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Abstract (EN)

[origin: WO9211752A1] The application relates to inocula of ectomycorrhizal fungi which are able to produce symbiotic mycorrhizal associations when incubated with both leafy and woody plants. With leafy plants, the association between plant root and fungi generally has endomycorrhizal morphology; with woody plants, the association generally has ectomycorrhizal morphology.

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ABSTRACT

The application relates to inocula of ectomycorrhizal fungi which are able to produce symbiotic mycorrhizal associations when incubated with both leafy and woody plants. With leafy plants, the association between plant root and fungi generally has endomycorrhizal morphology; with woody plants, the association generally has ectomycorrhizal morphology.

CLAIMS (OCR text may contain errors)

What is Claimed is:

1. The process of producing an ectomycorrhizal inoculant for either woody or herbaceous plants, comprising the steps of: obtaining mycelia of a selected ectomycorrhizal, microsclerotia-producing fungi, contacting propagule of said mycelia with a growth medium and a particulate carrier in vitro, maintaining said contact in the absence of living plants in darkness and without active aeration for a period of time effective to produce sclerotia, microsclerotia or the initials of sclerotia or microsclerotia among said mycelia, and obtaining an ectomycorrhizal inoculum containing sclerotia microsclerotia or the initials of sclerotia or micro- sclerotia.

2. The process of claim 1, wherein said obtained inoculum is effective to form endomycorrhizal symbiotic associations with the roots of herbaceous plants and mycor- rhizal symbiotic associations with the roots of woody plants.

3. The process of claim 1, wherein said maintaining is conducted for about three months at about room temperature.

4. The process of claim 1, wherein said mycelia of selected ectomycorrhizal fungi are selected from the fungus species consisting of Rhizopogon roseolus, Pisolithus tinctorius, Amanita muscaria, Astraeus hygrometricus, Cenococ- cum geophilum, Scleroderma aurantium, Athelia neuhoffii. Boletinellus merulioides, Hebeloma anthracophilu , Hebeloma crustuliniforme, Paxillus involutus, Piloderma bicolor, Rhizopogon nigrescens, Scleroderma albidum. Scleroderma polyrhizum, Suillus cothurnatus, Alpova pachyploeus, Boletus punctipes, and Lactarius deliciosus.

5. The process of claim 1, wherein said carrier is selected from the group consisting of perlite and modified clays.

6. The process of claim 5, wherein in said contact- ing and maintaining steps the weight ratio of said particulate carrier to said growth medium is at least 2:1.

7. The process of claim 6, wherein said carrier is perlite.

8. An ectomycorrhizal inoculant produced in vitro and containing sclerotia or microsclerotia or the initials of sclerotia or microsclerotia.

9. The inoculant according to claim 8, wherein said inoculant is effective to form endomycorrhizal symbiotic associations with herbaceous plants and mycorrhizal symbiotic associations with woody plants.
10. The ectomycorrhizal inoculant of claim 9, wherein said sclerotia or microsclerotia are of the fungus selected from the group consisting of Rhizopogon roseolus, Pisolithus tinctorius , Amanita muscaria, Astraeus hygrometricus, Cenococcum geophilum. Scleroderma aurantium, Athelia neuhoffii, Boletinellus merulioides, Hebeloma anthracophilum, Hebeloma crustuliniforme, Paxillus involutus, Piloderma bicolor, Rhizopogon nigrescens. Scleroderma albidum, Scleroderma polyrhizum, Suillus cothurnatus, Alpova pachyploeus, Boletus punctipes, and Lactarius deliciosus.

11. A tree seedling in contact with an ectomycor- rhizal inoculant produced in vitro and containing sclerotia or microsclerotia or the initials of sclerotia or microsclerotia.

12. The tree seedling of claim 11, wherein said tree is a pine tree.

13. A herbaceous seedling in contact with an ec- tomycorrhizal inoculant produced in vitro and containing sclerotia or microsclerotia or the initials of sclerotia or microsclerotia.

14. The herbaceous seedling of claim 13, wherein said seedling is selected from the group consisting of corn, wheat, onion and asparagus seedlings. AMENDED CLAIMS

[received by the International Bureau on 30 March 1992 (30.03.92); original claim 12 cancelled; original claims 1,2 and 4 amended; claims 3,5-7 unchanged; new claims 8-11, 18 and 19 added; claims 8,10,11 and 13 amended and renumbered as claims 12,

14,15 and 16; claims 9 and 14 unchanged but renumbered as claims 13 and 17 (4 pages)] 1. The process of producing a mycorrhizal inoculant of a normally ectomycorrhizal fungi for use with either woody or herbaceous plants, comprising the steps of: obtaining mycelia of an ectomycorrhizal fungus, contacting a propagule of said mycelia with a growth medium and a particulate carrier, and maintaining said contact in the absence of living plant matter in darkness and without aeration for a period of time effective to stress the mycelia, inducing it to produce at least microsclerotia or its initials among said mycelia, and obtaining an ectomycorrhizal inoculum containing at least microsclerotia or the initials of microsclerotia, which inoculum is capable of producing intracellular hyphae when contacted with herbaceous plants.

2. The process of claim 1, wherein said obtained inoculum is effective to form mycorrhizal symbiotic associations with the roots of woody plants.

3. The process of claim 1, wherein said maintaining is conducted for about three months at about room temperature.

4. The process of claim 1, wherein said mycelia of selected ectomycorrhizal fungi are from the fungus species consisting of Rhizopogon roseolus, Pisolithus tinctorius. Amanita muscaria, Astraeus hyσrometricus, Cenococcum geophilum, Scleroderma aurantium, Athelia neuhoffii, Boletinellus

merulioides. Hebeloma anthracophilum, Hebeloma crustuliniforme, Paxillus involutus, Piloderma bicolor, Rhizopogon niqrescens, Scleroderma albidu , Scleroderma polyrhizum, Suillus cothurnatus, Alpova pachyploeus. Boletus punctipes, and Lactarius deliciosus.

5. The process of claim 1, wherein said carrier is selected from the group consisting of perlite and modified clays. 6. The process of claim 5, wherein in said contactin and maintaining steps the weight ratio of said particulate carrie to said growth medium is at least 2:1.

7. The process of claim 6, wherein said carrier is perlite.

8. The process of claim 1 wherein the propagule used in the contacting step are obtained by xenically growing said mycelia in the dark in petri dishes containing a nutrient-containing solid medium.

9. The process of claim 8 wherein the contacting step comprises cutting plugs from said petri dishes on which said mycelia have been grown and xenically contacting the plugs with the growth medium and particulate carrier.

10. The process of claim 9 wherein said carrier is perlite and said solid medium is agar.

11. The method of claim 1 wherein the growth medium is Fowells and Krauss' pine nutrient solution modified by the addition of glucose and thiamine.

12. A mycorrhizal inoculant for either woody or herbaceous plants produced from mycelia of ectomycorrhizal fungi in the absence of living plant matter, said inoculant containing at least microsclerotia or the initials of microsclerotia and being capable of producing intracellular hyphae when contacted with herbaceous plants.

13. The inoculant according to claim 12, wherein said inoculant is effective to form endomycorrhizal symbiotic associa- tions with herbaceous plants and mycorrhizal symbiotic associations with woody plants. 14. The ectomycorrhizal inoculant of claim 12, wherein said mycelia of ectomycorrhizal fungi are of the fungus selected from the group consisting of Rhizopogon roseolus, Pisolithus tinctorius, Amanita muscaria, Astraeus hygrometricus, Cenococcum geophilum, Scleroderma aurantium, Athelia neuhoffii. Boletinellus merulioides, Hebeloma anthracophilum, Hebeloma crustuliniforme, Paxillus involutus, Piloderma bicolor, Rhizopogon nigrescens, Scleroderma albidum, Scleroderma polyrhizum, Suillus cothurnatus, Alpova pachyploeus. Boletus punctipes, and Lactarius deliciosus.

15. A tree seedling in contact with a mycorrhizal inoculant produced from mycelia of ectomycorrhizal fungi in the absence of living plant matter and containing at least microsclerotia or the initials of microsclerotia, said inoculant being capable of producing intracellular hyphae when contacted with a herbaceous plant.

16. A herbaceous seedling in contact with an ectomycorrhizal inoculant produced in the absence of living plant matter and containing at least microsclerotia or the initials of microsclerotia that produce intracellular hyphae of a mycorrhizal fungus.

17. The herbaceous seedling of claim 16, wherein said seedling is selected from the group consisting of corn, wheat, onion and asparagus seedlings.

18. A method of growing plants having mycorrhizae associated with their roots comprising the steps of: obtaining mycelia of an ectomycorrhizal, microsclerotia-producing fungus, contacting propagule of said mycelia with both a growth medium containing a nutrient solution comprising sugar and thiamine and a particulate carrier, maintaining said contact in the absence of living plant matter in darkness and without aeration for a period of time effective to stress the mycelia, inducing it to produce at least microsclerotia or its initials, obtaining an ectomycorrhizal inoculum from said mycelia which is capable of producing intracellular hyphae when contacted with herbaceous plants, germinating a seedling, contacting said seedling with said inoculum and a nutrient solution, and obtaining plants having a symbiotic association with said fungi.

19. The method of claim 18 wherein the nutrient solution is Fowells and Krauss' pine nutrient solution and the growth medium s pH balanced.

DESCRIPTION (OCR text may contain errors)

INOCULUM FROM ECTOMYCORRHIZAL FUNGI FORMING

ENDOMYCORRHIZAL INFECTION WITH HERBACEOUS PLANTS

Field of the Invention

This invention relates to mycology, more specifically to a process for the production of inoculants for herbaceous plants and to the inoculants so produced. Background of the Invention

The momenclature used in this application is intended to be consistent with Snell and Dick, A Glossary of Mycology, Harvard U. Press, Cambridge, MA (1957). Mycorrhizae are symbiotic associations between the hyphae of certain fungi and the absorbing organs of plants, typically the roots. Mycorrhizal fungi are classified accord¬ ing to the manner in which they infect roots. The two main types are ectomycorrhizae in which fungal hyphae penetrate the intercellular spaces between root cells without entering the interior of the cells, and endomycorrhizae where projections of the fungus enter the interior of the cell.

Ectomycorrhizae are generally associated with trees and other woody species and are formed by "higher fungi" that are found in a number of families of basidiomycetes and as- comycetes. Roots infected with ectomycorrhizal fungi are different from uninfected roots. They are short, swollen branched and lack root hairs.

Endomycorrhizae are generally associated with her baceous plants such as grasses, corn, onions and many more, however there are some trees that also form endomycorrhizae. Endomycorrhizae are formed from spores produced by "lowe fungi," classified as zygomycetes, and belong to one family, the

Endogonales. The fungi that produce these spores ar unknown. Superficially, the infected roots look normal, however, the infection can be detected by microscopic examina¬ tion. Such examination shows the hyphal projections of the fungus that have invaded the cells. These hyphae may have small branches (arbuscles) or swellings at their tips (vesicles). Many endomycorrhizae are called "Vesicular- Arbuscular Mycorrhizae" or VAM, because of the presence of vesicles and arbuscles inside root cells. Ectomycorrhizae generally do not have vesicles or arbuscles.

Ectomycorrhizal fungal inoculants for woody plants such as pine have been produced. See, for example, Litchfield et al., U.S. Pat. 4,327,181, "Aerobic Submerged Fermentation of

Sporulating Ectomycorrhizal Fungi" (1982) disclosing liquid culture of selected fungi to produce inoculants for broadcast over forest soil. Marx et al., "Growth and Ectomycorrhizal

Development of Loblolly Pine Seedlings in Fumigated Soil Infested with the Fungal Symbiot Pisolithus Tinctorium," Forest

Science Vol. 22, pp. 245-254 (1975), show the use of Pisolithus

Tinctorius cultured in an agar/vermiculite/peat moss medium in forest nurseries. See also Mosse et al., U.S. Pat. 4,294,037, "Production of Mycorrhizal Fungi" (1981) and Warner, U.S. Pat. 4,551,165, "Mycorrhizal Seed Pellets" (1985).

As far as known, however, inoculants suitable for leafy plants such as wheat or the common vegetables corn, onion, asparagus and the like have not heretofore been produced. Compare Watrud, "Spore Germination and Axenic Culture of Endomycorrhizae," writing at page 81 of Methods and Principles of Mycorrhizal Research, the American Phytopathol- ogical Society (1982): ". . . To date, successful axenic subculture of hyphae of vesicular-arbuscular mycorrhizae has yet to be reported. . . ." See also Hudson, Fungal Biology, pp. 218 and 219, Edward Arnold (1986); Smith and Douglas, The Biology of Symbiosis, pp. 152 and 153, Edward Arnold (1987); and Mugnier et al., U.S. Patent 4,599,312, "Method of Producing Endomycorrhizian Fungi with Arbuscules and Vesicles in Vitro" (1986). DETAILED DESCRIPTION OF THE INVENTION

It has now been found that fungal inoculants for herbaceous plants can be made by a versatile process in which mycelia of selected ectomycorrhizal fungi are initially grown from cultures on a solid medium. These mycelia, still in the solid medium (fungal plugs) , are then axenically added to perlite wetted with a nutrient solution and incubated in vitro. The incubation is conducted under the conditions described below for a period of time sufficient to allow the formation of sclerotia, microsclerotia or the initials of sclerotia or icrosclerotia. Generally, the incubation is conducted for between about 2 and about 4 months, preferably about 3 months. The inoculant obtained can be used with woody plants, with which the inoculant generally produces symbiotic associations having ectomycorrhizal morphology. The inoculant can also be

used with leafy plants, with which the inoculant generally produces symbiotic associations having endomycorrhizal morphol¬ ogy.

The solid medium used in the initial growth of mycelia is generally gelled agar. Modess Modification of Hagem Agar in the agar can be the nutrient. Other nutrients, such as a modified Melin and Rama Das Agar, can also be used. Three or four weeks in the dark at around 25°C provide sufficient time for initial growth.

Fungal plugs taken from the agar growth medium are axenically added to jars containing perlite and nutrient solution and incubated in vitro for about three months in the dark under quiescent and substantially anerobic conditions. In this connection, "quiescent" simply means without agitation. A useful nutrient solution is Fowells and Krauss's pine nutrient solution modified by the addition of glucose and thiamine (Fowells and Krauss, "The inorganic nutrition of Loblolly pine and Virginia pine with special reference to nitrogen and phosphorus" Forest Science 5:95-112 (1959)).

It should be noted that the inert carrier used in the nutrient solution is somewhat selective. The most satisfactory results have been obtained with perlite as an effective carrier although some modified clays can be used. Vermiculite has been found ineffective under the conditions disclosed. The propor¬ tion of carrier to liquid nutrient (carrier/nutrient weight ratio) is not critical but around at least 2/1 has been found satisfactory to provide an effective saturated support.

The inoculum prepared above can be used with leafy plants or by broadcast sowing with woody plants. In the former case, one axenically germinated seed can be placed on top of one teaspoon full of the fungus inoculum about three cen¬ timeters below the surface of a flower pot filled with perlite. The plants are then grown in the greenhouse for four months. The pots are saturated twice weekly with a low-phosphorus nutrient solution (Peter's Lo Phos Fertilizer).

Known ectomycorrhizal fungi that formed mycorrhizal infections having endomycorrhizal morphology with corn, wheat, onion, and/or asparagus plants include the following:

TABLE I Name Source

1. Rhizopoσon roseolus John Melhuish

(Melhuish #20) U.S.D.A. Forest Service

2 . Pisolithus tinctorius American Type Culture (ATCC #38054) Collection

3. Amanita muscaria John Melhuish

(Melhuish #21) U.S.D.A. Forest Service 4. Astraeus hyσrometricus American Type Culture

(ATCC #46449) Collection

5. Cenococcum qeophilum American Type Culture (ATCC #38052) Collection

6. Scleroderma aurantium American Type Culture (ATCC #58507) Collection

7. Athelia neuhoffii John Melhuish (Melhuish #47) U.S.D.A. Forest Service 8. Boletinellus merulioides John Melhuish

(Melhuish #64) U.S.D.A. Forest Service

9. Hebeloma anthracophilum John Melhuish (Melhuish #54) U.S.D.A. Forest Service

10. Hebeloma crustuliniforme John Melhuish

(Melhuish #53) U.S.D.A. Forest Service 11. Paxillus involutus Americah Type Culture (ATCC #46218) Collection

12. Piloder a bicolor John Melhuish (Melhuish #50) U.S.D.A. Forest Service

13. Rhizopogon nigrescens John Melhuish

(Melhuish #38) U.S.D.A. Forest Service

14. Scleroderma albidum American Type Culture

(ATCC #58021) Collection

15. Scleroderma polyrhizum John Melhuish

(Melhuish #68) U.S.D.A. Forest Service 16. Suillus cothurnatus John Melhuish (Melhuish #31) U.S.D.A. Forest Service

17. Alpova pachyploeus Dr. Donald Marx (Marx #258) U.S.D.A. Forest Service

18. Boletus punctipes John Melhuish

(Melhuish #15) U.S.D.A. Forest Service

19. Lactarius deliciosus American Type Culture

(ATCC #36647) Collection

While the reasons for the efficacy of the present inoculum and its preparation are not completely understood, it can theoretically be explained as follows. Fungi are able to propagate from a number of sources or propagules including spores, infected root fragments, mycelia, hyphae, etc. One class of propagule are sclerotia (Willets, "Sclerotium Forma¬ tion," Filamentous Fungi 3:197-213 (1978)), and these are believed to be involved here. Sclerotia are generally too small to be seen by the naked eye but they can be seen with a microscope. Oσcassionally, sclerotia are observable with the unaided eye. Structures of this type are also known as "micro¬ sclerotia" (Baard et al. "Structure and Lysis of Microsclerotia . . .," Trans. Br. Mycol. Soc. 77(2) :251-260 (1981)). The development of the mycelium in contact with the inert support and nutrient thus proceeds until sclerotia, microsclerotia, or their initials are produced. The initials of sclerotia are recognizable under the microscope as enlarged structures in fungal hyphae. Sclerotia are believed to develope from these swollen structures and may be considered a dimorphic form of the fungus. Under some environmental conditions, the fungus has long, thin, hair-like hyphae, while under other conditions the fungus may have the more rounded morphology of microsclero¬ tia. Operable fungi for the present inventions are those fungi which produce sclerotia or microsclerotia. Spore-like struc¬ tures may also be found in the inoculant.

The inoculant of the present invention has good shelf life. When stored at 24°C in the dark, inocula of the present invention have been found to remain effective for in excess of one year, usually for in excess of three years.

The following nonlimiting examples illustrate the invention.

EXAMPLES 1-6 A. Preparation of Inoculum Selected fungi (Cenococcum Geophilum, Pisolithus tinctorius, Astraeus hygrometricus A anita muscaria. Rhizopogon roseolus, and Scleroderma aurantium) were axenically grown from mycelia for three weeks at 24°C in the dark in petri plates on Hage 's nutrient agar modified by Modess (Modess, 0., 1941, Zur Kenntnis der Mykorrhizabildner von Kiefer und Fichte, Symbolae Bot. Upsalienses 5(1): 1-146) as shown in Table II.

Table II

Formulation* of Modess Modification of Hagem Agar

KH_2PO	0.5 g
$MgSO_4.7H_2O$	0.5 g
NH_4Cl	0.5 g
$FeCl_3$ (1% solution)	1.0 ml
Glucose	5.0 g
Malt extract	5.0 g
Agar	10.0 g
H_2O (distilled)	1000.0 ml

* Final pH ■ 4.7

Subsequently, 0.5 mm plugs were cut from the periphery of the fungal colonies and axenically added to separate test tubes containing 40 ml (8 g) of strained perlite and 20 ml of Fowells and Krauss's modified nutrient solution (Table II) . The test tubes containing the perlite and nutrient solution had previously been autoclaved at 120°C and 15 lbs pressure for 20 minutes. Inoculated test tubes were incubated for three months in the dark at 24"C. Table III

Modified Fowells and Krauss's Nutrient Solution Formulation*

NH4NO3 0.68 ml of 1 molar stock solution

KH_2PO_4 0.15 ml of 1 molar stock solution

$MgSO_4$ 0.95 ml of 1 molar stock solution

$CaCl_2$ 0.95 ml of 1 molar stock solution

NaCl 0.95 ml of 1 molar stock solution

KC1 2.92 ml of 1 molar stock solution

Ferric Citrate** 1.10 ml of stock solution

Combined minor elements*** 0.13 ml of stock solution

Thiamine 50 ug

Glucose 2.5 g

HC1 1.5 ml of IN solution

H_20 (distilled) 1000 ml

* Final pH - 5.7 *** Stock Solution of Minor Elements:

** Stock Solution of Ferric Citrate:		$CuS0_4.5H_2O$	8.0 MG
		$ZnS0_4.4H_2O$	21.8 mg
Ferric Citrate	2.5 g	$MnCl_2.4H_2O$	180.0 mg
Citric Acid	1.6 g	H_3BO_3	285.5 mg
H_2O	250 ml	$H_2MO_4.H_2O$	2.1 mg
		H_2O	100 ml

Five ml of Fowells and Krauss's nutrient solution was added to each test Tube and thoroughly mixed with the incubated inoculum before use.

B. Growth of Endomycorrhizal Structures in Leafy Plants Seeds of onion, wheat, corn and asparagus that had been soaked in distilled water overnight were washed for 1 minute in an aqueous solution containing 1% $HgCl_2$ and 1 ml of Tween 20 per liter and rinsed three times with sterile dis¬ tilled water. Seeds were then axenically placed in sterile petri plates containing moistened filter paper and allowed to germinate in the dark at 24°C. When the emerged radicles were between 1 cm and 3 cm long (within 1 week for all seeds), they were planted in the greenhouse in 4-inch pots filled with strained horticultural grade perlite. A hole was dug 3 cm from the surface, and one teaspoon full of the fungus inoculum was put in the hole with one germinated seed placed on top of the inoculum and covered with perlite. Twenty pots were prepared for each fungus being tested along with twenty control plants of each seed type. Each pot was placed on top of an inverted, empty 5-inch pot to eliminate cross-contamination on the green¬ house bench. All pots were saturated with the Fowells and Krauss's solution. The pots were watered daily and saturated twice a week with Peter's Lo Phos Fertilizer.

Each of the six fungi being tested was (C. qeophilum, P. tinctorius, A. hygrometricus, R. roseolus, S. aurantium and

A. muscaria) used as inoculum for the onion seeds. Only

Cenococcum geophiluπt and Pisolithus tinctorius were used to inoculate the corn, wheat, and asparagus seeds. Inoculated plants were grown under normal greenhouse conditions with the day length extended to 12 hours with incan¬ descent light bulbs.

After 8 weeks, plants were periodically removed from the greenhouse and the roots and perlite in which they were grown was examined as follows. Roots were individually washed in distilled water until free of perlite. The perlite that the plant grew in was agitated in 1 liter of distilled water and filtered along with the root washings through one layer of cheesecloth. This filtrate was then refiltered through nested 230 mesh and 325 mesh sieves. The retentate on the 325 mesh sieve was saved and examined microscopically. The cleaned roots were cleared and stained using the procedure of Kormanik et al. (Kormanik, Bryan and Schultz, Can. J. Microbiol. 26:536- 538 (1980)). This procedure allows the microscopic visualiza¬ tion of fungi and fungal propagules within the intact root.

The roots of the plants that were examined after 8 weeks in the greenhouse showed no apparent internal fungal infection. However, mycelia were present on the exterior of the roots. The perlite washings revealed the presence of microsclerotia in every instance. When plants were similarly examined after 16 weeks, endomycorrhizal infection was well established in plants treated with all six fungal inoculants, and numerous microsclerotia were present on and around the roots. Root samples from plants grown 16 weeks or more were excised and fixed, dehydrated, and embedded in epoxy resin according to methods in Pizzolato, T. D., 1978, "A tannic acid- ferric chloride-toluidine blue stain for wood amyloplasts embedded in epoxy resin," Forest Science 24:49-51. The fixed samples were subsequently stained in Alsops reagent for 1 minute at 130^{C}C by the methods of Alsop (1974) and Pizzolato (1984) (Alsop, D., 1974, "Rapid single solution polychrome and staining of semithin epoxy sections by polyethylene glycol 200 as a stain solvent," Stain Technology 49:265-272; Pizzolato, 1984, "Vascular system of the fertile floret of Anthoxanthum odoratu L.," Botanical Gazette 145(3) :358-371) . The light microscope sections of internal root cells obtained with these procedures revealed the presence of intercellular and intracel- lular hyphae, vesicles, and spore-like structures with all fungi and plants tested. Control plants yielded no evidence of infection or microsclerotia.

An examination of the fungal inocula used to infect the plants showed the presence of fungal hyphae that contained many swollen areas characteristic of the initials of sclerotia and microsclerotia. The inocula also contained spore-like structures. While microsclerotia have been observed in comparable inocula of the present invention, none were observed here. The failure to observe sclerotia or microsclerotia is not dispositive of their presence; it is not known how well these structures survive the preparation procedure for micro¬ scopic observation.

Results of these studies indicate that inocula produced from ectomycorrhizal fungi by procedures outlined above can be used to induce mycorrhizal infection in herbaceous plants. Furthermore, all the inocula produced were shown to form mycorrhizae with loblolly pine seedlings. EXAMPLES 7-19 A. Preparation of Inoculum

The preparation of inocula as in Examples 1-6 was substantially repeated except that different fungal species were employed and a different agar medium was used for the initial growth incubation. Selected fungi (Athelia neuhoffii,

Boletinellus merulioides, Hebeloma anthracophilum. Hebeloma crustuliniforme, Paxillus involutus, Piloderma bicolor,

Rhizopogon nigrescens, Scleroderma albidum. Scleroderma polyr- hizum, Suillus cothurnatus, lpova pachyploeus, Boletus punctipes, and Lactarius deliciosus) were axenically grown from mycelia for four weeks at 24°C in the dark in petri plates on a modification of Melin and Rama Das nutrient agar (Melin and

Rama Das "Influence of root metabolites on the growth of tree mycorrhizal fungi" Physiol. Plant. 7:851-858 (1954)).

TABLE IV Formulation of Modified Melin and Rama Das Agar KH_2P0_4 1.0 gm

$MgS0_4.7H_2O$ 0.5 gm NH Tartrate 5.0 gm

ZnS0 (1:500) 0.5 ml

Fe Citrate (1%) 0.5 ml

Thiamin 50.0 ug (microgram)

Glucose 20.0 gm Agar 10.0 gm

Distilled H_20 to 1000 ml

Subsequently, 0.5 mm plugs were cut from the periphery of the fungal colonies and two plugs from each fungus were axenically added to a jar containing 100 ml of strained perlite and 55 m of Fowells and Krauss's modified nutrient solution (Table III). The jars containing the perlite and nutrient solution ha previously been autoclaved at 120°C and 15 lbs. pressure for 20 minutes. The inoculated jars were incubated for 3 months in the dark at 24°C. B. Ectomycorrhizal Infection in Asparagus Plants

Asparagus seeds that had been soaked in distilled water overnight were washed for one minute in 1% HgCl with 1 ml of Tween 20 per liter and rinsed three times with sterile distilled water. Seeds were then axenically placed in sterile petri plates containing moistened filter paper and incubated in the dark at 24°C. Six days later, the germinated seeds were planted in 4-inch pots filled with strained horticultural grade 1 perlite. A hole was dug 3 cm from the surface, and one teaspoon full of the fungus inoculum was put in the hole with one terminated seed placed on top of the inoculum and covered with perlite. Ten pots were prepared for each fungus being tested along with twenty control plants. Each pot was placed on top

of an inverted empty 5 inch pot to eliminate contamina¬ tion on the greenhouse bench. All pots were then saturated with the Fowells and Krauss's solution. The pots were watered daily and saturated twice a week with Peter's Lo Phos Fer¬ tilizer. Inoculated plants were grown under normal greenhouse conditions. After 4 months, plants were removed from the greenhouse and the roots and perlite in which they were grown were examined as described above. An examination of the fungal innocula used to infect the plants showed the presence of fungal hyphae that contained many swollen areas characteristic of the initials of sclerotia and microsclerotia. The inocula also contained spore-like structures.

Each of these fungal inocula forms mycorrhizae with Virginia pine or Loblolly pine in axenic culture.

Results of these studies indicate that inocula produced from ectomycorrhizal fungi by procedures outlined above can be used to induce mycorrhizal infection in herbaceous plants.

In view of the present specification and appended claims, various additions, modifications, and omissions will be obvious to those skilled in the art and are within the inven- tion as claimed below.

CLASSIFICATIONS

International Classification	A01G1/04
Cooperative Classification	A01G1/048, C12R1/645
European Classification	A01G1/04F, C12R1/645

LEGAL EVENTS

Date	Code	Event	Description
Dec 31, 2005	PG25	Lapsed in a contracting state announced via postgrant inform. from nat. office to epo	**Ref country code:** IT **Free format text:** LAPSE BECAUSE OF NON-PAYMENT OF DUE FEES **Effective date:** 20051231
Oct 7, 2005	REG	Reference to a national code	**Ref country code:** FR **Ref legal event code:** ST
Aug 31, 2005	PG25	Lapsed in a contracting state announced via postgrant inform. from nat. office to epo	**Ref country code:** FR **Free format text:** LAPSE BECAUSE OF NON-PAYMENT OF DUE FEES **Effective date:** 20050831
Aug 24, 2005	GBPC	Gb: european patent ceased through non-payment of renewal fee	**Effective date:** 20041231
Jul 1, 2005	PG25	Lapsed in a contracting	**Ref country code:** DE

Date	Code	Event	Description
		state announced via postgrant inform. from nat. office to epo	**Free format text:** LAPSE BECAUSE OF NON-PAYMENT OF DUE FEES **Effective date:** 20050701
Mar 16, 2005	REG	Reference to a national code	**Ref country code:** ES **Ref legal event code:** FD2A **Effective date:** 20040102
Dec 31, 2004	PG25	Lapsed in a contracting state announced via postgrant inform. from nat. office to epo	**Ref country code:** GB **Free format text:** LAPSE BECAUSE OF NON-PAYMENT OF DUE FEES **Effective date:** 20041231
Aug 31, 2004	EUG	Se: european patent has lapsed	
Aug 13, 2004	REG	Reference to a national code	**Ref country code:** CH **Ref legal event code:** PL
Jun 30, 2004	PGFP	Postgrant: annual fees paid to national office	**Ref country code:** DE **Payment date:** 20040630 **Year of fee payment:** 14 **Ref country code:** FR
Jun 28, 2004	PGFP	Postgrant: annual fees paid to national office	**Ref country code:** GB **Payment date:** 20040628 **Year of fee payment:** 14
Jan 2, 2004	PG25	Lapsed in a contracting state announced via postgrant inform. from nat. office to epo	**Ref country code:** ES **Free format text:** LAPSE BECAUSE OF NON-PAYMENT OF DUE FEES **Effective date:** 20040102
Jan 1, 2004	PG25	Lapsed in a contracting state announced via postgrant inform. from nat. office to epo	**Ref country code:** SE **Free format text:** LAPSE BECAUSE OF NON-PAYMENT OF DUE FEES **Effective date:** 20040101
Dec 31, 2003	PG25	Lapsed in a contracting state announced via postgrant inform. from nat. office to epo	**Ref country code:** CH **Free format text:** LAPSE BECAUSE OF NON-PAYMENT

Date	Code	Event	Description
			OF DUE FEES **Effective date:** 20031231 **Ref country code:** LI **Ref country code:** LU
Jun 30, 2003	BERE	Be: lapsed	**Owner name:** *JANERETTE CAROL A. **Effective date:** 20021231
Jan 17, 2003	PGFP	Postgrant: annual fees paid to national office	**Ref country code:** ES **Payment date:** 20030117 **Year of fee payment:** 13
Jan 8, 2003	PGFP	Postgrant: annual fees paid to national office	**Ref country code:** CH **Payment date:** 20030108 **Year of fee payment:** 13
Dec 31, 2002	PG25	Lapsed in a contracting state announced via postgrant inform. from nat. office to epo	**Ref country code:** AT **Ref country code:** BE **Free format text:** LAPSE BECAUSE OF NON-PAYMENT OF DUE FEES **Effective date:** 20021231
Dec 30, 2002	PGFP	Postgrant: annual fees paid to national office	**Ref country code:** LU **Payment date:** 20021230 **Year of fee payment:** 13
Dec 17, 2002	PGFP	Postgrant: annual fees paid to national office	**Ref country code:** SE **Payment date:** 20021217 **Year of fee payment:** 13
Feb 18, 2002	PGFP	Postgrant: annual fees paid to national office	**Ref country code:** BE **Payment date:** 20020218 **Year of fee payment:** 12
Jan 1, 2002	REG	Reference to a national code	**Ref country code:** GB **Ref legal event code:** IF02
Dec 27, 2001	PGFP	Postgrant: annual fees paid to national office	**Ref country code:** AT **Payment date:** 20011227 **Year of fee payment:** 12
Oct 21, 1998	26N	No opposition filed	

Date	Code	Event	Description
Apr 1, 1998	NLV1	Nl: lapsed or annuled due to failure to fulfill the requirements of art. 29p and 29m of the patents act; no legal effect from	
Mar 6, 1998	ET	Fr: translation filed	
Mar 1, 1998	REG	Reference to a national code	**Ref country code:** ES **Ref legal event code:** FG2A **Ref document number:** 2111001 **Country of ref document:** ES **Kind code of ref document:** T3 **Format of ref document f/p:** P
Feb 27, 1998	REG	Reference to a national code	**Ref country code:** CH **Ref legal event code:** NV **Representative=s name:** BUGNION S.A.
Jan 29, 1998	ITF	It: translation for a ep patent filed	**Owner name:** UFFICIO BREVETTI RICCARDI & C.
Dec 4, 1997	REF	Corresponds to:	**Ref document number:** 69031659 **Country of ref document:** DE **Date of ref document:** 19971204 **Format of ref document f/p:** P
Oct 31, 1997	REG	Reference to a national code	**Ref country code:** CH **Ref legal event code:** EP
Oct 29, 1997	REF	Corresponds to:	**Ref document number:** 159642 **Country of ref document:** AT **Date of ref document:** 19971115 **Kind code of ref document:** T **Format of ref document f/p:** P
Oct 29, 1997	AK	Designated contracting states:	**Kind code of ref document:** B1 **Designated state(s):** AT BE CH DE DK ES FR GB GR IT LI LU NL SE
Oct 29, 1997	PG25	Lapsed in a contracting state announced via postgrant inform. from	**Ref country code:** DK **Free format text:** LAPSE

Date	Code	Event	Description
		nat. office to epo	BECAUSE OF NON-PAYMENT OF DUE FEES **Ref country code:** GR **Ref country code:** NL **Free format text:** LAPSE BECAUSE OF FAILURE TO SUBMIT A TRANSLATION OF THE DESCRIPTION OR TO PAY THE FEE WITHIN THE PRESCRIBED TIME-LIMIT **Effective date:** 19971029
Sep 6, 1995	17Q	First examination report	**Effective date:** 19950724
Jan 12, 1994	A4	Supplementary search report	**Effective date:** 19931123
Jan 12, 1994	AK	Designated contracting states:	**Kind code of ref document:** A4 **Designated state(s):** AT BE CH DE DK ES FR GB GR IT LI LU NL SE
Oct 20, 1993	AK	Designated contracting states:	**Kind code of ref document:** A1 **Designated state(s):** AT BE CH DE DK ES FR GB GR IT LI LU NL SE
Oct 20, 1993	17P	Request for examination filed	**Effective date:** 19930702