Research note

A Simple New Technique for Ectomycorrhizal Formation between *Cantharellus* and *Dendrocalamus strictus*

Rohit Sharma,[1,3] Ram C. Rajak,[2] Akhilesh K. Pandey[1]

【Summary】

In vitro ectomycorrhizal formation is important in establishing an ectomycorrhizal relationship between a host tree and mushroom mycelia. Moreover, axenic conditions are considered essential for the synthesis of ectomycorrhiza under laboratory conditions. There have been several attempts in the past to evolve new methods of ectomycorrhizal formation between pine seedlings and fungal mycelia, but there have been few reports with angiosperm trees. In an attempt to design equipment that would require limited space and yet permit the observation of ectomycorrhiza formation, we developed a flask- filter paper technique for *in vitro* ectomycorrhizal formation between *Cantharellus tropicalis* and *Dendrocalamus strictus*. The characteristic feature of this technique is that root and shoot parts extend within a flask forming typical ectomycorrhiza in aseptic synthesis. Hence, this technique enabled *Dendrocalamus* plants to grow axenically and develop an ectomycorrhizal association with *Cantharellus* mycelium. This method is simple and will help in the *in vitro* study of other mycorrhizal fungi which form ectomycorrhiza with other angiosperm trees.

Key words: artificial synthesis, *Cantharellus tropicalis*, *Dendrocalamus strictus*, ectomycorrhiza, new technique.

Sharma R, Rajak RC, Pandey AK. 2009. A simple new technique for ectomycorrhizal formation between *Cantharellus* and *Dendrocalamus strictus*. Taiwan J For Sci 24(2):141-8.

[1] Mycological Research Laboratory, Department of Biological Sciences, RD University, Jabalpur- 482 001, M.P. India. 印度RD大學真菌研究實驗室。

[2] SGH Centre for Biotechnology, B- 96 Priyadarshini Housing Society, Dumna Road, Jabalpur- 482 001, M.P. India. 印度SGH生物工藝學中心。

[3] Corresponding author, email:rsmushrooms@rediffmail.com 通訊作者。

Received February 2009, Accepted May 2009. 2009年2月送審 2009年5月通過。

研究簡報

雞油菌與印度實竹外生菌根合成的簡易新技術

Rohit Sharma,[1,3] Ram C. Rajak,[2] Akhilesh K. Pandey[1]

摘 要

活體外的菌根合成技術是建立宿主植物與真菌共生關係的重要一環，實驗室裡，無菌狀態的環境是外生菌根合成試驗的最基本要求。在過去已有諸多研究探討松苗與真菌菌絲間的菌根合成研究，但對於被子植物的菌根合成則甚少提及。本研究設計一套『三角瓶-濾紙』技術，可經由活體外觀察雞油菌(*Cantharellus tropicalis*)與印度實竹(*Dendrocalamus strictus*)的外生菌根合成，讓根、莖進入無菌的三角瓶中形成典型的外生菌根，因此可明確觀察到印度實竹在無菌狀態下與雞油菌菌絲形成外生菌根。本項簡易技術可以用在其它菌根菌與其它被子植物的活體外菌根合成試驗。

關鍵詞：人工合成、雞油菌、印度實竹、外生菌根、新技術。

Rohit Sharma, Ram C. Rajak, Akhilesh K. Pandey。2009。雞油菌與印度實竹外生菌根合成的簡易新技術。台灣林業科學24(2):141-8。

Extensive research is needed to enhance our knowledge of interactions within the fungal community, through field studies on the spatial distribution of mycelia of species of different groups, to explore the potential for manipulating the ectomycorrhizospheric environment for biotechnological purposes (Cairney and Meharg 2002, Whipps 2004, Cairney 2005). *In vitro* fungal plant co-culture systems have been used to analyze the biological functions of several ectomycorrhizal symbioses. Several devices were developed for *in vitro* ectomycorrhiza synthesis for physiological, biochemical, and structural experiments under aseptic conditions (Moore et al. 1989, Danell 1994, Cairney and Chambers 1999, Vaario et al. 1999, Vaario et al. 2000, Yamada et al. 2001, Danell 2002, Theodorou and Reddell 2006). The equipment used is either bulky or does not permit the investigation of a large number of experimental units at the same time to allow observation of ectomycorrhizal development of the extraradical phase. Moreover, maintenance of aseptic conditions for long periods of time is not always easy thus making non-aseptic techniques popular.

These inconveniences or inadequacies made the non-aseptic growth pouch technique (Fortin et al. 1980, Wong and Fortin 1989) attractive for many studies. But aseptic techniques are required for several studies of root-fungal interactions, involving ectomycorrhizal-specific proteins (Hilbert and Martin 1988). At present, the most convenient aseptic methods are large tubes (Molina 1979, Yang and Wilcox 1984) and Petri dishes (Chilvers et al. 1986, Duddridge 1986) to maintain aseptic conditions for the entire seedlings (Molina 1979, Chilvers et al. 1986) or for only the roots (Yang and Wilcox 1984, Duddridge 1986). However, these methods are slow, as ectomycorrhizal development requires over 4 wk after inoculation. Good progress has been made with the synthesis of pine ectomycorrhiza (Marx and Kenney 1982, Marx et al. 1982), but not much has been made with angiosperm ectomycorrhiza, as angiosperm roots inherently are somewhat

more difficult to work with. Also, techniques for synthesizing mycorrhiza which work well with pines are more difficult to apply to angiosperms such as eucalypts, bamboo, sal, dipterocarps, etc.

Cantharellus tropicalis is one of the most common edible wild mushrooms of India. It was described as an ectomycorrhizal fungus following its observed association with *Dendrocalamus strictus* (Sharma 2008). Several attempts failed to form ectomycorrhiza with *Dendrocalamus* when previously described techniques were used. In an attempt to design equipment that is not bulky and requires limited space, forms rapid ectomycorrhiza, and yet permits observation of ectomycorrhiza formation, we developed a filter paper-flask technique. It avoids limitations of previous methods and incorporates better features of other aseptic techniques. This technique enables bamboo shoots to grow well, and roots make contact with *Cantharellus* mycelium in a short time. Root and shoot parts extend within the flask, and typical ectomycorrhiza form. The purpose of this study was to create a new aseptic system for routine ectomycorrhiza formation and develop a new, simple, and flexible inoculation technique for synthesizing ectomycorrhiza of bamboo seedlings in flasks which should be applicable to similar plants with small seedlings.

The fungal symbiont, *C. tropicalis* was isolated in September 2004 from mycorrhizal fruiting bodies collected from pure *D. strictus* forests in Balaghat, Madhya Pradesh, India through mushroom tissue culture (Straatsma and van Griensven 1986). The pure culture was maintained on modified MMN medium (pH 5.8) in the dark at 26±2℃. Seeds of *D. strictus* were collected from natural tropical forests of the State Forest Research Institute, Jabalpur and Government Forest Nursery, Seoni, Madhya Pradesh. Seeds of *D. strictus* were surface-sterilized, planted aseptically on moist-chamber Petri plates, and incubated for 3~4 d at 26±2℃ in the dark until the radicles had grown to 1 cm.

The test tube or flask system consists of wide-mouth test tubes (25×150 mm) or Erlenmeyer flasks (150/250 ml), containing modified MMN media. The test tubes and flasks were autoclaved (121±2℃ and 15 psi for 20 min) and cooled to room temperature (26±2℃). To inoculate fungus in this synthetic system, 9-mm-diameter fungal plugs of *C. tropicalis* were aseptically placed on medium and incubated at 26±2℃. After 3~4 d, when fungal mycelium had begun to colonize the medium, sprouted seeds were introduced aseptically into flasks and test tubes. The seedlings were placed with large forceps on a platform formed by filter paper due to imbibition of the media (Fig. 1, Fig. 2 A,B). The mouth was closed with a cotton plug to avoid contamination by other microorganisms. When seedlings grow, root extends downwards while permitting fungal access to roots through the filter paper and allowing non-destructive observations.

The test tube or flask systems were incubated for 1 mo in a plant growth chamber (with a relative humidity of 75%, a temperature of 26±2℃, and a photoperiod of 12:12-h light: dark) which was covered with a sheet of cardboard to reduce root exposure to light. Roots from the mycorrhizal synthesis system and control system were removed for fungal colonization of bamboo roots under a binocular stereomicroscope. Preparation of roots for microscopy was similar to the procedure used by Massicotte et al. (1986) and examined with an Nikon Eclipse E800 microscope (Japan) fitted with a Nikon H-II camera. To check fungal viability on colonized host roots, several lateral root tips from each combination of mycorrhizal synthesis system were inoculated

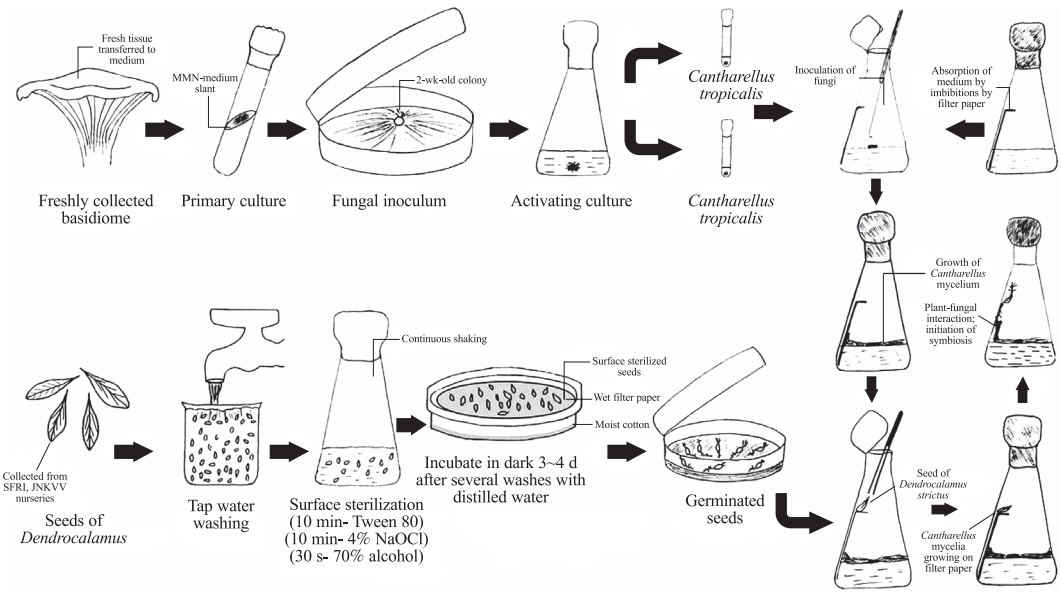

Fig. 1. Simple, new filter paper-flask technique used for *in vitro* ectomycorrhiza formationl between *Cantharellus tropicalis* and *Dendrocalamus strictus*.

onto MMN agar medium plates, and the culture morphology was observed.

The filter paper-flask technique rapidly formed ectomycorrhiza within 2 wk after inoculation and 3 d after interaction between the host and fungus. It maintains the root and shoot system under aseptic conditions. The asepsis of the technique was also checked by transferring segments of uncontaminated roots to medium used for the growth of ectomycorrhizal fungi. No microbial growth from these root segments was observed. MMN was found to be satisfactory as an inert medium carrier. The mycelium grew slowly through the MMN medium in the flask. After 2 wk of inoculation, short lateral roots had developed from the roots, some of which were in contact with hyphae arising from the fungal inoculum. Contact of *C. tropicalis* mycelium with a short root primordium of a germinated seedling took place within 3~4 d, and early morphological manifestations of ectomycorrhizal formation occurred within 7 d. At this time, a mantle and initial Hartig net were clearly evident. At 2~3 wk, dichotomous mycorrhiza had developed on *D. strictus*. Seedlings were then removed from the flasks for inspection, and sections were cut for microscopic observation.

Ectomycorrhiza which had initially developed on the lateral roots along the main roots were obvious in 3~4 d. Externally, ectomycorrhiza lacked root hairs and consisted of a light-brown mantle with slightly woven hyphae. The mantle was compact but undifferentiated. Microscopic examination of sections confirmed a multi-layered mantle and a well-developed Hartig net. Epidermal cells showed some radial elongation and were separated by Hartig net hyphae; these are features that are characteristic of ectomycorrhiza formed on intact seedlings roots of this host fungus combination. Lateral root formation occurred in 6 of 10 seedlings incubated on MMN medium. A network of black extraradicle hyphae formed around the short lateral root bases. The roots stopped elongating and failed to form root hairs, and the fungus

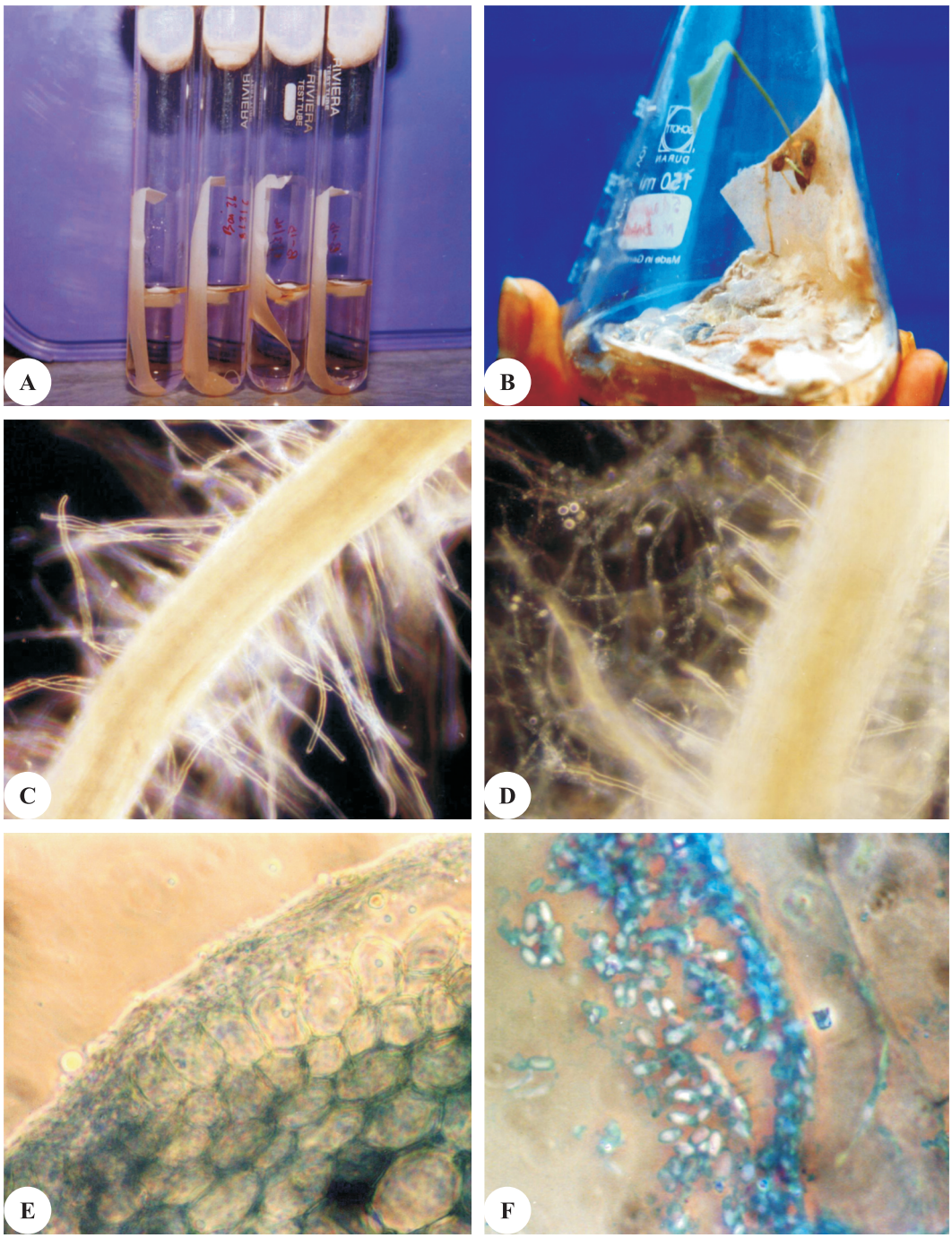

Fig. 2. New technique for aseptic ectomycorrhizal synthesis. A,B. Test tube and flask setup for the new method of *in vitro* synthesis; C. uninoculated *Dendrocalamus strictus* roots with root hairs; D. *D. strictus* roots inoculated with *C. tropicalis* and fewer root hairs; E. cross-section of ectomycorrhiza showing mantle and Hartig net; F. cross-section of ectomycorrhiza showing cystidia.

had completely enveloped the surface of host roots at 2~3 mm on either side of the root. A macroscopic view of *C. tropicalis* ectomycorrhizal formation with *D. strictus* is shown in Fig. 2D, and typical Hartig net development is shown in Fig. 2E, F. There are no previous studies of mycorrhizae formed with *C. tropicalis* in pure culture which can be compared with our results.

The filter paper-flask method also permitted observation of developing external mycelium before and after ectomycorrhizal formation. Fortin et al. (1980) suggested the exudation of substances stimulating the growth of *P. tinctorius* and *Cenococcum graniforme* which should also be investigated for this association in future studies. However, this technique has some elements in common with other methods including a culture technique that used a Petri plate lined with paper (Chilvers et al. 1986, Wong and Fortin 1989) from which the present technique evolved; a plastic pouch technique (Fortin et al. 1980) uses paper to support and nourish roots; and a cellulose thimble technique (Littke et al. 1980) uses paper to help fungal growth and transmission. Moreover, Vaario et al. (2000) described a simple *in vitro* system for the synthesis of *Abies firma- Cenococcum geophilum* ectomycorrhiza. Theodorou and Riddell (2006) studied 11 species of mycorrhizal fungi from stands of either *Eucalyptus* spp., *Allocasuarina* spp., or *Pinus radiata* D. Don and tested them in an aseptic system for their abilities to initiate ectomycorrhiza with *Allocasuarina littoralis* (Salisb.) L. Johnson, *Casuarina equisetifolia* ssp. *equiselifolia* L., and *C. cunninghamiana* Miq. Moreover, Vaario et al. (1999) first reported the *in vitro* aseptic synthesis of *Abies firma* Sieb, et Zucc. with *Pisolithus tinctorius* (Pers.) Coker & Couch and improved the technique for aseptic synthesis of ectomycorrhiza of *A. firma* a slow-growing species *in vitro*, and *Pisolithus tinctorius* using a novel culture medium and both sterilized and re-rooted seedlings.

The proposed technique is not only useful for the production of sufficient mycorrhizal seedlings for biochemical and physiological studies of ectomycorrhizal formation but can produce mycorrhizal material for practical forestry applications. The reported technique is also being investigated in relation to the inoculation of pot-cultured seedlings and variations of ectomycorrhizal formation among different angiosperm trees and their ectomycorrhizal associations. Moreover, this technique permitted the accurate evaluation of the colonization process of *Cantharellus* sp. The host plant-fungus association reported here will help in studies on the development of its fruit bodies and also opens possibilities for physiological work to investigate reasons for changes in natural *Cantharellus* populations and also the effects of different organic sources on the ectomycorrhizal formation capacity of *Cantharellus*.

ACKNOWLEDGEMENTS

The authors thank the Department of Biotechnology, New Delhi, India for financial assistance as major research project no: BT/PR3916/PID/20/153/2003 and a Junior Research Fellowship to Dr. Rohit Sharma. The authors are also thankful to the head of the Department of Biological Sciences, R.D. University, Jabalpur, India for laboratory facilities.

LITERATURE CITED

Cairney JWG. 2005. Basidiomycete mycelia in forest soils: dimensions, dynamics and roles in nutrient distribution. Mycol Res 109(1):7-20.

Cairney JWG, Chambers SM. 1999. Ectomycorrhizal fungi-key genera in profile. Heidelberg, Berlin: Springer-Verlag. 369 p.

Cairney JWG, Meharg AA. 2002. Interactions between ectomycorrhizal fungi and soil saprotrophs: implications for decomposition of organic matter in soils and degradation of organic pollutants in the rhizosphere. Can J Bot 80(8):803-9.

Chilvers GA, Douglas PA, Lapeyrie FF. 1986. A paper sandwich technique for rapid synthesis of ectomycorrhizas. New Phytol 103: 397-402.

Danell E. 1994. Formation and growth of the ectomycorrhiza of *Cantharellus cibarius*. Mycorrhiza 5: 89-97.

Danell E. 2002. Current research on chanterelle cultivation in Sweden. In: Hall I, Wang Y, Danell E, Zambonelii A, editors. Edible mycorrhizal mushrooms and their cultivation. Christ Church, New Zealand: Crop and Food Research. p 1-4.

Duddridge JA. 1986. The development and ultrastructure of ectomycorrhizae III. Compatible and incompatible interactions between *Sullus grievllei* (Klotzsch) Sing, and species of ectomycorrhizal hosts *in vitro* in the absence of exogenous carbohydrate. New Phytol 103:457-64.

Fortin JA, Piche Y, Lalonde M. 1980. Technique for the observation of early morphological changes during ectomycorrhiza formation. Can J Bot 58:361-5.

Hilbert JL, Martin F. 1988. Regulation of gene expression in ectomycorrhizas-I. Protein changes and the presence of ectomycorrhiza-specific polypeptides in the *Pisolithus-Eucalyptus* symbiosis. New Phytol 110:339-46.

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 12:575-8.

Marx DH, Kenney DS. 1982. Production of ectomycorrhizal fungus inoculum. In: Schenck NC, editor. Methods and principals of mycorrhizal research. St. Paul (MN): American Phytopathological Society. p 131-46.

Marx DH, et al. 1982. Commercial vegetative inoculum of *Pisolithus tinctorius* and inoculation techniques for development of ectomycorrhizae on container-grown seedlings. For Sci 28:373-400.

Massicotte HB, Peterson RL, Ackerley CA, Piche Y. 1986. Structure and ontogeny of *Ainus crispa-Alpova diplophloeus* ectomycorrhizae. Can J Bot 64:177-92.

Molina R. 1979. Pure culture synthesis and host specificity of red alder mycorrhizae. Can J Bot 57:1223-8.

Moore LM, Jansen AE, Van Griensven LJLD. 1989. Pure culture synthesis of ectomycorrhizas with *Cantharellus cibarius*. Acta Bot Neerland 38(3):273-8.

Sharma R. 2008. Studies on ectomycorrhizal mushrooms of MP and Chattisgarh, Ph.D. thesis, Rani Durgavati University, Jabalpur, India.

Straatsma G, van Griensven LJLD. 1986. Growth requirements of mycelial cultures of the mycorrhizal mushroom *Cantharellus cibarius* Fr. Trans Br Mycol Soc 87:135-41.

Theodorou C, Reddell P. 2006. *In vitro* synthesis of ectomycorrhizas on Casuarinaceae with a range of mycorrhizal fungi. New Phytol 118(2):279-88.

Vaario LM, Gill WM, Tanaka M, Ide Y, Suzuki K. 2000. Aseptic ectomycorrhizal synthesis between *Abies firma* and *Cenococcum geophilum* in artificial culture. Mycoscience 41(4):395-9.

Vaario LM, Tanaka M, Ide Y, Gill WM, Suzuki K. 1999. *In vitro* ectomycorrhiza formation between *Abies firma* and *Pisolithus tinctorius*. Mycorrhiza 9(3):177-83.

Whipps JM. 2004. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Can J Bot 82:1198-227.

Wong KKY, Fortin JA. 1989. A Petri dish technique for the aseptic synthesis of ectomycorrhizae. Can J Bot 67:1713-6.

Yamada A, Ogura T, Ohmasa M. 2001. Cultivation of mushrooms of edible ectomycorrhizal fungi associated with *Pinus densiflora* by *in vitro* mycorrhizal synthesis-II, morphology of mycorrhizas in open pot soil. Mycorrhiza 11:67-81.

Yang CS, Wilcox HE. 1984. Technique for observation of mycorrhizal development under monoxenic conditions. Can J Bot 62:251-4.