and a solubility in the inoculant between 100 and 500 $mg.L^{-1}$.