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Full Length Article

Cultivation of *Amanita princeps* and *Gyrodon suthepensis* for Mycorrhizations with *Castanopsis acuminatissima* and their Effects on the Host Plants

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Abstract

The aim of this research was to investigate optimal culture conditions of *Amanita princeps* and *Gyrodon suthepensis* for inoculum production of the edible ectomycorrhizal fungi (ECM), and to evaluate the effects of mycelial inoculation of the ECM fungi on mycorrhizations with seedlings of *Castanopsis acuminatissima* (Blume) A.DC. and the growth of the host plants. Mycelia of *A. princeps* and *G. suthepensis* were isolated from basidiocarps of the ECM mushrooms in Northern Thailand. Mycelia of *A. princeps* grew well on potato dextrose agar (PDA) and modified Melin Norgans (MMN) agar at pH 6 and at 28^{o}C in the dark condition. Mycelia of *G. suthepensis* grew well on PDA and Pachlewski (PACH) agar supplemented with 1.0 g L^{-1} yeast extract at 25^{o}C and at pH 6 and 7. The dark condition or the daily light and dark condition did not have significant effect on mycelial growth of *G. suthepensis*. ECM roots of *C. acuminatissima* inoculated with *A. princeps* possessed thick mantle hyphae and formed cortical Hartig net, while ECM roots of the plants inoculated with *G. suthepensis* possessed thin mantle hyphae and formed epidermal Hartig net. The ECM fungi significantly increased survival and growth of the host plants. *G. suthepensis* was more effective in increasing the growth of *C. acuminatissima* seedlings than *A. princeps*. The results of this research can be used for production of ECM host seedlings for forest restoration. © 2019 Friends Science Publishers

Keywords: Ectomycorrhizal fungi; Edible mushrooms; Mycelial cultivation; *Castanopsis acuminatissima*

Introduction

Amanita princeps and *Gyrodon suthepensis* are edible ectomycorrhizal (ECM) fungi. They belong to the phylum Basidiomycota. ECM fungi in the genus *Amanita* are distributed worldwide (Lambert *et al*., 2018). *A. princeps* belongs to the family Amanitaceae in the order Agaricales. It is a gilled mushroom and a common wild edible mushroom in Northern Thailand and sold in markets in Southeast Asia during rainy seasons (Chandrasrikul *et al*., 2008; Sanmee *et al*., 2008). *G. suthepensis* is an edible pored mushroom. It was reported as new species found in Northern Thailand. This fungus belongs to the family Paxillaceae in the order Boletales (Kumla *et al*., 2017). The beneficial effects of ECM associations are that these fungi increase uptake of mineral nutrients to roots of host plants and in return receive organic nutrients from photosynthesis of the host plants (López *et al*., 2008). Cultivation of ECM mushrooms is difficult because of the slow *in vitro* growth of the mycelia on culture media. Therefore, study about optimal cultivation for inoculum production of each species of ECM fungi is still necessary. Roots of forest trees may be colonized by indigenous ECM fungi, and these may be edible or poisonous mushrooms. It is of course beneficial to use mycorrhizations of known edible ECM fungi before tree planting in forests (Godbout and Fortin, 1990). Most ECM mushrooms are produced from mycorrhizations with host plants (Savoie and Largeteau, 2011). Reintroducing edible ECM fungi during reforestation is necessary because the local inoculum potential of the soil has been decreased during the disturbance of the forest.

Castanopsis acuminatissima (Blume) A.DC. is an evergreen tree that belongs to the family Fagaceae, which is one of the ECM host families (Brundrett *et al*., 1996). Fagaceae plants in Northern Thailand distribute in evergreen, mixed evergreen-deciduous, and mixed evergreen-conifer forests at about 760–2100 m above sea level (Forest Restoration Research Unit, 2000). Many tree species in Fagaceae are potential framework species for forest restoration in Northern Thailand (Blakesley *et al*., 2002). *C. acuminatissima* is one of the Fagaceae, which is used in reforestation program in Thailand (Fern, 2019). Forest planting is important to extend the forest areas to restore disturbed forests in ecosystems. ECM fungi have important

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roles for survival and growth of tree seedlings when transplanted to deforested areas (Hall *et al*., 2003). Objectives of this research were to investigate optimal cultivation of *A. princeps* and *G. suthepensis* for inoculum production, and to evaluate the effects of mycelial inoculation of the ECM fungi on mycorrhizations with seedlings of *C. acuminatissima* and the growth of the host plants.

Materials and Methods

Isolation of Tissues from Basidiocarps of *A. princeps* and *G. suthepensis*

Basidiocarps of *A. princeps* and *G. suthepensis* were collected from a tropical mixed evergreen-deciduous forest in Chiang Mai province, Northern Thailand. The fruiting bodies had adhering soil removed by a dissecting knife. Small pieces from inside of the stalks and inside the caps of the mushrooms were aseptically removed by sterilized fine forceps and placed on modified Melin Norkans (MMN) agar at pH 5.8 in 9-cm diameter Petri dishes. The culture plates were incubated for mycelial growth in the dark condition at 28°C for two months.

Effect of Culture Media on the Mycelial Growth

Six formulas of culture agar were tested for mycelial growth of *A. princeps* and *G. suthepensis*: (1) potato dextrose agar (PDA), (2) PDA supplemented with 1.0 g L^{-1} yeast extract, (3) MMN, (4) MMN supplemented with 1.0 g L^{-1} yeast extract, (5) Pachlewski (PACH) agar and (6) PACH supplemented with 1.0 g L^{-1} yeast extract. All the culture media were adjusted to pH 5.6. Each solid culture medium in a Petri dish was inoculated with a 5-mm-diameder plug of the ECM hyphae. Five replications were done for each culture medium. The culture plates were incubated at 28°C for one month, and the colony diameters were measured.

Effect of Temperature on Mycelial Growth of the ECM Fungi

Mycelia of *A. princeps* and *G. suthepensis* were cut (5-mm-diameder per piece) from colony edges. The 5-mm-diameter plugs of hyphae of *A. princeps* and *G. suthepensis* were inoculated to the optimum formula of culture agar (from the previous experiment) with one piece per 9-cm diameter Petri dish. The culture plates were incubated at 25°C, 28°C and 30°C. Five replications were performed for each treatment. One month after inoculation, colony diameters of the ECM fungi were measured.

Effect of pH and Light Conditions on the Growth of the ECM Fungi

The suitable culture media from the previous experiments for the growth of *A. princeps* and *G. suthepensis* were adjusted to pH 5, 6 and 7. For each pH, one piece of 5-mm-diameter plug of the mycelia from colony edges of the ECM fungi was inoculated on the culture agar in a 9-cm diameter Petri dish. All Petri dishes were incubated at the optimum temperature for each the ECM fungi in the dark condition, and in the daily light and dark condition for one month. The experiment was performed with five replicates per treatment. After one month, colony diameters of *A. princeps* and *G. suthepensis* for each treatment were measured.

Inoculum Production

Inoculum production of *A. princeps* and *G. suthepensis* was undertaken in 250 mL of culture media in each 500 mL flask. Twenty pieces of 5-mm-diameter plugs of the mycelia from colony edges of the ECM fungi were inoculated in each flask containing the optimum culture media and the optimum pH for the ECM fungi. The culture flasks of the ECM fungi were incubated on a reciprocal shaker at 130 rpm for two months at the optimum conditions for each of the ECM species.

Seedling Preparation

Seeds of *C. acuminatissima* were washed in tap water to remove floating damaged seeds before surface sterilization. The seeds were surface sterilized by soaking in 1% NaOCl for five min, followed by 70% ethanol for 30 s, then washed with three changes of water. The sterilized seeds were cultivated in trays containing a sterilized mixture of soil: peat (2:1 v/v) and watered once a day in the partial shade of a greenhouse.

Transplanting and Inoculation of the ECM Fungi

Three-month-old seedlings of *C. acuminatissima* were transplanted into 1 kg of sterilized soil in growing bags (8×18 cm) with one plant per bag. For inoculation, mycelia of *A. princeps* and *G. suthepensis* in the inocula were fragmented and homogenized in sterilized blenders for 10 s. The mycelium slurry (10 mL) of each ECM fungi was inoculated at the planting hole before transplanting seedlings of *C. acuminatissima*. For the control treatment, each planting hole had 10 mL of the culture medium added without mycelium of the ECM fungi before transplanting of the seedlings. The experiment was performed with 30 seedlings per treatment. Seedling bags were placed in a greenhouse and watered once a day.

Assessment of ECM Formation and the Growth of *C. acuminatissima* Seedlings

Six months after inoculation, the *C. acuminatissima* seedlings were collected for evaluation of height, stem diameter and shoot and root dry weight. Roots of the *C. acuminatissima* seedlings were carefully washed and

separated from shoots. Shoots of the seedlings were dried at 60°C for 48 h and placed in desiccators before weighing to assess the shoot dry weights. Roots samples of *C. acuminatissima* seedlings were assessed under a stereomicroscope to observe and count ECM root tips. Percentage of ECM root length was done by gridline intersection method according to Brundrett *et al.* (1996). Some root tips were selected for cross section. The root sections were cleared with 3% KOH at 50°C for 10 min and stained with 0.05% trypan blue at 50°C for three min. The stained root sections were mounted on microscopic slides in lactoglycerol for observation under a compound microscope. After assessment of ECM formation, roots of *C. acuminatissima* seedlings were dried at 60°C for 48 h and weighed to determined root dry weight.

Statistical Analysis

Data of the experiments were analyzed by one-way analysis of variance (ANOVA) in SPSS version 23. The means of data were compared by Duncan's Multiple Range Test ($P \leq 0.05$).

Results

Mycelial Growth of the ECM fungi on Culture Media

Tissue culture from basidiocarps of *A. princeps* and *G. suthepensis* were successfully isolated from inside of the mushroom caps. Mycelia of *A. princeps* grew well on PDA, PDA supplemented with 1.0 g L^{-1} yeast extract, MMN and MMN supplemented with 1.0 g L^{-1} yeast extract. PACH agar was not suitable for the mycelial growth of *A. princeps*. Adding 1.0 g L^{-1} yeast extract to the original formula of all the culture media did not have a clearly significant effect on the mycelial growth of *A. princeps*. However, colony diameters of *A. princeps* on MMN supplemented with 1.0 g L^{-1} yeast extract were a little larger than colony diameters of this fungus on the original formulas of PDA and MMN. Mycelia of *G. suthepensis* grew faster than mycelia of *A. princeps*. Colony diameters of *G. suthepensis* were about two times larger than colony diameters of *A. princeps* on all of the culture formulas. The most suitable culture medium for mycelial growth of *G. suthepensis* was PDA and also PDA supplemented with 1.0 g L^{-1} yeast extract. Mycelia of *G. suthepensis* also grew well on PACH agar supplemented with 1.0 g L^{-1} yeast extract. MMN agar was not suitable for the mycelial growth of *G. suthepensis* when compared with the other culture media (Fig. 1). Colonies of *A. princeps* on the culture media were white to whitish cream color, and colonies of *G. suthepensis* on the culture media were yellowish brown to brown color. Both mycelia of *A. princeps* and *G. suthepensis* had clamp connections at the septa of hyphae. Mycelia of *G. suthepensis* produced intercalary hyphal swellings, and secreted yellowish brown to brown pigment to the culture media.

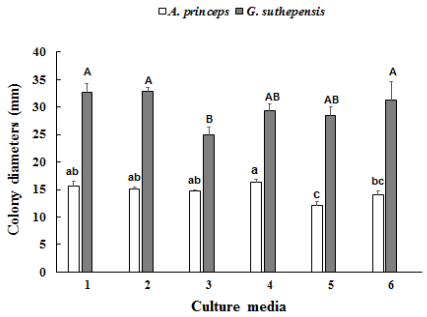

Fig. 1: The mycelial growth of *A. princeps* and *G. suthepensis* (1) PDA, (2) PDA supplemented with 1.0 g L^{-1} yeast extract, (3) MMN, (4) MMN supplemented with 1.0 g L^{-1} yeast extract, (5) PACH, (6) PACH supplemented with 1.0 g L^{-1} yeast extract. Columns with different letters are significant different of the means. Bars are ± standard error of the means

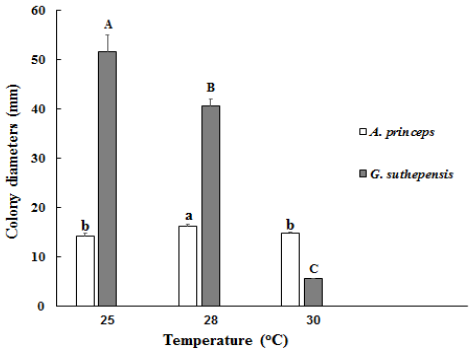

Fig. 2: Colony diameters of *A. princeps* and *G. suthepensis* at the different temperatures. Columns with different letters are significant different of the means. Bars are ± standard error of the means

Effects of Temperature, pH and Light Conditions on Mycelial Growth of the ECM Fungi

Temperature had a significant effect ($P<0.05$) on mycelial growth of both *A. princeps* and *G. suthepensis*. The optimal temperature for mycelial growth of *A. princeps* was 28°C. The largest expansion of mycelial growth of *A. princeps* was found at 28°C. Colony diameters of *A. princeps* incubated at 25°C and 30°C were not significantly different. The optimal temperature for mycelial growth of *G. suthepensis* was 25°C, and the mycelial growth of *G. suthepensis* was very low at 30°C (Fig. 2). The optimal conditions for mycelium growth of *A. princeps* were at pH 6 in the dark condition (Fig. 3a). Mycelia of *G. suthepensis* grew well at pH 6 and 7. The dark condition or the daily light and dark condition did not have a significant effect on mycelial growth of *G. suthepensis*. However, colony diameters of *G. suthepensis* incubated in the daily light and dark condition were a little larger than those incubated in the dark condition at pH 6. pH 5 was not suitable for mycelial growth of both *A. princeps* and *G. suthepensis*. Colony diameters of *A. princeps* and *G. suthepensis* on the culture media at pH 5 were smaller than colony diameters of the ECM fungi on the culture media at

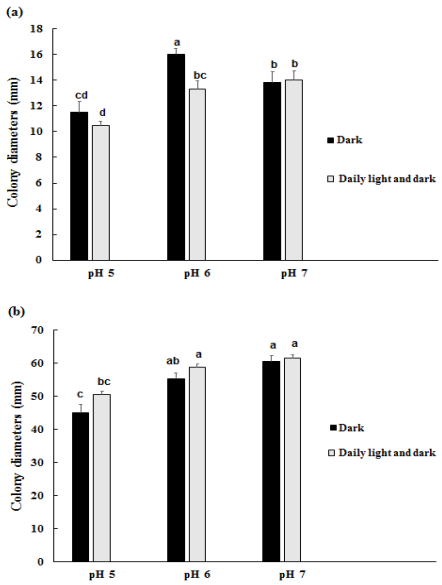

Fig. 3: Mycelial growth of (**a**) *A. princeps* and (**b**) *G. suthepensis* at different pH and light conditions. Columns with different letters are significant different of the means. Bars are ± standard error of the means

pH 6 and 7 (Fig. 3b). Therefore, inoculum production of *A. princeps* and *G. suthepensis* was done according to the most optimal conditions for mycelial growth of the ECM fungi. Inoculum production of *A. princeps* was cultured in MMN broth supplemented with 1.0 g L^{-1} yeast extract at pH 6 and incubated on a reciprocal shaker at 130 rpm for 2 months in the dark condition. PDB at pH 6 was chosen for the culture medium for inoculum production of *G. suthepensis*, and the culture was incubated on a reciprocal shaker at 130 rpm for two months under the daily light and dark condition.

ECM Formation and Survival of *C. acuminatissima* Seedlings

Roots of *C. acuminatissima* inoculated with mycelia of *A. princeps* and *G. suthepensis* appeared as ECM roots. Seedlings of *C. acuminatissima* inoculated with *A. princeps* and *G. suthepensis* had a percentage of survival much higher than for the plants without inoculation with the ECM fungi. Survival of *C. acuminatissima* seedlings inoculated with the ECM fungi was about 2.4 times that of the uninoculated seedlings. Roots of *C. acuminatissima* seedlings inoculated with *G. suthepensis* had ECM root tips and the percentage of root length colonization was significantly higher than for those inoculated with *A. princeps* (Table 1). The ECM roots associated with *A. princeps* and *G. suthepensis* were covered with mantle hyphae around the ECM roots, while roots of the uninoculated plants did not have mantle hyphae around the roots. Branching patterns of ECM roots of *C. acuminatissima* associated with *A. princeps* and *G. suthepensis* were simple or irregularly pinnate. The color of the ECM roots of *C. acuminatissima* associated with *A. princeps* and *G. suthepensis* were pale grayish brown to pale brown. Cross-sections of ECM root tips of *C. acuminatissima* inoculated with hyphae of *A. princeps* and *G. suthepensis* clearly displayed mantle hyphae and Hartig net hyphae, but cross-sections of root tips of uninoculated *C. acuminatissima* did not show any mantle and Hartig net hypha (Fig. 4a). The Hartig net hyphae of *A. princeps* penetrated between cortical cells (Fig. 4b), whereas the Hartig net hyphae of *G. suthepensis* grew between the epidermal cells and only the first row of cortex (Fig. 4c).

Growth of *C. acuminatissima* Seedlings

Growth of *C. acuminatissima* was significantly increased ($P < 0.001$) by inoculation with *A. princeps* and *G. suthepensis*. Height and stem diameter of *C. acuminatissima* seedlings inoculated with *A. princeps* and *G. suthepensis* were significantly larger than for the uninoculated plants. Shoot dry weight of *C. acuminatissima* seedlings inoculated with *G. suthepensis* had the highest growth (Table 2). In this experiment, root dry weight of the uninoculated plants was not significantly different from root dry weight of the inoculated plants. Shoot per root dry weight ratio of the uninoculated plants was about 1.8, and shoot per root dry weight ratios of the plants inoculated with *A. princeps* and *G. suthepensis* were about 2.9 and 2.7, respectively.

Discussion

In this experiment, mycelia of *A. princeps* grew well on both PDA and MMN agar. However, the largest colony diameter of *A. princeps* was found on MMN supplemented with 1.0 g L^{-1} yeast extract. Yeast extract contains amino acids, trace elements and vitamin B complex that may be useful for the growth of the ECM fungus (Reed and Nagodawithana, 1991). Vaario *et al*. (2018) reported that *Tricholoma matsutake* obtains both C and N from protein sources in soil organic matter. The suitable culture medium for mycelial growth of *G. suthepensis* was PDA, which is most widely used for cultivation of many fungi. Mycelia of *G. suthepensis* also grew well on PACH agar supplemented with 1.0 g L^{-1} yeast extract. MMN agar was not suitable for the mycelial growth of *G. suthepensis* compared with the other culture media. The original formula of MMN contains 1% glucose as the main carbon source, while the original formulas of PDA and PACH contain 2% D-glucose, which may be a suitable concentration of the carbon source for mycelial growth of *G. suthepensis*.

The optimal temperatures for the growth of *G. suthepensis* and *A. princeps* were 25°C and 28°C, respectively. Mycelial growth of *G. suthepensis* was more sensitive to changing of temperature compared with *A. princeps*. The growth of mycelia of *G. suthepensis* was very low at 30°C. Many species of ECM fungi have optimal temperatures for growth in the range of 25–28°C such as *Boletus edulis*, *Lactarius deliciosus* and *L. insulsus* (Xu *et*

Table 1: Ectomycorrhizal (ECM) colonization of *A. princeps* and *G. suthepensis* associations with *C. acuminatissima* and survival of the host plants

ECM fungi	Root length colonization (%)	ECM root tips per plant	Survival of the plants (%)
None	0 c	0 c	37
A. princeps	27 b	2,953 b	87
G. suthepensis	32 a	3,891 a	90

Means ± standard error of the means in the same column followed by different letters are significantly different ($P \leq 0.05$)

Table 2: Effects of *A. princeps* and *G. suthepensis* on the growth of *C. acuminatissima*

ECM fungi	Height (cm)	Stem diameter (mm)	Shoot dry weight (g)	Root dry weight (g)
None	42.04±3.86 c	2.61±0.23 c	3.50±0.87 b	2.00±0.88 a
A. princeps	54.50±2.65 b	3.29±0.18 b	5.78±0.44 ab	2.03±0.25 a
G. suthepensis	66.70±2.29 a	4.74±0.18 a	7.13±0.65 a	2.66±0.34 a

Means ± standard error of the means in the same column followed by different letters are significantly different ($P \leq 0.05$)

al., 2008). Optimal temperature depends on the species or strains of ECM fungi. Daza *et al*. (2006) reported that optimal temperatures of the four strains of *A. caesarea* were in the range of 24–28°C. However, some species of ECM fungi such as *Phlebopus portentosus* and *Pisolithus orientalis* grew well at 30°C (Sanmee *et al*., 2010; Kumla *et al*., 2016), while *mycelium of Hydnum repandum* could not grow at 30°C (Peksen *et al*., 2013). Effects of visible light on mycelial growth depend on the species of the fungi. Weitz *et al*. (2001) reported that darkness was optimum for the mycelial growth of *Omphalotus olearius* and *Panellus stipticus* but darkness and 12 h light and 12 h dark per day did not have any effect on the mycelial growth of *Armillaria mellea* and *Mycena citricolor*. The mycelial growth of these fungi was significantly decreased under light condition compared to under dark condition all the time of the culture. Hence, many researchers incubated mycelia of ECM fungi in darkness (Sanmee *et al*., 2010; Kibar and Peksen, 2011; Islam and Ohga, 2013).

Roots of *C. acuminatissima* inoculated with mycelia of *A. princeps* and *G. suthepensis* appeared as ECM roots, where extra-radical ECM hyphae, mantle sheath and Hartig net hyphae were found. Root tip surfaces of the uninoculated plants dried more easily than ECM root tips of the inoculated plants. Mantle hyphae help to absorb water and nutrients to the roots of the host plants, and mantle hyphae also help to protect the roots from water loss. Branching patterns of ECM roots of *C. acuminatissima* associated with *A. princeps* and *G. suthepensis* were simple or irregularly pinnate. Features of branching pattern of ECM root tips are dependent on associations of particular ECM fungi and the host plants. Kumla *et al*. (2017) reported new species of *G. suthepensis* where they found the fungus associated with *Betula alnoides* in the forests of Northern Thailand, and branching patterns of the ectomycorrhizae were simple or monopodial-pinnate. ECM roots of *Eucalyptus* spp. associated with ECM fungi may be unbranched or pinnate branching, while ECM roots

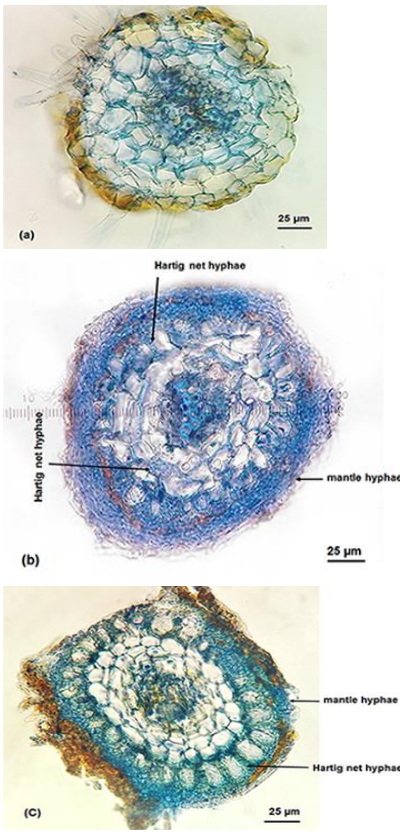

Fig. 4: Cross section of root tips of *C. acuminatissima*
(**a**) Root tips of uninoculated plant, (**b**) Ectomycorrhizal (ECM) root tips of the plant associated with *A. princeps*, (**c**) ECM root tips of the plant associated with *G. suthepensis*

of *Pinus* spp. associated with ECM fungi appear as dichotomous branching of ECM root tips (Brundrett *et al*., 1996). These results showed that ECM roots of *C. acuminatissima* associated with *A. princeps* had cortical Hartig net, which has rarely been found for ECM roots of angiosperms. Many other angiosperms associated with ECM fungi had epidermal Hartig net, in which the Hartig net hyphae penetrated to only the epidermal layer (Brundrett *et al*., 1996; Smith and Read, 2002). Hartig net hyphae are the main areas of nutrient exchange between the ECM fungal hyphae and the roots of the host plant (Prakash *et al*., 2015).

For growth response to ECM association of the host plant, growth of *C. acuminatissima* was significantly increased by inoculation with *A. princeps* and *G. suthepensis*. *C. acuminatissima* seedlings inoculated with *G. suthepensis* had the highest growth. Brundrett *et al*. (1996) reported that the efficacy of ECM fungi for growth of the host plants depended on associations between species of the ECM fungi, the host plants and the environmental conditions. Percentage of root length colonization and ECM root tips of *C. acuminatissima* seedlings inoculated with *G. suthepensis* were significantly higher than those of the seedlings inoculated with *A. princeps*. This experiment

showed that growth of *C. acuminatissima* seedlings was related to the percentage of root length colonization and ECM root tips. ECM colonization enhances plant growth by increasing the absorptive area of mantle hyphae and the extra-radical ECM hyphae surrounding the root system can considerably increase the uptake of water and nutrients for growth of the host plant (Hall *et al*., 2003; Dominguez-Nunez *et al*., 2008). ECM associations can optimize plant nutrition and may contribute to the maintenance of tropical forests (Corrales *et al*., 2018).

Conclusion

The optimal culture media for inoculum production of *A. princeps* was MMN broth supplemented with 1.0 g L^{-1} yeast extract at pH 6 and incubated at 28°C in the dark condition, and for inoculum production of *G. suthepensis* was PDB at pH 6 and incubated at 25°C in the daily light and dark condition. Mycorrhizations of *A. princeps* and *G. suthepensis* markedly increased survival and growth of *C. acuminatissima* seedlings. *G. suthepensis* was especially the most effective in increasing growth of host seedlings. This research provides information for inoculum production of *A. princeps* and *G. suthepensis* for mycorrhizations with *C. acuminatissima* or other ECM host species for forest restoration.

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