Tropical Agricultural Research Vol. 21(2): 189 - 197 (2010)

Effective Spore Density of *Glomus mosseae,* Arbuscular Mycorrhiza (AM), for Inoculation of Rooted Cuttings of Black Pepper (*Piper nigrum* Linn.)

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ABSTRACT. *Introduction of mycorrhizal associations is a complementary alternative to improve fertilizer absorption efficiency of black pepper (Piper nigrum Linn.). Therefore, as an initial step, this experiment was carried out to select a suitable spore density of the Arbuscular Mycorrhize (AM) fungus Glomus mosseae for inoculation of rooted cuttings of black pepper in the nursery. Four mycorrhizal inoculum levels of G. mosseae, namely 25 g (T1), 75 g (T2), 150 g (T3) and 300 g (T4) were tested with a control (T5) after quantification of the initial spore density values. The respective quantities of inoculum were incorporated separately to standard size polythene bags (21 cm diameter x 13 cm height; gauge 150) filled with a sterilized standard potting mixture. A two nodal cutting of black pepper (local selection GK 49) was planted in each polythene bag and weeding and watering were done as required. Observation of darkly stained AM hyphae at the 2nd month after inoculation in all the inoculated treatments confirmed the success of inoculation. The heaviest infection of mycorrhizal fungi was observed in roots of T3 treatment showing root colonization, along with mycelium (100 %), vesicles (94 %), and spores (769/50 g) at the 6th month after inoculation. Significantly higher ($p<0.05$) shoot biomass (5.07 g) and root length (2740 cm) were observed at the 6th month in the plants in T2 than in the control (3.28 g and 1952 cm of roots). Incorporation of the AM at the rate of 75 g for one standard size polythene bag before planting rooted cuttings of black pepper was suitable to obtain good quality planting materials for field planting at the 6th month of growth in the nursery.*

INTRODUCTION

Mycorrhizae are highly evolved symbiotic associations formed between soil fungi and plant roots. Arbuscular Mycorrhizae (AM) is the most common subgroup coming under endomycorrhizae. The AM fungi inoculated to crop plants colonize the plant root system and increase the growth and yield of crop plants including pepper (Rao, 1993; Thanuja, 2002, Durgapal *et al.*, 2002;). The AM associated improvement of plant growth is attributed to various mechanisms such as increased uptake of nutrients and water, production of plant growth promoting substances, tolerance to drought and salinity and resistance to plant pathogens (Dalpe and Monreal, 2004). *Glomus mosseae,* an AM fungus, forms large asexual chlamydospores at the hyphal tips, usually one per tip, which are highly infective to genera of herbaceous and woody plants in a wide range of conditions. It has a high reproduction

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ability mediated through the production of spores. At maturity, the spore contents are separated from the attached hypha by a septum or by occlusion with deposits of wall material. Spores are borne singly in soil and also formed in the root cortex or in sporocarps (James and Schenck, 1984). The *G. mosseae* can form mycorrhizal associations with many plant species with significant effects (Xioutang, 1994). Moreover, there is a considerable potential for the development of inoculum of *G. mosseae* for tree crops.

Black pepper (*Piper nigrum* Linn.) is one of the most important export agricultural crops in Sri Lanka and is considered as the king of the spices (Anon., 2003). One of the main problems faced by the pepper farmers is the high cost of production due to increasing trends of using inorganic fertilizer. The problem becomes more complex, as black pepper is a high nutrient demanding crop. Among the various fertilizers applied, phosphorous fertilizers have a very low absorption efficiency in plants, due to its fixation and low mobility in soil (Liu *et al*., 2004). Therefore, it is important to develop cost effective methods to fulfill the nutrient demand of black pepper. As organic farming is also becoming popular among pepper growing farmers, inoculation and promotion of mycorrhizal associations would be a complementary alternative to increase the efficiency of fertilizer absorption under black pepper cropping systems with special reference to phosphorous fertilization. Therefore, as an initial step towards this goal, the present experiment was carried out with the objective of selecting a suitable spore density of AM fungus *Glomus mosseae* for inoculation of rooted cuttings of black pepper at the nursery stage.

MATERIALS AND METHODS

This experiment was carried out in the nursery of the Research Station of the Department of Export Agriculture (DEA), Matale, Sri Lanka from March to October, 2008. Four mycorrhizal inoculum levels of *G. mosseae* (25 g, 75 g, 150 g and 300 g) were tested with a control after quantification of initial spore density value of each inoculum using the wet sieving and decanting technique with sucrose centrifugation (Brundrett *et al*., 1996). Inoculum levels and corresponding quantifications are shown in Table 1.

Table 1. Inoculum levels and corresponding quantitative values assigned for each treatment

Treatment code	Inoculum level (g) per polythene bag	Quantity of potting mixture (g/polythene bag)	Mean number of spores/ polythene bag	Mean AM Spore density (No. of spores/g of potting mixture)
T1	25	875	265	0.3
T2	75	825	795	0.88
T3	150	750	1590	1.77
T4	300	600	3180	3.53
T5-Control	0	900	0	0

N= 5; standard size polythene bags (21 cm diameter x 13 cm height; gauge 150)

The experiment was laid out in a randomized complete block design with 5 replicates. Each replicate consisted of 5 polythene bags having one cutting in each bag (black polythene bag,

21 cm diameter × 13 cm height; gauge 150). The sterilized (autoclaved at 121 °C for 15 minutes) standard potting mixture consisting of equal parts of top soil, cow dung, coir dust and sand was used (Anon, 2003). The initial nutrient composition and the pH of the standard potting media were measured. The quantity of corresponding inoculum (mycorrhizal spores and structures with sorghum (*Sorghum bicolor* L.) roots and moist soil were obtained by harvesting AM (*Glomus mosseae*) regeneration bed that was assigned to each treatment (Table 1). The innoculum was well mixed with the standard potting mixture required to fill rest of the polythene bags. Each inoculum treatment was incorporated into 25 polythene bags. A two-nodal cutting of black pepper (*Piper nigrum* Linn.) local selection GK 49 was planted in each polythene bag and these pots were kept in an airtight humid chamber for rooting. In order to find out the equivalent dry weight of a moist soil sample, the moisture factor was adopted using a sample for determination of moisture content on a dry basis. The humid chamber was opened at the end of the 3rd week after planting. The cuttings were gradually hardened by opening the polythene cover for few hours every day for about two weeks, and the cover was totally removed at the 5th week after planting. General agronomic management practices were adopted throughout the growing period as per recommendations of the DEA (Anon, 2003).

Casualties of cuttings were counted at the time of complete opening of the humid chamber. Thus, number of leaves, height of the new shoots and the diameter of new shoots were recorded at 2nd, 4th and 6th month after inoculation. Leaf area, root length and root and shoot dry weights were measured using destructive methods at the 2nd, 4th and 6th month after inoculation by uprooting five rooted cuttings from each treatment. The roots of the uprooted plants were thoroughly washed with running water. Thereafter, the roots were carefully separated using a surgical scissor and the total root length of each sample was measured using the modified line intersection method described by Tennant (1975). The shoot and root dry weights of the plants were also taken after drying the plant samples at 70 °C for 48 hours.

In order to ascertain the success of AM inoculation, pepper root samples were stained using the procedure described by Jarstfer and Silvia (2001). The root samples from each treatment were cut into 1 cm segments and were treated with 10% KOH overnight (about 12 hours). Then, they were rinsed in 30% H_2O_2 at 50 °C. As soon as the roots were bleached white, they were rinsed with several changes of tap water. Then the roots were dipped in freshly prepared 0.1% Trypan blue for two hours and de-stained by two changes of tap water. Finally, the root segments were mounted on glass slides and covered with glass slips and observed under a light microscope (10 × 40).

The percentage mycorrhizal colonization of roots of successfully inoculated rooted cuttings using root samples was measured using an arbitrary scale as proposed in the modified technique described by Philips and Hayman (1970). The per cent of AM colonization was assessed by counting either the presence or absence of hyphae and vesicles and arbuscules in each segment of root and was expressed as percentage of colonization according to the method described by Kapoor and Paroda (2005).

Rhizosphere soils under each inoculum level were also assessed for AM spore population by the wet sieving and decanting method (Brundrett *et al*., 1996). The spores were counted using a stereomicroscope. The data of different observations were statistically analysed using GLM procedures of the SAS package (SAS, 1999).

RESULTS AND DISCUSSION

Initial chemical properties of the potting media were 0.19 % total nitrogen, 2.2 mg kg^{-1} available phosphorous, 1109 mg kg^{-1} exchangeable potassium, and 576 mg kg^{-1} magnesium with a pH of 5.55 (1:2.5 water), although, chemical properties of the potting media were not tested after the experiment, as it is beyond the objectives of this experiment. The number of live cuttings at the opening of the humid chamber at 21 days after planting revealed that the overall success was very high with 75 % survival, irrespective of treatment effects.

Darkly stained AM hyphae were observed in the samples of rooted cuttings at the 2nd month after inoculation at all inoculum levels except in T5 (control). Neither vesicles nor arbuscules were seen in the roots of control plants. This confirms the successful inoculation of *G. mosseae* in this experiment. Furthermore, extra-radical, intra-cellular and inter-cellular hyphae were observed during the 6 month period of inoculation (Plates 1a and 1b).

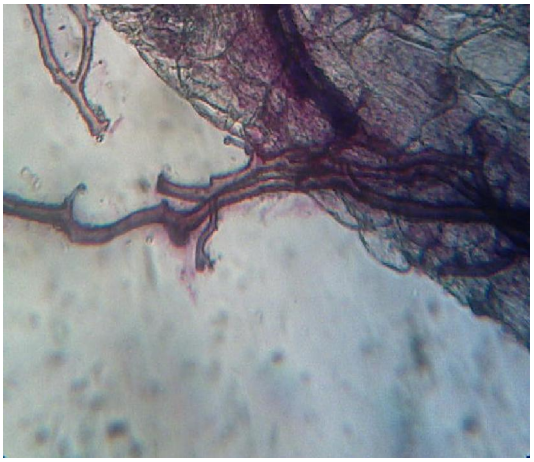

Plate 1a. Stained black pepper root segment at 2nd month after inoculation showing fungal hyphae in root tissues

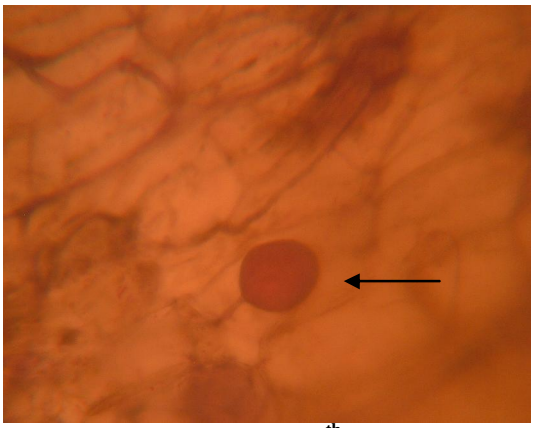

Plate 1b. Stained black pepper root segment at 4th month after inoculation showing vesicle formation (indicated by an arrow) within root tissues

Different mycorrhizal structures such as globose and sub-globose vesicles with hyphal connections were observed in the root samples of treated cuttings. These structures were recognized with reference to the illustrations of Brundrett *et al*., (1996) and Brown and King (1982). Preddy *et al*., (2003) also reported observing similar mycorrhizal structures in root sample of different genotypes of turmeric. It indicates that the time taken to produce vesicles and arbuscules is longer in black pepper roots than the time taken for the same process in *S. bicolor*, which was only two months (Kumari *et al*., 2008). This is comparable with studies of Brundrett *et al.,* (1996). The heaviest infection of mycorrhizal fungi was observed in roots of cuttings in treatment T3 having the maximum root colonization, along with mycelium, vesicles, and spores at the 6th month after inoculation (Table 2). Replacing one third of the nutrient-rich potting media with inoculum (top soil base material) in T4 may be one of the reasons for relatively low infection of AM in T4 when compared to that in T3. The introduction of high number of infective propagules at T4 could result in relatively low infection density in the pepper rhizosphere due to competitive effect.

Table 2. Mycorrhizal colonization of root samples and spore density of rhizosphere soil at 6th months after inoculation

Inoculum level	Root colonization % (Mycorrhizal structures)	Mean no. of Vesicles	Mean no. of spores/50g of media
T1	90	0	239
T2	100	10	305
T3	100	94	769
T4	100	07	524
T5 (control)	0	0	0

N=5

Shoot biomass

The shoot biomass of plants increased with time irrespective of inoculation treatments (Fig. 1). The treatment effect was not significant ($p>0.05$) for shoot dry weight until the 4th month after inoculation. Shoot dry weight became significant ($p<0.05$) at the 6th month after inoculation with the maximum shoot biomass (5.07 g) in the treatment T2 (75 g inoculum) (Fig. 2). This suggests the possibility of a temporary growth retardation in the inoculated cuttings at the initial stages of AM infection, which gradually disappeared at the 6th month. According to Linderman and Hendrix (1982), the AM fungi initially act as parasites, absorbing organic nutrients from its host plant until it develops effective nutrient and water absorbing external mycelium.

Initial competition due to AM infection in pepper rooted cuttings at higher inoculation levels has also been reported by Wimalaratne (2005). Xioutang (1994) found that inoculation of AM fungi increased the shoot dry weight and root dry weight of *Mangifera indica* plants.

Root length

The root length of cuttings increased with time irrespective of inoculation treatments (Fig. 2). In contrast to shoot biomass, the treatment effect was significant ($p<0.05$) for root length from the 4th month of inoculation. During 4th to 6th month of inoculation, a significant root elongation was observed and the order of magnitude with reference to treatments also changed showing almost similar root length values for each of the inoculated treatments. All the inoculated rooted cuttings had greater root length values than the non-inoculated plants at

the 4th and 6th month and the minimum root length was observed in the non-inoculated control (T5). Nevertheless, the differences in root length among T2, T3 and T4 were not significant. The narrow differences in root as well as shoot growth response to different inoculation levels may be attributed to the high nutrient status of the standard potting mixture. In general, the mycorrhizal induced growth improvements in higher plants are prominent under low nutrient conditions. The root length improvement observed in this study indicates the effectiveness of AM inoculation on initial growth of the rooted cuttings of black pepper at the nursery stage. Thanuja (2002) also reported the beneficial effects of inoculation with AM fungi, which resulted in enhanced rooting and root growth in black pepper cuttings.

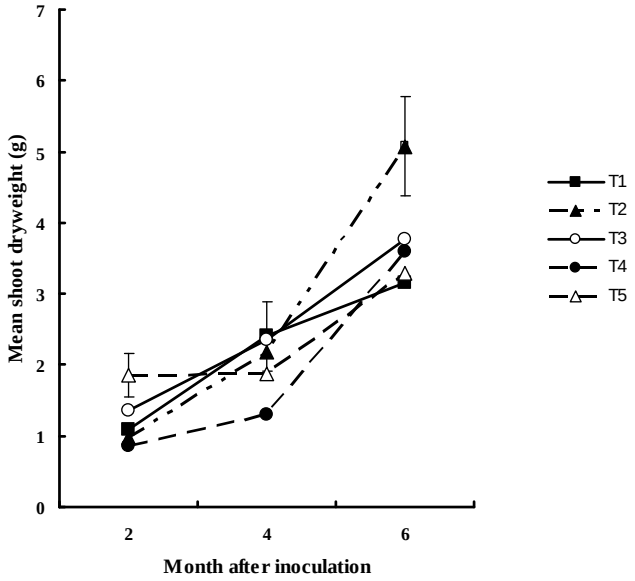

Fig. 1. Effect of AM inoculation on mean shoot biomass of black pepper (each vertical bar indicates LSD at 0.05 probability)

The improvement in rooting of black pepper cuttings would be beneficial in acquisition of drought resistance and to reduce transplanting shock at the post-nursery field establishment stage of black pepper cultivation. Improvement in nutrient absorption capacity as well as enhancement of plant vigor can also be expected with improved root growth in rooted cuttings of black pepper.

Other growth parameters

The mean values of other growth parameters at the 2nd, 4th and 6th month after inoculation are shown in Table 3. Significant changes were not observed ($p>0.05$) in root biomass, leaf area and number of leaves among the treatments. Similarly, some of the other selected growth parameters also did not show any trend in response to treatments, showing mean values of 0.39 cm, 0.54 cm and 39.2 cm for new shoot diameter, old shoot diameter and height of new shoot, respectively, at the 6th month after inoculation. This indicates that, these growth

parameters may not be useful as sensitive indicators for a nursery stage study of rooted cuttings of black pepper.

The field planting of rooted cuttings of black pepper is done by farmers using 4-6 months old nursery plants, and this depends on factors such as onset of rainfall in the growing season, availability of well developed rooted cuttings in the nurseries and availability of labour and other resources. Overall observations of this experiment indicated that, adoption of mycorrhizal inoculations seems to be a beneficial practice for pepper nursery management.

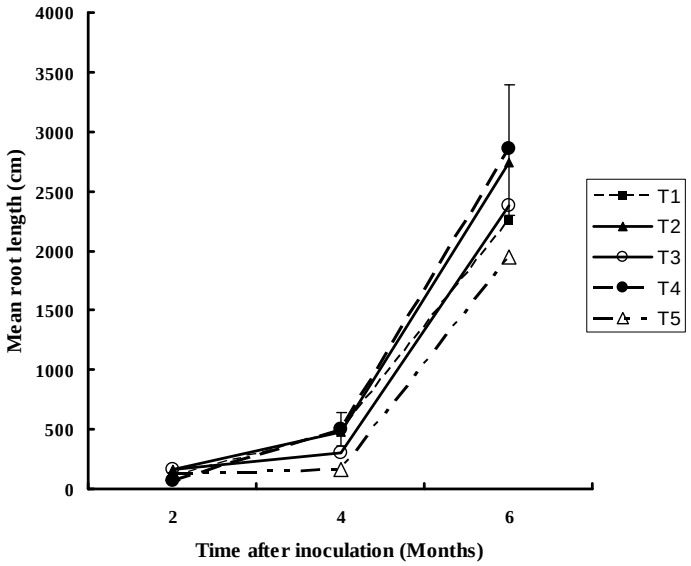

Fig. 2. Effect of AM inoculation on mean root length of black pepper with time after inoculation, (each vertical bar indicate LSD at 0.05 probability)

Table 3. The mean value of other growth parameters at 2nd, 4th and 6th month of treatment

Parameter	Treatments / Observation time	T1	T2	T3	T4	T5	LSD
Mean Root biomass (g)	2nd month	0.556	0.506	0.533	0.387	0.463	0.1612
	4th month	0.563	0.568	0.659	0.522	0.413	0.279
	6th month	0.454	0.650	0.498	0.542	0.477	0.303
Mean Leaf area (cm^2)	2nd month	20	13	31	15	13	20
	4th month	271	252	258	259	158	125
	6th month	247	259	301	248	238	155
Mean Number of leaves/plant	2nd month	2	3	2	2	2	1
	4th month	7	7	5	6	6	3
	6th month	7	10	8	8	8	4

CONCLUSIONS

Inoculation of pepper rooted cuttings with inoculum containing the mycorrhizal (*G. mosseae)* spores and fungal structures with sorghum (*Sorghum bicolor* L.) roots and soil, led to a successful inoculation of black pepper rooted cuttings (*Piper nigrum* Linn). Incorporation of 75 g of inoculum (795 mean number of spores) at the preparation of nursery bags was found to be adequate for a potting mixture of approximately 900 g, which is required to fill one standard polythene bag of size of 21 cm diameter x 13 cm height, to enhance root length and shoot dry weight of the plants. Further experiments to reduce the dependency of the high value components such as cow dung and coir dust in the standard potting mixtures along with mycorrhizal inoculum as a supplementary potting media component is proposed.

ACKNOWLEDGEMENTS

Authors wish to thank Ms. H.L.D. Lakmini for her valuable contribution in sample preparation and collection of literature as a part of her undergraduate studies. Mr. J.B. Palipane, a retired Research Officer is also acknowledged for the provision of initial AM inoculums and Mr. A. Jayasingha is acknowledged for technical assistance provided for this experiment. Financial assistance was by the NSF research grant RG/2007/Ag/01 and by the Department of Export Agriculture, Sri Lanka.

REFERENCES

Anonymous (2003). Technical bulletin on Pepper cultivation. Technical Bulleting No: 4. Department of Export Agriculture, 1095, Peradeniya.

Brown, M.F. and King, E.J. (1982). Morphology and histology of Vesicular-Arbuscular Mycorrhizae. Anatomy and cytology. Pp. 69-71. *In:* Schenck N.C.(Ed). Principles and Methods of Mycorrhizal Research. The American Phytopathological Society. St.Paul, Minn. U.S.A.

Brundrett, M. N., Bougher, B., Dell, T., Groove and Malajczuk, N. (1996). Working with Mycorrhizas in Forestry and Agriculture. Canberra: Australian centre for international Agricultural Research; GPO box 1571. pp 141-183.

Dalpe, Y. and Monreal, M. (2004). Arbuscular Mycorrhiza Inoculum to Support Sustainable Cropping Systems, online http:// www. Plantmanagementnetwork org/ pub /cm /review/ 2004/ amfungi.

Durgapal, A., Pandey, A. and Palni, L.S. (2002). The use of rhizosphere soil for improved establishment of conifers at nursery stage for application in plantation programmes. The J. Sust. Forest. **15**(3): 57-73

James, M.T. and Schenck, N.C. (1984). Taxonomy of the fungi forming endomycorrhizae-A Vesicular Arbuscular Mycorrhizal fungi (Endogonales). pp 1-10. *In:* Schenck N.C. (Ed). Principles and Methods of Mycorrhizal Research. The American Phytopathological Society. St. Paul, Minn. U.S.A.

Jarstfer, A.G. and Sylvia, D.M. (2001). Isolation, culture and detection of arbuscular mycorrhizal fungi. PP.535-542. *In:* Hurst C.J. (Ed). Manual of environmental Microbiology. American society of Microbiology. Washington D.C. online http:// cropsoil. psu.edu /sylvia/PDF-PUBS/Manual%20of%20Enviro%20Micro-2.pdf

Kapoor, K.K. and Paroda, S. (2005). Experimental Soil Microbiology, Postgraduate lecture notes, Department of Microbiology, CCS, Haryana Agricultural University, Hisar 125004, India. pp 45-46.

Kumari, I.S., Mala, W.J., Jayasinghe, A., Sumanasena, H.A. (2008). Effect of host crop on mass propagation of vesicular Arbuscular mycorrhizae (*Glomus mosseae*). Proceedings of Section B, Annual Sessions of Sri Lanka Association for Advancement of Science. **64**(1): 38.

Linderman, R.G. and Hendrix, J.W. (1982). Evaluation of plant response to colonization by vesicular-arbuscular mycorrhizal fungi. A. Host variables. pp 69-71. *In:* Schenck N.C.(Ed). Principles and Methods of Mycorrhizal Research. The American Phytopathological Society. St.Paul, Minn. U.S.A.

Liu, Q., Loganathan, P., Hedley, M. J. and Skinner, M. F. (2004). The mobilisation and fate of soil and rock phosphate in the rhizosphere of ectomycorrhizal *Pinus radiata* seedlings in an Allophanic soil. Plant and Soil. **264**: 219-229.

Philips, J.M. and Hayman, D.S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular Arbuscular Mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. **55**:158-161

Preddy, M.N., Charitha Devi, M. and Sridevi, N.V. (2003). Evaluation of turmeric cultivars for AM colonization, Indian Pathology. **56**(4): 456-466.

SAS. (1999). SAS institute Inc., Software/Release 8, Cary, NC, USA.

Rao, N.S.S. (1993). Biofertilizers in Agriculture and Forestry. Science publishers. Inc. Post Office Box 699, Enfield, New Hampshire 03748, united state of America.

Tennant, D. (1975). A test of a modified line intersects method of estimating root length. Journal of Ecology, **63**:995-1001.

Thanuja, T.V. (2002). Induction of rooting and root growth in Black pepper cuttings (Piper nigrum L.) with the inoculation of Arbuscular Mycorrhizae. Journal of Science and Horticulture. **92** (3-4): 339-346

Wimalaratne, H.G.M.C. (2005). Effect of Vesicular-Arbuscular Mycorrhiza (AM) on shoot and root development of black pepper (*Piper nigrum* Linn) rooted cuttings. B.Sc. project report, Department of Crop science, Faculty of Agriculture, University of Peradeniya, Sri Lanka.

Xioutang, L. (1994). Inoculation of forest and fruit trees with Vesicular Arbuscular Mycorrhizal fungi in Guangxi province, China. PP.114-118. *In:* Brundrett, M., Dell, B., Malajuzuk, N., Mingqin, G. (Eds). Mycorrhizas for plantation forestry in Asia. Australian Centre for International Agricultural Research GPO Box 1571, Canbera,