Mycorrhiza (1999) 8:263–266 

ORIGINAL PAPER

**Luciana da Silva Rodrigues**
**Maria Catarina Megumi Kasuya · Arnaldo Chaer Borges**

# Viability of ectomycorrhizal fungus mycelium entrapped in calcium alginate gel

Accepted: 11 October 1998

**Abstract** We studied the viability of fragmented mycelium of *Pisolithus tinctorius* and *Paxillus involutus* entrapped in calcium alginate gel to determine the efficacy of this method of producing ectomycorrhizal fungus inoculum. Fungi were grown in MMN solution at 28 °C before being fragmented in a blender and subsequently entrapped in calcium alginate. We tested different ratios of alginate and mycelium suspension to 0.7 M $CaCl_2$. The ratio 8:10 resulted in well-formed beads of the highest viability for *Paxillus involutus* (99%) and for *Pisolithus tinctorius* (75%). *Paxillus involutus* mycelium was more than 90% viable when entrapped mycelium was 10 to 50 days old, and *Pisolithus tinctorius* attained its highest viability (55%) for 20- to 40-day-old mycelium. Gel entrapped *Paxillus involutus* mycelium grew well at all temperatures after 30 days of storage, but viability significantly decreased after 60 days storage at 6 °C on dry filter paper. For gel-entrapped *Pisolithus tinctorius* mycelium, viability was highest when stored at 25 °C in 0.7 M $CaCl_2$. Entrapment of *Paxillus involutus* fragmented mycelium in calcium alginate beads under the conditions that we propose can be used successfully to produce inoculum.

**Key words** *Hebeloma crustuliniforme* · Inoculum production · Inoculum storage · *Pisolithus tinctorius* · *Paxillus involutus*

L.S. Rodrigues
Departamento de Microbiologia,
Universidade Federal de Sergipe,
Aracaju, Sergipe, Brazil

M.C.M. Kasuya (✉) · A.C. Borges
Departamento de Microbiologia,
Universidade Federal de Viçosa,
Viçosa, Minas Gerais, Brazil, 36571-000
Fax: +55-31-899-2573, e-mail: mkasuya@mail.ufv.br

## Introduction

The mutualistic association of ectomycorrhizal fungi (EM) with roots of many forest trees is essential for plant health and nutrition (Molina and Trappe 1982). Moreover, artificial inoculation with EM fungi can guarantee uniformity in producing healthy seedlings even if natural inoculum is present (Marx 1991).

The most-recommended form of inoculum of selected EM fungi is vegetative mycelium (Marx 1991). The mycelium can be slurried (Boyle and Robertson 1987) and grown in a mixture of vermiculite and sphagnum (Marx et al. 1982, 1984), or may be entrapped in a polymeric gel such as calcium alginate (Le Tacon et al. 1985). The latter has proven to be a promising method of EM inoculum production for some species of *Hebeloma* (Le Tacon et al. 1985; Mauperin et al. 1987) and *Laccaria* (Mortier et al. 1989). However, care must be taken to guarantee the quality and viability of the inoculum (Mauperin et al. 1987). Temperature and humidity are important factors that need special attention during bead storage (Le Tacon et al. 1985; Mauperin et al. 1987; Kuek et al. 1992).

Even though *Pisolithus tinctorius* inoculum has been commercially produced in vermiculite and sphagnum (Marx et al. 1982, 1984), we found no published information on *Pisolithus tinctorius* or *Paxillus involutus* mycelium entrapped in alginate gel. Our work was designed to find the best technique to maintain high viability of fragmented mycelia of *Pisolithus tinctorius* and *Paxillus involutus* entrapped in a calcium alginate gel. We investigated the effects of the ratio of alginate and mycelium suspension to $CaCl_2$, age of entrapped mycelium, and storage conditions on fragmented mycelium viability.

## Materials and methods

*Pisolithus tinctorius* (Mich.: Pers.) Coker and Couch (Pt 90A) was obtained from the collection of the Laboratory of Mycorrhizal

Associations (BIOAGRO, Department of Microbiology, Federal University of Viçosa, Viçosa, Brazil) and *Paxillus involutus* (Batsch.: Fr.) Fr. and *Hebeloma crustuliniforme* (Bull.: St. Am.) Quél. from the collection of the Laboratory of Forest Mycology, INRA (Nancy, France). The latter fungus was used only as a control in a storage condition test. We grew the fungi in 50 ml of modified Melin-Norkrans (MMN) liquid medium (Marx 1969) in 125-ml Erlenmeyer flasks incubated at 28 °C. Each flask was inoculated with five agar discs (6-mm diameter) containing mycelium taken from the edge of a colony grown on MMN agar for 20 days. After incubation for different periods, 5 g of fresh mycelium was washed, resuspended in 200 ml of sterile water, and fragmented in a blender by four successive 1-s pulses. We added an equal volume of sodium alginate at a concentration of 2% (Mauperin et al. 1987) to the suspension of fragmented mycelium, and this mixture was dropped into a 0.7 M $CaCl_2$ solution where beads of approximately 4 mm diameter formed. Beads were left in the 0.7 M $CaCl_2$ to cure/polymerize for 45 min at room temperature (Mauperin et al. 1987).

### Volumetric proportion of alginate and mycelium suspension to 0.7 M $CaCl_2$

To verify the best volumetric proportion of alginate and mycelium suspension to 0.7 M $CaCl_2$, suspensions of fragmented 23-day-old mycelium of *Pisolithus tinctorius* and *Paxillus involutus* were dropped into 0.7 M $CaCl_2$ in the following volumetric proportions: 10:10, 8:10; 6:10; 4:10; 2:10, and 1:10. After 45 min of curing, 100 beads were transferred to 4 MMN agar plates, with 25 beads/plate; each treatment was replicated 3 times. The number of colonies formed from beads after 18 days incubation at 28 °C was used to determine the percent viability of mycelium in the beads.

### Age of entrapped mycelium

To investigate the effects of culture age on viability, mycelia of *Pisolithus tinctorius* and *Paxillus involutus* that were 10, 20, 25, 30, 40, 50, or 60 days-old were entrapped in calcium alginate beads. In this experiment, the proportion of alginate and mycelium suspension to 0.7 M $CaCl_2$ was 8:10. One hundred beads were used to determine the percent viability of the entrapped mycelium as before with 3 replicates per treatment. We measured residual glucose remaining in the MMN medium of the source liquid cultures by the Merck GOD-PAP method at intervals during growth of the mycelia that were to be fragmented.

### Storage conditions

To test the effects of storage conditions on mycelium viability in cured beads, we produced beads as before in 8:10 alginate and mycelium suspension:0.7 M $CaCl_2$ for *H. crustuliniforme*, *Pisolithus tinctorius* and *Paxillus involutus* that were 33, 36, and 40 days old, respectively. One hundred beads were stored per Petri plate in either 3.5 ml of water, 3.5 ml of 0.07 M $CaCl_2$, or on dry filter paper. We sealed the plates with parafilm, and incubated them at either 6 °C or 25 °C. After 30 and 60 days, 25 beads of each combination were placed on 4 replicate MMN agar plates. We used the number of colonies formed from the beads after 28 days incubation at 28 °C to determine the viability of the mycelium they contained.

### Statistical analysis

We analyzed our viability data after arcsine transformation of percentages by analysis of variance and *post-hoc* Tukey test. We determined statistical significance by $a = 0.05$. We analyzed the data for residual glucose remaining in the liquid MMN medium used for mycelium production by curvilinear regression.

## Results

### Volumetric proportion of alginate and mycelium suspension to 0.7 M $CaCl_2$

The best ratios of alginate and mycelium suspension to 0.7 M $CaCl_2$ for gel entrapment were 10:10 and 8:10. These resulted in approximately 99% viability for *Paxillus involutus,* and 55% and 75%, respectively, for *Pisolithus tinctorius* (Fig. 1). Ratios of 6:10, 2:10, and 1:10 affected the viability of both fungi, reducing it on average to 50% for *Paxillus involutus* and 30% for *Pisolithus tinctorius*.

### Age of entrapped mycelium

*Paxillus involutus* consistently showed more than 90% viability when the mycelium used for fragmentation had been grown for 10 to 50 days (Fig. 2). *Pisolithus tinctorius* attained an average 55% viability when its mycelium was 20 to 40 days old (Fig. 2). After 40 days, viability of *Pisolithus tinctorius* mycelium in calcium alginate dropped to 10%. In the liquid medium on which

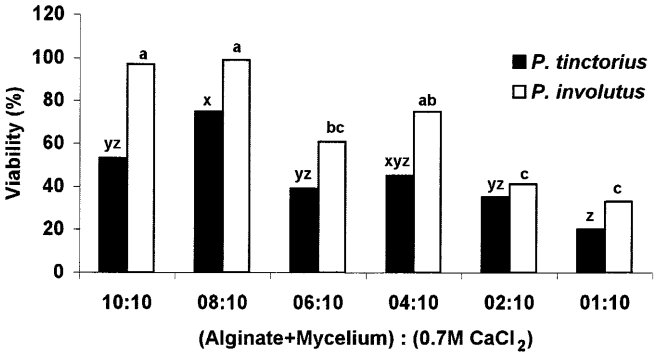


**Fig. 1** Viability of fragmented mycelium of *Pisolithus tinctorius* and *Paxillus involutus* entrapped in calcium alginate beads formed at different ratios of alginate and mycelium suspension to 0.7 M $CaCl_2$. Within a species, bars topped by the same letter do not differ significantly at $P = 0.05$ (Tukey HSD test)

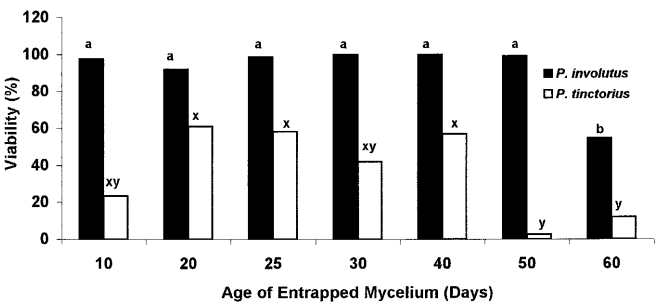


**Fig. 2** Viability of mycelial fragments of *Pisolithus tinctorius* and *Paxillus involutus* of different ages entrapped in calcium alginate beads. Within a species, bars topped by the same letter do not differ significantly at $P = 0.05$ (Tukey HSD test)

we grew the mycelia to be fragmented, *Paxillus involutus* utilized glucose faster than did *Pisolithus tinctorius* (Fig. 3), reflecting the rapid growth of *Paxillus involutus*.

Storage conditions

*Hebeloma crustuliniforme* had 100% viability after 60 days of storage under all conditions. Mycelium of *Paxillus involutus* was highly viable under tested conditions with the exception of alginate bead-entrapped mycelium stored on dry filter paper at 6 °C for 60 days (Fig. 4A). For *Pisolithus tinctorius*, storage in 0.07 M $CaCl_2$ solution at 25 °C was best (Fig. 4B). We did not determine the viability of *Pisolithus tinctorius* in alginate beads stored on dry filter paper.

## Discussion

Mauperin et al. (1987) suggested that prolonged exposure to calcium ions increases polymerization, improves bead stability, and thereby enhances viability of entrapped mycelium. From our work, however, it seems that a high concentration of $CaCl_2$ does not substitute for prolonged exposure. Both fungi that we tested were sensitive to high concentrations of $CaCl_2$ (Fig. 1). Abundant calcium ions at high proportions of $CaCl_2$ may have reacted with L-guluronic components of alginic acid, and may have caused excessive polymerization. Such a situation would be deleterious because it would diminish pore formation in the alginate beads (Cheetham and Bucke 1984). High concentrations of calcium ions may also have affected the stability of fungus membranes, thereby altering fungus physiology during storage (Griffin 1994). Although the viability of fragmented mycelium in our experiment was similar for high ratios (10:10 and 8:10) of alginate and mycelium suspension to 0.7 M $CaCl_2$ (those with low concentrations of calcium ions), the beads formed at 8:10 were better polymerized and more uniform in size and shape than those formed at 10:10. Therefore, we used the 8:10 ratio in subsequent experiments.

The viability of *Paxillus involutus* mycelium declined after 60 days growth in culture prior to entrapment in beads (Fig. 2), a full 20 days after having exhausted glucose in the medium (Fig. 3). Mycelium of *Pisolithus tinctorius* had only about 25% viability after 10 days growth (Fig. 2). At that time, residual glucose in the MMN medium was 2.5 g/l (Fig. 3), suggesting that the fungus had not begun vigorous growth. The viability of *Pisolithus tinctorius* was relatively high after 20–40 days growth prior to entrapment, but then diminished markedly after 50 days (Fig. 2). This decreased viability was in accord with residual glucose in the medium which was practically nil after 50 days (Fig. 3). Thus, the age of mycelium at entrapment can affect the viability of mycelium in alginate beads. It is necessary to determine the appropriate age of mycelium to be fragmented and entrapped, because this can also affect the efficiency of mycorrhiza formation (Littke et al. 1980; Marx 1981; Lapeyrie and Bruchet 1985).

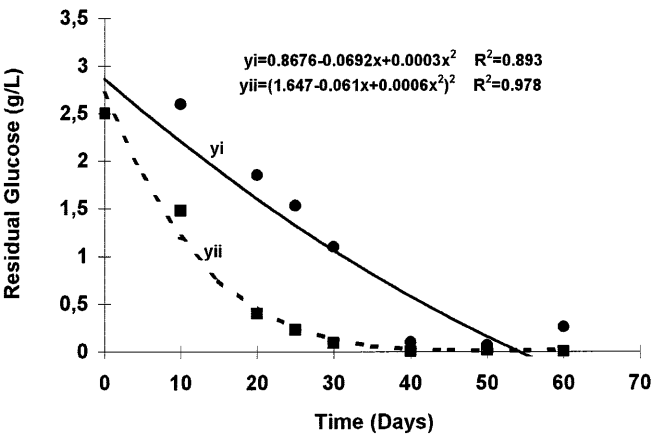


**Fig. 3** Best fit regression curves of residual glucose in the MMN liquid medium used to produce mycelium for entrapment in alginate beads of *Pisolithus tinctorius* (*solid line; yi*) and *Paxillus involutus* (*dashed line; yii*) as a function of time of growth in the medium

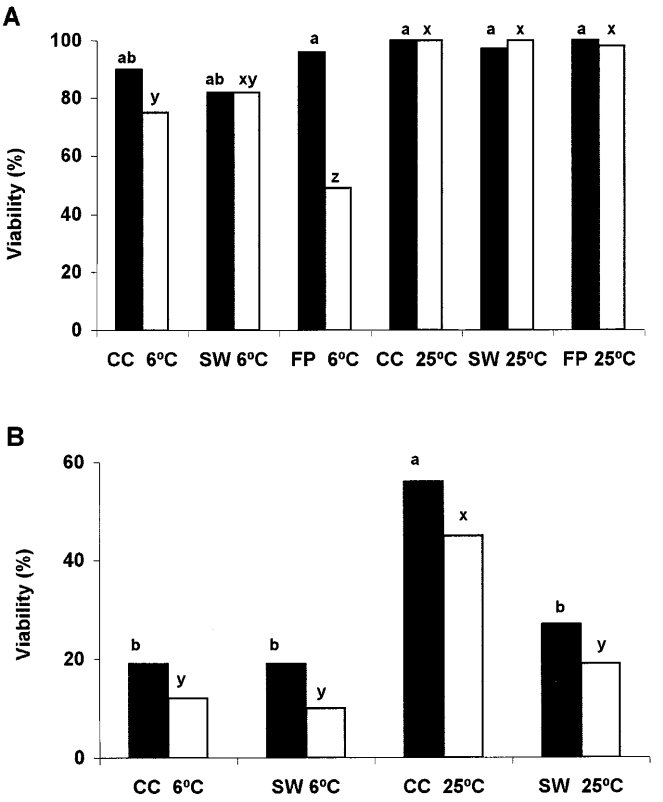


**Fig. 4** Viability of entrapped mycelia of *Paxillus involutus* (**A**) and *Pisolithus tinctorius* (**B**) stored on dry filter-paper (*FP*), in 0.07 M $CaCl_2$ (*CC*) or in sterile water (*SW*), at 6 °C or 25 °C for 30 days (■) and 60 days (□). Bars topped by the same letter do not differ significantly at $P=0.05$ (Tukey HSD test)

*Hebeloma crustuliniforme*, which we used as a control, was 100% viable for all temperatures and storage conditions that we tested. Our results agree with those of Mauperin et al. (1987), who showed that this fungus had very high survival and could be stored at 4 °C for up to 5 months.

Although Lapeyrie and Bruchet (1985) and Hung and Molina (1986) suggested that storage of mycelium in vermiculite and sphagnum at low temperature results in higher viability than storage at room temperature, entrapped mycelium of *Paxillus involutus* in our experiment had highest viability when stored at 25 °C (Fig. 4A). Similarly, survival of *Pisolithus tinctorius* entrapped in calcium alginate beads was best when stored in 0.07 M $CaCl_2$ at 25 °C (Fig. 4B). Although storage dishes were sealed with parafilm, best viability with storage at 25 °C may have been a consequence of partial desiccation of beads stored at 6 °C. Humidity can affect the viability of fragmented mycelium entrapped in alginate beads (Le Tacon et al. 1985). After 60 days at 6 °C, the best storage conditions for *Paxillus involutus* were in either distilled water or 0.07 M $CaCl_2$ (Fig. 4).

In all of our experiments, *Paxillus involutus* had higher viability than *Pisolithus tinctorius*, suggesting that the former fungus is more resistent to fragmentation and entrapment than the latter. Mycelium of *Paxillus involutus* produced many structures that appeared to be clamydospores, which may have contributed to its high viability. The sensitivity of *Pisolithus tinctorius* to fragmentation may result from the low frequency and/or fragility of septa in hyphae. If septa are disrupted during fragmentation of mycelium, cell contents will be lost and viability diminished.

Our study shows that entrapment of *Paxillus involutus* fragmented mycelium in calcium alginate beads can be successfully used for inoculum production under the conditions proposed in this study. However, *Pisolithus tinctorius* does not show such promise.

**Acknowledgements** The authors are very thankful to FINEP (Financiadora de Estudos e Projetos, Brazil) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) for financial support, and to Dr. Raymond Pacovsky (ESALQ/USP) and Dr. David Janos for critical reading of the manuscript and English improvement.

## References

Boyle CD, Robertson WJ (1987) Use of mycelial slurries of mycorrhizal fungi as inoculum for commercial tree seedling nurseries. Can J For Res 17:1480–1486

Cheetham PSJ, Bucke C (1984) Immobilization of microbial cells and their use in waste water treatment. Soc Appl Bacteriol 19:219–235

Griffin DH (1994) Fungal physiology, Wiley, New York

Hung LL, Molina R (1986) Temperature and time in storage influence the efficacy of selected isolates of fungi in commercially produced ectomycorrhizal inoculum. For Sci 32:534–545

Kuek C, Tommerup IC, Malajczuk N (1992) Hydrogel bead inocula for the production of ectomycorrhizal eucalypts for plantations. Mycol Res 96:273–277

Lapeyrie FF, Bruchet G (1985) Some factors influencing viability of ectomycorrhizal fungal inoculum. New Phytol 100:585–593

Le Tacon F, Jung G, Mugnier J, Michelot P, Mauperin C (1985) Efficiency in a forest nursery of an ectomycorrhizal fungus inoculum produced in a fermenter and entrapped in polymeric gels. Can J Bot 63:1664–1668

Littke WR, Bledsoe CS, Nadkarni NM, Edmonds RL (1980) Technique for rapid mycorrhizal colonization of container-grown Douglas-fir by *Hebeloma crustuliniforme*. Soil Biol Biochem 12:575–578

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Marx DH (1981) Variability in ectomycorrhizal development and growth among isolates of *Pisolithus tinctorius* as affected by source, age, and reisolation. Can J For Res 11:168–174

Marx DH (1991) The practical significance of ectomycorrhizae in forest establishment. In: The Marcus Wallenberg Foundation Symposia No. 7, 1991, Stockholm. Ecophysiology of ectomycorrhizae of forest trees. Marcus Wallenberg Foundation, Stockholm, pp 54–90

Marx DH, Cordell CE, Kenney DS, Mexal JG, Artman JD, Riffle JW, Molina RJ (1982) Commercial vegetative inoculum of *Pisolithus tinctorius* and inoculation techniques for development of ectomycorrhizae on container-grown tree seedlings. For Sci 28:373–400

Marx DH, Cordell CE, Kenney DS, Mexal JG, Artman JD, Riffle JW, Molina RJ (1984) Commercial vegetative inoculum of *Pisolithus tinctorius* and inoculation techniques for development of ectomycorrhizae on bare-root tree seedlings. For Sci 25:101

Mauperin CH, Mortier F, Garbaye J, Le Tacon F, Carr G (1987) Viability of an ectomycorrhizal inoculum produced in a liquid medium and entrapped in a calcium alginate gel. Can J Bot 65:2329–2336

Molina R, Trappe JM (1982) Patterns of ectomycorrhizal host specificity and potential among Pacific Northwest conifers and fungi. For Sci 28:423–458

Mortier F, Le Tacon F, Garbaye J (1989) Effect of dose and formulation of *Laccaria laccata* inoculum on mycorrhizal infection and growth of Douglas fir in a nursery. Agric Ecosyst Environ 28:351–354