

Responses of *Castanopsis tribuloides* and *Pinus kesiya* seedlings to mycelial inoculation of *Russula alboareolata* and *Amanita princeps*

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Abstract

Castanopsis tribuloides and *Pinus kesiya* are ectomycorrhizal (ECM) host plants. They are used for reforestation in degraded forestlands in northern Thailand. Objective of this research was to compare the effects of mycelial inoculation of *Russula alboareolata* and *Amanita princeps*, edible ECM mushrooms, on *C. tribuloides* and *P. kesiya* seedlings by evaluating ECM formation, growth response and nutrient accumulation. The study period was from 2018 to 2020. Two-month old seedlings of *C. tribuloides* and *P. kesiya* were inoculated with mycelium slurries of *R. alboareolata* and *A. princeps*. The effects of the ECM fungi on the host plants were evaluated at six months after inoculation. ECM roots of *C. tribuloides* associated with *R. alboareolata* and *A. princeps* were irregularly pinnate, whereas ECM roots of *P. kesiya* inoculated with the fungi were dichotomous branching patterns. Survivals of *C. tribuloides* seedlings in the uninoculated, *R. alboareolata* and *A. princeps* treatments were 66.7, 76.7 and 97.2% respectively. Survivals of *P. kesiya* seedlings in both the uninoculated and inoculated treatments were about 95%. Inoculation with the ECM fungi significantly increased the growth and nutrient accumulations of *C. tribuloides* and *P. kesiya* seedlings. The increase in dry weight was greater for inoculated plants of *C. tribuloides* than for those of *P. kesiya*. For *C. tribuloides*, the increase was significantly greater for plants inoculated with *R. alboareolata* than for those inoculated with *A. princeps*. For *P. kesiya*, there was no difference in the increase in dry weight between plants inoculated with each of the fungal species. Seedlings of *C. tribuloides* and *P. kesiya* associated with the edible ECM fungi may be beneficial for reforestation.

Keywords: Ectomycorrhizas, Edible mushrooms, Fagaceae, Pinaceae

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Introduction

Forests are natural resources of plants, animals and other forest products. In recent years, many forest areas have decreased. Replanting has significantly contributed to restore the forest ecosystems. *Castanopsis tribuloides* (Sm.) A. DC. (Fagaceae) is one of framework tree species for forest replanting in degraded tropical forestlands in northern Thailand (Blakesley et al., 2002). *C. tribuloides* is a medium-sized, evergreen tree, up to 18 m tall. Seeds of *C. tribuloides* are edible (Forest Restoration Research Unit, 2000). *Pinus kesiya* Royle ex. Gordon (Pinnaceae) is a pioneer species preferred for reforestation on highland sites in Thailand (Fern, 2014). *C. tribuloides* and *P. kesiya* are widely distributed in the mixed evergreen and deciduous forest, the evergreen forest, and the mixed evergreen and pine forest in the tropical regions of Asia (Forest Restoration Research Unit, 2000; Schmidt and Nguyen, 2004; Fern, 2014). Both *C. tribuloides* and *P. kesiya* are ectomycorrhizal (ECM) host plants.

Mycelia of ECM fungi around roots of the host plants increase the absorptive areas of rooting systems of the plants, thereby increasing the uptake of mineral elements (Dosskey et al., 1990; Finlay, 2004). The ECM fungi, in turn, receive nutrients from photosynthesis of the host plants (Dosskey et al., 1990; Brundrett et al., 1996). This interaction of ECM fungi with roots of the host plants typically have beneficial effects on growth of the host plants (Smith and Read, 1997). However, the response of the host plants to ECM formation depends on the suitability of the ECM fungi to the host plants and environment. Most ECM fungi need host plants to stimulate the production of fruiting bodies (Ohta and Fujiwara, 2003; Sanmee et al., 2010). *Russula alboareolata* Hongo and *Amanita princeps* Corner & Bas are edible gilled ECM mushrooms, they belong to the orders Russulales and Agaricales, respectively. Their fruiting bodies provide food for human and wild animals. The ECM mushrooms are collected for food and sold by local villagers during rainy seasons in Thailand (Chandrasrikul et al., 2008). Forest trees may be colonized by indigenous ECM fungi that may be edible or poisonous mushrooms. Therefore, it is beneficial to use seedlings associated with known edible ECM fungi before planting in forests. The objectives of this research were to compare the effects of mycelial inoculation of *R. alboareolata* and *A. princeps* on mycorrhization of *C. tribuloides* and

P. kesiya seedlings, and to evaluate subsequent survival, growth and mineral nutrition of the host plants in a greenhouse.

Material and Methods

Isolation and inoculum production of *R. alboareolata* and *A. princeps*

Basidiocarps of *R. alboareolata* and *A. princeps* were collected from the forest at Doi Suthep-Pui National Park in Chiang Mai province, Thailand. Isolates of *R. alboareolata* and *A. princeps* obtained aseptically from tissues inside basidiocarps were placed on modified Melin Norkans (MMN) agar (Marx, 1969) at pH 6 in Petri dishes. The culture plates were incubated in the dark at 28°C for one month. Five-mm-diameter plugs of hyphae from pure cultures of *R. alboareolata* and *A. princeps* were subcultured on MMN agar supplemented with 1.0 g/L yeast extract in 9 cm Petri dishes and incubated in the dark at 28°C for one month to increase mycelia of the fungi. For inoculum production, the mycelia from colony edges of each species were cut and inoculated in MMN broth supplemented with 1.0 g/L yeast extract at pH 6 in Erlenmeyer flasks. The culture flasks were incubated on a reciprocal shaker at 130 rpm for two months at room temperature (28-32°C) and in the dark. After that, mycelial inocula of *R. alboareolata* and *A. princeps* were fragmented in sterilized blenders for 10 s.

Seedling preparation

Seeds of *C. tribuloides* and *P. kesiya* were washed and surface sterilized by immersing in 0.6% NaOCl for 5 min and in 70% ethanol for 30 s. Then, the seeds were washed in sterile water three times. The surface-sterilized seeds of *C. tribuloides* and *P. kesiya* were sown in trays containing a sterilized mixture of soil, peat and sand (2:1:1, v/v) and placed in a greenhouse. The seeds were watered once daily for germination of the seeds and then the growth of the seedlings.

Inoculation of the hyphae of the ECM fungi to *C. tribuloides* and *P. kesiya* seedlings

Two-month-old seedlings of *C. tribuloides* and *P. kesiya* were transferred to 1000 g sterilized soil in the growing bag (8×18 cm) with one plant per bag. The soil used in this experiment (pH 5.5) had 7.19% organic matter, 0.36% total nitrogen, 8.24 mg/kg



available phosphorus, 280.00 mg/kg exchangeable potassium, 82.50 mg/kg exchangeable calcium, 34.14 mg/kg exchangeable magnesium and 26.46 mg/kg extractable sulfur. Treatments were applied to the culture medium before planting. The ECM treatments consisted of 20 mL of the mycelial inoculum slurry added to each hole made to receive a seedling. The uninoculated plants were treated with 20 mL of the culture medium without fungal mycelium. Seedlings inoculated with the ECM fungi and uninoculated treatments of *C. tribuloides* and *P. kesiya* were replicated 30 times. The seedlings were placed in a greenhouse and watered once daily.

Evaluation of ECM formations and the growth of *C. tribuloides* and *P. kesiya* seedlings

At six months after transplanting, seedlings of *C. tribuloides* and *P. kesiya* were measured for height (with a ruler at the soil line to the height of the stem) and stem diameter (with a pair of callipers at one cm above root collar). Roots of *C. tribuloides* and *P. kesiya* seedlings were carefully washed. Shoots of *C. tribuloides* and *P. kesiya* seedlings were separated from roots. The proportion of ECM roots in the root samples was estimated for the percentage of root length colonization by the gridline intersection method according to Brundrett et al. (1996). Shoots and roots were dried at 60°C for 2 days before weighing to evaluate the shoot and root dry weights. The dried shoots of *C. tribuloides* and *P. kesiya* seedlings were also evaluated for the contents of nitrogen (N) by the Kjeldahl method, phosphorus (P) by the dry ashing and molybdovanado-phosphoric acid method and potassium (K) by the dry ashing and atomic absorption spectrophotometer method. The percentage of increased growth associated with mycorrhizal colonization was calculated as the following formula: $[(\text{Total dry weight of inoculated plant} - \text{Total dry weight of uninoculated plant}) / \text{Total dry weight of inoculated plant}] \times 100$.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA). SPSS program 23.0 for Windows was used to conduct the ANOVA. Significant differences were determined to compare means of the treatments at $p \leq 0.05$ with Duncan's Multiple Range Test. The period of this research was performed from 2018 to 2020.

Results

Mycelia of *R. alboareolata* and *A. princeps*

Hyphae of *R. alboareolata* were pale brown, without clamp connections. Hyphae of *A. princeps* were white, and the hyphae have clamp connections. Colonies of *R. alboareolata* on MMN agar supplemented with 1.0 g/L yeast extract were whitish cream to pale brown (Fig. 1a). Colonies of *A. princeps* were white on MMN agar supplemented with 1.0 g/L yeast extract (Fig. 1b).

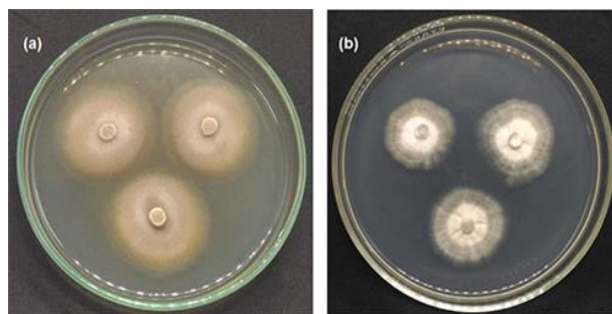


Figure-1. Colonies of *Russula alboareolata* (a) and *Amanita princeps* (b) cultured on MMN agar supplemented with 1.0 g/L yeast extract

Associations between roots of the host plants and the ECM fungi

All seedlings of *C. tribuloides* and *P. kesiya* inoculated with *R. alboareolata* and *A. princeps* formed ECM roots, while roots of the uninoculated seedlings were not found to have ECM roots and easily dry after harvesting (Fig. 2a and Fig. 3a).

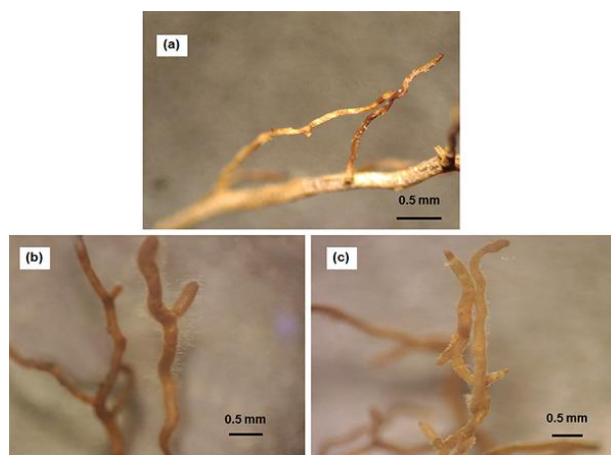


Figure-2. Roots of *Castanopsis tribuloides* seedlings (a) non-ectomycorrhizal roots, (b) ectomycorrhizal (ECM) roots inoculated with *Russula alboareolata*, and (c) ECM roots inoculated with *Amanita princeps*

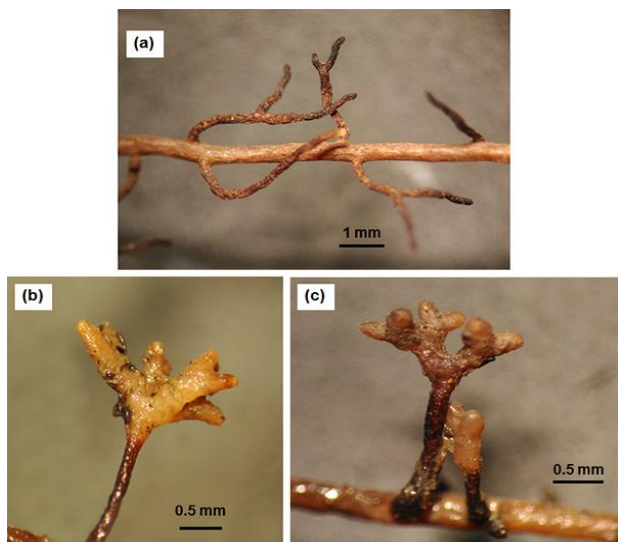


Figure-3. Roots of *Pinus kesiya* seedlings
 (a) non-ectomycorrhizal roots, (b) ectomycorrhizal (ECM) roots inoculated with *Russula alboareolata*, and (c) ECM roots inoculated with *Amanita princeps*

Branching patterns of the ECM roots of *C. tribuloides* associated with *R. alboareolata* and *A. princeps* were irregular pinnate (Fig. 2b and Fig. 2c). Branching patterns of ECM roots of *P. kesiya* associated with the ECM fungi were dichotomous (Fig. 3b and Fig. 3c). Root length colonizations of *C. tribuloides* seedlings inoculated with *R. alboareolata* and *A. princeps* were 30.2% and 29.8% respectively. Root length colonizations of *P. kesiya* seedlings inoculated with *R. alboareolata* and *A. princeps* were 19.9% and 18.9% respectively. Inoculation with *R. alboareolata* and *A. princeps* significantly increased survival of *C. tribuloides* seedlings. Survival of *C. tribuloides* seedlings of the uninoculated treatment, inoculated with *R. alboareolata* and *A. princeps* treatments were 66.7%, 76.7% and 97.2% respectively. Whereas both of the inoculated and uninoculated treatments of *P. kesiya* seedlings survived about 95% (Table 1).

Table-1. Survival of *Castanopsis tribuloides* and *Pinus kesiya* seedlings

ECM fungi	Survival of seedlings (%)	
	<i>C. tribuloides</i>	<i>P. kesiya</i>
None	66.7 ^c	94.5 ^a
<i>R. alboareolata</i>	76.7 ^b	95.4 ^a
<i>A. princeps</i>	97.2 ^a	95.0 ^a

The means in the same column with different letters are significantly different ($p \leq 0.05$).

Growth of *C. tribuloides* and *P. kesiya* seedlings

Heights, stem diameters and dry weights of both *C. tribuloides* and *P. kesiya* seedlings inoculated with *R. alboareolata* and *A. princeps* were significantly higher than those of the uninoculated seedlings (Table 2, Fig. 4 and Fig. 5). The increase in dry weight was greater for ECM-inoculated seedlings of *C. tribuloides* than for those of *P. kesiya*. The increase was significantly greater for *C. tribuloides* seedlings inoculated with *R. alboareolata* than inoculated with *A. princeps*. The percentage of increased growth of *C. tribuloides* seedlings inoculated with *R. alboareolata* and *A. princeps* compared with the uninoculated treatment were 86.8% and 82.4%, respectively. For *P. kesiya*, there was no difference in the increase in dry weight between plants inoculated with each of the fungal species. The percentage of increased growth of *P. kesiya* seedlings inoculated with *R. alboareolata* and *A. princeps* compared with the uninoculated treatment were 44.8% and 42.6%, respectively.

Table-2. Heights and stem diameters of *Castanopsis tribuloides* and *Pinus kesiya* seedlings

ECM fungi	Height (cm)		Stem diameters (mm)	
	<i>C. tribuloides</i>	<i>P. kesiya</i>	<i>C. tribuloides</i>	<i>P. kesiya</i>
None	21.3±5.96 ^b	17.9±2.48 ^b	2.7±0.51 ^b	2.3±0.30 ^b
<i>R. alboareolata</i>	64.2±7.64 ^a	21.4±3.66 ^b	5.3±0.79 ^a	3.0±0.54 ^a
<i>A. princeps</i>	58.9±16.28 ^a	20.8±2.47 ^a	5.6±0.80 ^a	3.0±0.48 ^a

Means±standard error of the means in the same column with different letters are significantly different ($p \leq 0.05$).

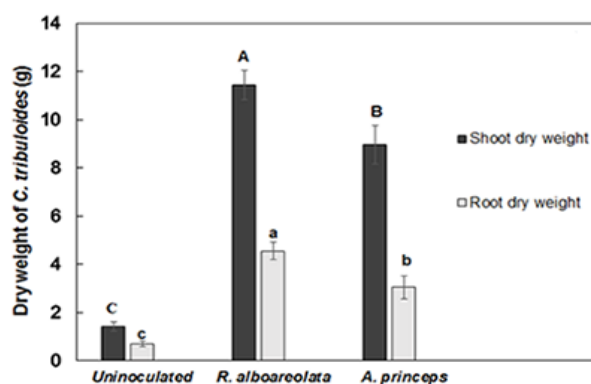


Figure-4. Shoot and root dry weights of *Castanopsis tribuloides* seedlings

Shoot dry weights with different capital letters and root dry weights with different lower-case letters are significantly different ($p \leq 0.05$) of the means with±standard error of the means.

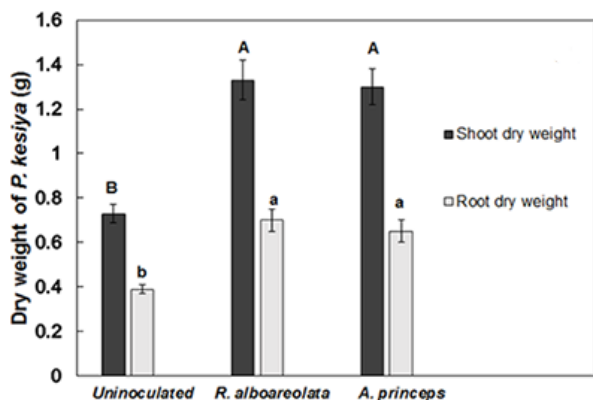


Figure-5. Shoot and root dry weights of *Pinus kesiya* seedlings

Shoot dry weights with different capital letters and root dry weights with different lower-case letters are significantly different ($p \leq 0.05$) of the means with \pm standard error of the means.

Nutrient contents of *C. tribuloides* and *P. kesiya* seedlings

The *C. tribuloides* and *P. kesiya* seedlings inoculated with *R. alboareolata* and *A. princeps* had shoot N, P and K contents significantly higher than those of the uninoculated seedlings. However, accumulations of P and K contents of *C. tribuloides* seedlings inoculated with *R. alboareolata* were significantly higher than those of *C. tribuloides* seedlings inoculated with *A. princeps* (Table 3). The N, P and K contents of the *P. kesiya* seedlings inoculated with *R. alboareolata* and *A. princeps* were not significantly different between species of the ECM fungi (Table 4).

Table-3. Nutrient accumulations (mg/plant) in shoots of *Castanopsis tribuloides* seedlings

ECM fungi	N content	P content	K content
None	20.69 \pm 1.06 ^b	1.40 \pm 0.08 ^c	12.41 \pm 0.72 ^c
<i>R. alboareolata</i>	80.95 \pm 2.96 ^a	11.52 \pm 0.48 ^a	109.43 \pm 11.43 ^a
<i>A. princeps</i>	77.02 \pm 8.42 ^a	7.89 \pm 0.78 ^b	86.00 \pm 5.98 ^b

Means \pm standard error of the means in the same column with different letters are significantly different ($p \leq 0.05$).

The P contents of *C. tribuloides* seedlings inoculated with *R. alboareolata* and *A. princeps* were about 8.2 and 5.6 times that of the uninoculated *C. tribuloides* seedlings, respectively. The K accumulations in the *C. tribuloides* seedlings inoculated with *R. alboareolata* and *A. princeps* were about 8.8 and 6.9 times that of the uninoculated *C. tribuloides*

seedlings, respectively. While the N contents of *C. tribuloides* seedlings inoculated with *R. alboareolata* and *A. princeps* were nearly 4 times that of the uninoculated *C. tribuloides* seedlings. The N, P and K contents of the *P. kesiya* seedlings inoculated with *R. alboareolata* and *A. princeps* were about 1.5, 2.1 and 1.7 times that of the uninoculated *P. kesiya* seedlings, respectively (Table 3 and Table 4)

Table-4. Nutrient accumulations (mg/plant) in shoots of *Pinus kesiya* seedlings

ECM fungi	N content	P content	K content
None	5.22 \pm 0.27 ^b	0.22 \pm 0.01 ^b	11.73 \pm 0.64 ^b
<i>R. alboareolata</i>	8.09 \pm 0.58 ^a	0.48 \pm 0.01 ^a	19.70 \pm 1.15 ^a
<i>A. princeps</i>	7.90 \pm 0.24 ^a	0.44 \pm 0.02 ^a	19.13 \pm 0.47 ^a

Means \pm standard error of the means in the same column with different letters are significantly different ($p \leq 0.05$).

Discussion

Characteristics of ECM root tips can be used for preliminary investigation of ECM fungi associated with host plants. For *C. tribuloides* seedlings, features of branching patterns of ECM roots of *C. tribuloides* associated with *R. alboareolata* and *A. princeps* were irregularly pinnate. Whereas characteristics of ECM root tips of *P. kesiya* associated with *R. alboareolata* and *A. princeps* were dichotomous branching patterns. Most mycorrhizal root tips of Pinaceae are dichotomously branched such as *P. radiata* roots associated with *Suillus brevipes*, *Amanita muscaria* and *Boletus edulis* (Brundrett et al., 1996), and *P. kesiya* associated with *Pisolithus orientalis* (Kumla et al., 2016). Features of branching patterns of ECM root tips are dependent on associations of particular host plants and ECM fungi. Branching pattern of ECM roots of *Eucalyptus globulus* associated with *Laccaria laccata* was pinnate (Brundrett et al., 1996). Feature of ECM roots of *Intsia bijuga* associated with *Scleroderma* sp. was monopodial pinnate in the young ectomycorrhizal roots, but the feature of ECM roots was becoming irregular pinnate in the older ectomycorrhizal roots (Nugroho et al., 2010). Smith and Pfister (2009) reported that they found ECM roots in the form of tuberculate ECM, which was an association between *Quercus* sp. (Fagaceae) and *Boletus rubropunctus*.

Mantle sheath hyphae of the ECM fungi that enveloped on the outside of the ECM roots could protect the roots against water loss (Smith and Read, 1997; Pietro et al., 2007; Bücking et al., 2012). Inoculation with *R. alboareolata* and *A. princeps* significantly increased survival of *C. tribuloides* seedlings. External hyphae and mantle sheath hyphae of the ECM root tips helped to increase the areas of water and nutrient uptake, which helped to increase survival and growth of *C. tribuloides* seedlings. Efficacy of ECM fungi for growth of the host plants depends on associations between ECM fungi, the host plants and environmental conditions (Brundrett et al., 1996; Smith and Read, 1997). Growth of *C. tribuloides* and *P. kesiya* seedlings was significantly increased by mycorrhization with *R. alboareolata* and *A. princeps*. Especially, shoot dry weight and root dry weight of *C. tribuloides* seedlings inoculated with *R. alboareolata* were higher than those of the seedlings inoculated with *A. princeps*. This experiment showed that *R. alboareolata* had high efficiency for increasing growth of *C. tribuloides* seedlings. The increase in growth of the *R. alboareolata* inoculated *C. tribuloides* seedlings were nearly two times higher than the *P. kesiya* seedlings. The efficacy of *R. alboareolata* forming ECM with *C. tribuloides* implies a more suitable association between this ECM fungus and the host plant within the nursery growing conditions.

Accumulation of nutrient contents of host plants was dependent on the associations between the ECM fungi and the seedling species (Smith and Read, 1997). Shoot nutrient contents of N, P and K in *C. tribuloides* and *P. kesiya* inoculated with *R. alboareolata* and *A. princeps* were increased. In particular, the accumulation of N, P and K contents in *C. tribuloides* seedlings were much greater than in *P. kesiya* seedlings. ECM fungi are known to take up both organic and inorganic nitrogen sources such as amino acids and NH_4^+ from the soil and translocate to the host plants at the interface areas of Hartig net hyphae and epidermal and cortical cells (Chalot et al., 1994; Daza et al., 2006). Most of ECM fungi increase P uptake, especially in fungal species with external hyphae of the long-distance exploration type (Lehto and Zwiazek, 2011). Whereas some ECM fungi have been reported to increase K accumulation in host plants. For example, *Acacia spirorbis* and *Eucalyptus globules* inoculated with ECM *Pisolithus albus* increased K contents in shoots of the host plants (Jourand et al., 2014). *Hebeloma cylindrosporum*

could increase K accumulation in *Pinus pinaster* in K deficient soil (Garcia and Zimmermann, 2014). However, some ECM fungus such as *Tuber melanosporum* was reported that it did not increase K accumulation in *Quercus petraea*, *Q. faginea* and *Pinus halepensis* seedlings compared with the uninoculated plants (Dominguez-Nunez et al., 2008; Garcia et al., 2014). In this experiment, the results showed that *R. alboareolata* had higher efficiency than *A. princeps* for P and K uptakes to *C. tribuloides* seedlings. The EMC roots formed by *R. alboareolata* and *C. tribuloides* seedlings were very effective in uptake of P and K. The results of this research suggest that the inoculated seedlings may be helpful for growth and survival of the plants in the early stages of planting in degraded forest areas with the low amount of ECM fungi in natural ecosystems. However, many biotic and abiotic factors in the areas have effects on competition among ECM fungi, and these factors interact with each other may have negative or positive effects on mycorrhiza formation (Mamoun and Olivier, 1993; Kennedy, 2010).

Conclusion

The edible ECM fungi, *R. alboareolata* and *A. princeps*, associated with *C. tribuloides* and *P. kesiya* seedlings were effective in increasing the growth and N, P and K accumulations of the host plants. The growth responses of the inoculated *C. tribuloides* seedlings were greater than those of the inoculated *P. kesiya* seedlings. *R. alboareolata* was more effective than *A. princeps* for the growth of *C. tribuloides* seedlings. There was no difference in the increase growth between *P. kesiya* seedlings inoculated with each of the fungal species. Inoculation of *C. tribuloides* and *P. kesiya* seedlings with mycelia of the edible ECM fungi formed specific ECM associations, which may be beneficial for reforestation.

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Conflict of Interest: None.

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Contribution of Authors

Youpensuk S: Conceived idea, designed research methodology, collected and analysed data and article write up
Wanwaen S: Literature review, collected and analysed data and article write up

