Hydrogel bead inocula for the production of ectomycorrhizal eucalypts for plantations

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Inocula of eleven eucalypt ectomycorrhizal fungi, produced by the culture of mycelia within hydrogel beads, were found to be of high efficacy as propagules. The beads were discrete units approximately 2.5 mm in diameter. SEM examination revealed a dense profusion of intact mycelia within the beads. All beads produced mycelial outgrowth within 1 to 3 days when plated on agar medium and a single bead was sufficient for the initiation of mycorrhiza in the roots of aseptic seedlings and micropropagated plantlets. Depending on fungal species, the beads can be stored for at least 7 months without losing their capacity as propagules.

The successful application of ectomycorrhizal research in plantation forestry depends on the availability of a range of fungi capable of improving the economics of tree production in various environments, and the ability to supply the fungi as inocula. The lack of practical and efficacious forms of inocula has been a limiting factor in the use of ectomycorrhizal fungi in plantation forestry. Inocula of ectomycorrhizal fungi are usually comprised of biomass and carrier material. For inocula to be efficacious and practical they should meet certain criteria (Tommerup, Kuek and Malajczuk, 1987; Malajczuk *et al*., in press). The biomass should be of an appropriate genotype, be produced via axenic culture, and have a consistent physiological status appropriate for the initiation of mycorrhiza. The carrier material, if used, should be associated with a consistent amount of biomass and protect the biomass against physical and chemical stress during production and handling procedures. The inoculum should be in a form which is practical for large scale production and use, allows close control of production parameters, is low in volume and cost effective.

Many existing or advocated types of inocula only partially satisfy the criteria. For example, with spore inocula (Marx *et al*., 1984; Cordell *et al*., 1987) it is not possible to inoculate with a specific fungal isolate due to the inherent variability among spores, and spore collection may be difficult due to the sparse production of sporocarps. Inocula based on culture on carriers such as cereal grain or vermiculite (Marx & Kenney, 1982; Cordell *et al*., 1987; Le Tacon *et al*., 1988) are produced via solid substrate culture. This method of culture is subject to low culture uniformity, difficulties in the control of culture conditions and scale up. The inocula produced often contain undesirable levels of residual carbohydrates which can adversely affect the success of inoculation (Marx, 1980). Inocula consisting of pre-grown mycelia incorporated into gels (Le Tacon *et al*., 1985; Mauperin *et al*., 1987; Deacon & Fox, 1988) are usually produced by using homogenized mycelia, thus resulting in poor physiological status of the propagules. Apart from the possible need for homogenization prior to use, production of inocula consisting of mycelial suspensions (Gagnon, Langlois and Fortin, 1988; Boyle, Robertson and Salonius, 1988) involves the cumbersome separation of the biomass from the spent culture medium and removal of residual, undesirable nutrients.

To achieve a higher quality of inoculum form and manufacturing process, development of *Mycobead*[*] was initiated to make available a range of genera and species of ectomycorrhizal fungi for the plantation industry. *Mycobead* is produced by the submerged aerobic culture of mycelia entrapped within alginate beads. An assessment of the efficacy of any inoculum must involve an examination of its effectiveness as a source of propagules, as this is a fundamental determinant of efficacy. Accordingly, this communication describes a scanning

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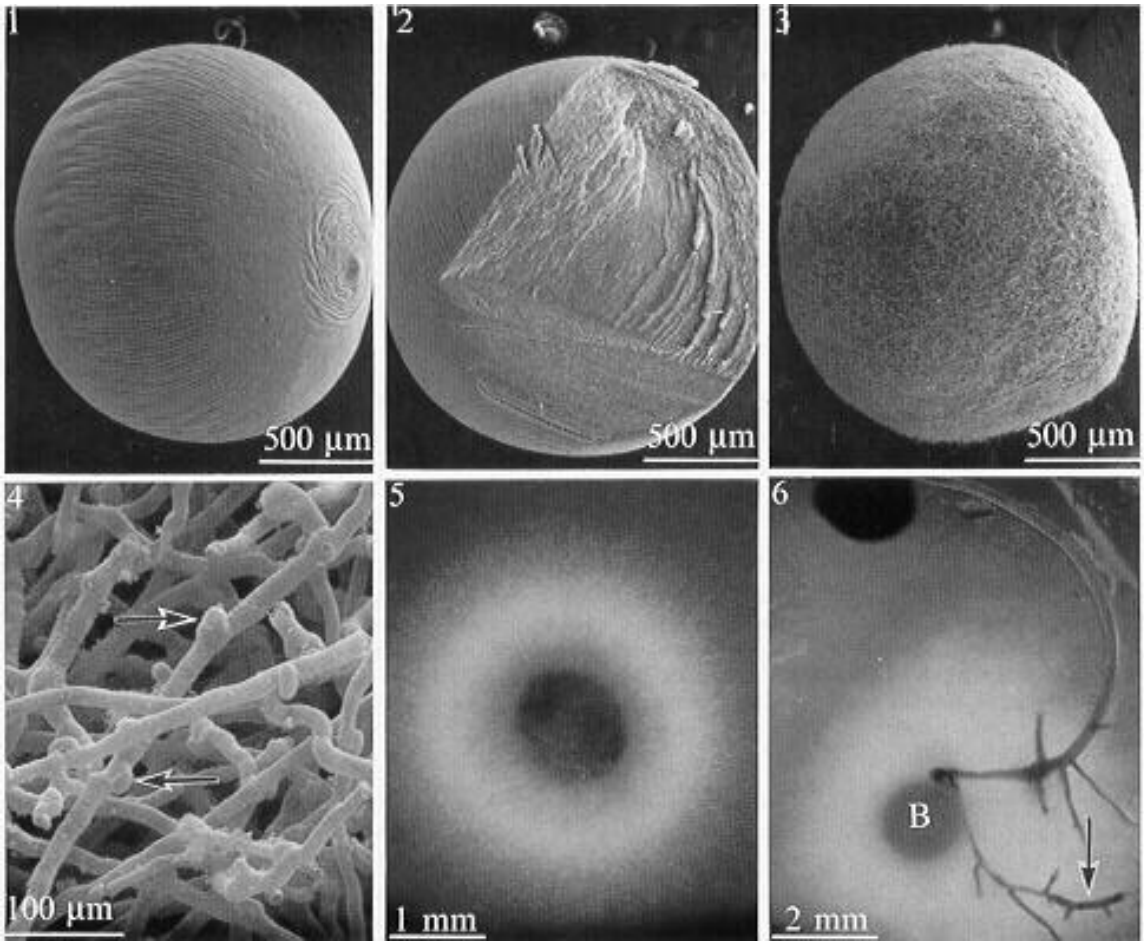

Fig. 1. SEM of a hydrogel bead containing mycelia of *Laccaria laccata* E439. **Fig. 2.** SEM of bead inoculum of *Laccaria laccata* E439 fractured to reveal the internal structure. **Fig. 3.** SEM showing dense mycelium of *Laccaria laccata* E439 after hydrogel material was removed by dissolution. **Fig. 4.** Detail of Fig. 3 showing intact hyphae and clamp connections (arrowed). **Fig. 5.** Growth of mycelium from a *Setchelliogaster* sp. nov H1023 bead after 9-day incubation on MMN agar. **Fig. 6.** Mycorrhiza (arrowed) of *Eucalyptus diversicolor* 10 days after inoculation of a 4-day old seedling with a *L. laccata* E439 bead (B) and incubation on an MMN agar plate.

electron microscopic examination of *Mycobead* to examine the spatial distribution of mycelia within the gel matrix; together with tests on the quality and uniformity of *Mycobead* as propagules, its ability to initiate mycorrhizal roots, and the effect of storage time on its propagule quality.

MATERIALS AND METHODS

Fungi and inocula

Fungal isolates used for the preparation of *Mycobead* inocula were from the culture collection of the Commonwealth Scientific and Industrial Research Organization's (CSIRO) Division of Forestry. The collection comprises isolates made Australia-wide under partial funding by the Western Australian Chip and Pulp Co. Pty. Ltd., a National Biotechnology grant and an Australian Centre for International Agricultural Research grant. The basidiomycete isolates used were verified by the CSIRO to be mycorrhizal on eucalypts. *Mycobead* inocula of three isolates of *Laccaria laccata* (Scop. ex Fr.) Berk. & Br., E439, E2013, and E2058, *Laccaria* sp. E1045, two isolates of *Pisolithus tinctorius* (Per.) Coker & Couch, H53 and H1101, *Descolea maculata* Boug. E1115, *Elaphomyces* sp. H4318 and H4142, *Hebeloma westraliense* Boug., Tomm. & Mal. E2067, and *Setchelliogaster* sp. nov. H1023, were produced and supplied by Biosynthetica Pty. Ltd. The beads were supplied immediately after manufacture as axenic material suspended in sterile deionized water, stored at 4°C, and always used within 24 h of manufacture. Technical information from the manufacturer stated that all *Mycobead* products are cultured until the carbohydrate substrate (glucose) is completely exhausted, and none is present in the suspension water.

To verify this, the water used for the suspension of the beads was tested for glucose using glucose oxidase-peroxidase assay (Sigma Kit No. 510) immediately after the beads were delivered by the manufacturer.

SEM examination of a hydrogel bead inoculum

Beads of *L. laccata* E439 were prepared for examination as previously described (Kuek & Armitage, 1985).

Growth of mycelia from beads

A sample of 100 beads from different batches of inocula of the various fungi was plated on modified Melin-Norkrans (MMN) agar (Marx, 1969) and incubated at 25^{o} for 10 days.

Storage life at 4^{o}C

Mycobead in sterile, deionized water was stored at 4^{o} and sampled at monthly intervals. At each each sampling, 100 beads were aseptically withdrawn from batches consisting of 2400 *L. laccata* E439, 3000 *L. laccata* E2058, 3000 *Elaphomyces* sp. H4318, 12000 *H. westraliense*, 9000 *P. tinctorius* H53, and 3200 *Setchelliogaster* sp. nov. beads respectively, plated on MMN agar and incubated at 25ºC for 10 days. The viability of the beads at each sampling time was assessed by determining from the plates, the percentage of beads which were colony forming units (C.F.U.).

Initiation of mycorrhiza of aseptic seedlings

Seeds of *Eucalyptus diversicolor* F. Muell. and *Eucalyptus globulus* Labill. were surface decontaminated by immersion for 15 min. in 5% sodium hypochlorite (6.3 g L^{-1} chlorine) and those of *Eucalyptus camaldulensis* Dehn. for 5 mins. The seeds were then placed on 1% agar containing 0.05% glucose and incubated in the dark at 25ºC for 5 days and 1 day in the light for *E. diversicolor* and *E. globulus*, and 8 days and 1 day respectively for *E. camaldulensis*. Seedlings were then transferred to MMN agar (altered to reduce glucose to 1 g L^{-1}) and a bead of inoculum was placed next to each root system. *L. laccata* E439 inoculum was used for *E. diversicolor*, and *H. westraliense* inoculum for *E. camaldulensis* and *E. globulus*. Plates were incubated in a plant-growth cabinet with a 12 h light (220 E m $^{-2}$ás^{-1} at 25ºC for 10 - 17 days and then examined for the development of mycorrhiza.

Initiation of mycorrhiza of micropropagated plantlets

Plantlets of *E. camaldulensis* CML 507 (supplied by Alcoa of Australia Ltd.) which had been on rooting medium for 14 days were inoculated in two ways with *H. westraliense*: (i) a bead was placed into each of 20 jars (250 mL) containing 20 mL of MMN agar (glucose reduced 0.5 g L^{-1}), incubated for 7 days at 25ºC to allow growth of mycelia from the beads, and then a plantlet was transplanted on top of each fungal colony; (ii) similarly, a plantlet was transplanted into each of 20 jars containing MMN agar (0.5 g L^{-1} glucose) and a bead was placed on the agar next to the roots of each plantlet. The plantlets were examined for the development of mycorrhiza 10 days after transplanting.

RESULTS

Bead morphology

Beads of all the fungi were discrete, spheroidal particles with no mycelia or hyphae visible either on the beads or in the suspending liquid. Bead were uniform in size with diameters averaging 2.5 mm, and

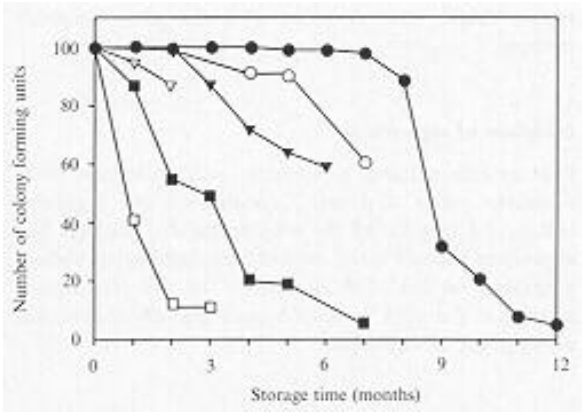

Fig. 7. Effect of storage time of bead inocula of *L. laccata* E439 (▼), *L. laccata* E2058 (○), *H. westraliense* E2067 (●), *P. tinctorius* H53 (▽), *Elaphomyces* sp. H4318 (□), and *Setchelliogaster* sp. nov. H1023 (■) on the number of colony forming units in 100-bead samples.

were denser than the suspending water. The color of inocula of each fungal isolate corresponded with that of mycelium grown on
agar. The beads could be compressed to flat discs without rupturing. No glucose was detected in the suspending water of any of the batches supplied.

SEM

Mycelia were confirmed to be absent on the surface of the beads (Fig. 1). Fractured beads revealed the entire bead to be of a solid structure (Fig. 2). Dissolution of the hydrogel material revealed dense development of mycelium within the beads (Fig. 3) and that the hydrogel matrices were evenly colonized up to just below the bead surfaces. Hyphae appeared to be whole and intact (Fig. 4) with no evidence of fragmented hyphae as would be the case if the beads only contained homogenized mycelium.

Growth of mycelia from beads

Immobilized mycelia of all fungi in the *Mycobead* form were able to grow through the surface of the beads and form colonies on the agar on which they were plated within 1 to 3 days (Fig. 5). Mycelial growth on the surfaces of beads of *L. laccata* E439 and *H. westraliense* could be observed at X20 magnification as early as 24 h. The ability to produce colonies on agar was found in each of over 9000 beads sampled from different production batches of all types of *Mycobead*.

Storage

All freshly manufactured beads of the fungi tested were c.f.u. on MMN agar. However, storage resulted in a reduction in the percentage of colony forming units (Fig. 7). The degree and rapidity of the

reduction depended on the fungal species. Storage for 5 months did not significantly affect *L. laccata* E2058 and *H. westraliense*, and bead viability was still high after 7 months for the latter. In contrast, the least storable was *Elaphomyces* whose viability was down to 40% C.F.U. after 1 month storage.

Initiation of mycorrhiza. Bead inocula initiated mycorrhiza on all seedlings of *E. diversicolor* within 10 days, and *E. camaldulensis* and *E. globulus* within 17 days (Fig. 6). All the micropropagated plantlets had mycorrhiza initiated within 10 days of transplanting/inoculation. Inoculation at the time of transfer of the plantlets or inoculation 7 days prior to transfer made no difference to the development of mycorrhiza.

DISCUSSION

Several properties of *Mycobead* distinguish it from existing forms of fungal inocula and contribute to its uniformly high performance as propagules. The immobilized mycelia comprised intact hyphae and were optimally located for subsequent outgrowth. Such location and presence of intact hyphae is only possible by the culture of the fungi within the beads rather than the encapsulation of pre-grown mycelium, and underlines the superiority of *Mycobead* as an inoculum form. The containment of mycelia within beads also confers physical protection to the cells and presents a physical form which allows for ease and accuracy in dispensing dosages, such as via a fluid drill.

The ability of mycelia of all isolates to rapidly grow out from beads indicated a high degree of viability of the cells. Such viability results in high effectivity per unit inoculum volume or weight. The 100% initiation of mycorrhiza achieved with single beads suggests that small dosages will be sufficient for efficient production of mycorrhizal seedlings. This means that the volume of inoculum required for any number of inoculations will be comparatively small. This is an advantage in production, storage, transport and usage. Even at an inoculum dose ten times (ten beads per seed) higher than that which was used in this study, the amount of inoculum required for 10000 seedlings would occupy only two litres.

The axenic production of *Mycobead* via submerged aerobic culture allows the production of specific isolates under culture conditions which are more uniform, reproducible and controllable than solid substrate culture. Thus, cell biomass and physiology can be optimized. With *Mycobead*, the problem of the introduction of gratuitous carbon sources to the rhizosphere at inoculation (Marx, 1980) is avoided because alginate is relatively inert and the amount of residual carbohydrates from the culture medium is controlled by adjusting the culture conditions. *Mycobead* is comparable to or better than other forms of inocula in storage life. There are limitations in comparison due to differences in fungal species, and to the different amounts of unutilized substrates remaining in the inoculum produced via different production processes. Nevertheless, compared to solid-substrate-cultured inocula (Hung & Molina, 1986), the storage life of *Mycobead* of *L. laccata* is comparable, whilst that of *H. westraliense* is better. With respect to mycelial slurries, *Mycobead* of *H. westraliense* sp. was comparable, whilst that of *P. tinctorius* is better than slurry inocula of the respective fungi (Boyle, Robertson and Salonius, 1988). The propagule quality of *Mycobead* of *H. westraliense* after 5 months storage was high and comparable to inoculum of pre-grown mycelium *Hebeloma crustuliniforme* incorporated in gels (Mauperin *et al*., 1987). However, whilst the latter inoculum was not tested beyond 5 months, *Mycobead* was stored for 7 months without significant loss of propagule quality. It should be possible to increase the shorter storage life of *Mycobead* of some species by adjusting the incubation period for growth prior to storage, and the physicochemical composition of the storage fluid.

Mycobead is a new inoculum form which satisfactorily fulfills the criteria for efficacious and practical inocula. The extension of these experiments to studies on inoculation in soil under controlled environments and subsequently in commercial potting mixes under nursery production conditions, will be reported elsewhere.

ACKNOWLEDGEMENTS

The study was funded by Biosynthetica Pty. Ltd. Peta Oats, Jenny Atthowe and Tim Fallows are thanked for their contributions in the execution of the experiments. NM, ICT and CK received a supporting grant from the Australian Special Rural Research Council.

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