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STUDY ON INOCULATION OF ECTOMYCORRHIZAL FUNGI ON *PINUS KESIYA* IN THAILAND

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ABSTRACT

Ectomycorrhizal short roots of 4 commercial tree species in the pine and dipterocarp forests ; *Pinus kesiya*, *P. merkusii*, *Shorea obtusa*, and *Dipterocarpus obtusifolius* were collected and isolated to prepare pure mycelial culture using the technique recommended by Marx (1969). Different techniques of inoculation were experimented on *Pinus kesiya* seedlings in different growing media and environmental conditions. The results showed that water suspension inocula was possible in semi-controlled environment. Pure mycelial culture, without leaching, by placing one tea spoon of inocula immediately beneath the root system of the transplanted seedlings gave better growth and survival than the controls. Seedlings inoculated with leached inoculum at 2 tea spoons per pot showed better survival and growth than the controls. Different growing media were tried in the pot culture at different amounts and the results showed that forest soil with pH 4.5 and high organic matter content was better than mixed soil and forest soil with pH 6.4 and low organic matter. Environmental conditions used in the studies consisted of three types of nursery ; the glasshouse, the lathe house and the plastic net screen outdoor. The disadvantage of the lathe house and the plastic net screen was the amount of rainfall which is uncontrollable. It was observed that air temperature was rather high troughout the study ; minimun temperature was 19.5 °C in January and maximum temperature was 39.0 °C in March. This high temperature was

suspected to hinder ectomycorrhizal symbiosis. Field planting trial was carried out on the adversed site with erratic climatic conditions. The preliminary result showed that inoculated seedlings survived and developed better than the controls. All the controls died after outplanting for 4 months whilst the inoculated seedilings survived 18.18 % after 3 years. Fruiting body has not yet appear to help identifying the ectomycorrhiza species.

บทคัดย่อ

งานวิจัยเรื่องนี้ได้เริ่มโดยเพาะเลี้ยงเชื้อราจากรากที่มีเชื้อราของไม้ ๔ ชนิด คือ สนสามใบ, สนสองใบ, เต็ง และพลวง โดยอาศัยเทคนิคที่แนะนำ โดย Marx (1969) แล้วนำเอาเชื้อบริสุทธิ์ที่เลี้ยงได้ไปเพาะเชื้อให้แก่กล้าไม้สนสามใบที่ปลูกในวัสดุปลูกและสภาพแวดล้อมต่าง ๆ กัน ผลการทดลองพบว่า ถ้าเอาเชื้อที่เลี้ยงในอาหารในหลอดแก้วไปละลายน้ำแล้ว นำไปรดที่รากของกล้าไม้ ในสภาพแวดล้อมที่ควบคุมได้ ปรากฏว่าได้ผลพอสมควร แต่ไม่เหมาะกับการผลิตกล้าไม้จำนวนมาก ถ้านำเชื้อราที่เลี้ยงในอาหารเลี้ยงเชื้อแล้ว แต่ไม่ได้ล้างอาหารที่ยังตกค้างออกนำไปเพาะแก่กล้าไม้ ปรากฏว่าให้ผลในการเพิ่มการเจริญเติบโตและการรอดตายดีกว่าไม่ได้เพาะเชื้อเลย การเพาะเชื้อโดยใช้เชื้อที่ล้างอาหารออกแล้วเพียงสองช้อนชาต่อต้นก็ให้ผลดีกว่าการไม่เพาะเชื้อทั้งด้านการเจริญเติบโตและการรอดตาย ในการทดลองใช้วัสดุเพาะชำต่าง ๆ กันในกระถาง ผลปรากฏว่าการใช้ดินป่าไม้ที่มี pH 4.5 และมีอินทรีย์วัตถุมากจะได้ผลดีกว่าดินผสม หรือดินป่าไม้ที่มี pH 6.4 และมีอินทรีย์วัตถุ สำหรับสภาพแวดล้อมของการทดลองมีทั้งเรือนกระจก เรือนไม้ระแนง และตาข่ายไนลอน ซึ่งมีข้อดีข้อเสียต่างกันคือ เรือนกระจกจะทำให้อุณหภูมิสูง ส่วนเรือนไม้ระแนงและตาข่ายไนลอนจะไม่สามารถควบคุมการให้น้ำได้ ฤดูการทดลองมีผลกระทบต่อการเพาะเชื้ออยู่มาก เพราะอุณหภูมิสูงเกินไป เช่น ๓๙° ซ จะไม่เอออำนวยต่อการติดเชื้อ จากการนำกล้าไม้ที่เพาะเชื้อและไม่เพาะเชื้อไปทดลองปลูกที่เขาบิน จังหวัดราชบุรี ซึ่งมีฝนตกเฉลี่ยเพียง๗๐๐มม.

ต่อปี และดินขาดความอุดสมบูรณ์อย่างมาก ปรากฏว่ากล้าไม้สนสามใบที่ไม่ได้เพาะเชื้อจะตายหมดภายใน ๔ เดือนหลังจากปลูก ส่วนกล้าไม้ที่เพาะเชื้อจะรอดตายมากแล้วค่อย ๆ ตายไปจนเหลือ ๑๘.๑๘ เปอร์เซนต์หลังจากปลูกไปแล้ว ๓ ปี เนื่องจากดินฟ้าอากาศวิปริตมาก การออกเห็ดจึงไม่มีให้เห็น ทำให้ผู้วิจัยไม่สามารถจะบอกชนิดของเชื้อราที่ใช้เพาะให้แก่กล้าไม่ได้

INTRODUCTION

Many studies from various parts of the world have pointed out that many forest trees form symbiotic root associations called mycorrhizae with various soil–inhabiting fungi. In many tree species the fungus mainly grows on the outside of the feeder roots and the association is called ectomycorrhizae. It is generally recognized that if forest tree species such as *Pinus* are to grow normally, it is essential that ectomycorrhiza fungi develop on their roots.

Mikola (1980) stated that the transport of soil or living seedlings for mycorrhizal inoculation is not at all a satisfactory method. Although the technique itself is easy, there are several drawbacks, so inoculation with fungal spores or pure cultures has been suggested. Marx (1980) reviewed many reports and concluded that the use of pure mycelial cultures of ectomycorrhizal fungi has been repeatedly recommended as the most biologically sound method of inoculation but technical difficulties in isolating, culturing, and inoculating mycorrhizal fungi are still encountered.

No research on pure culture inoculation on pine seedlings was conducted in Thailand until IFS agreed to support this study in 1978.

MATERIALS AND METHODS

Isolation of ectomycorrhizae was made from mycorrhizal short roots recommended by Marx and Barnett (1974). Isolations were made from 4 tree species; *Pinus merkusii*, *P. kesiya*, *Dipterocarpus obtusifolius*, and *Shorea obtusa* using the technique recommended by Marx (1969). Forty isolates were thus collected and cultured in Hagem slant agar in test tubes and incubated at 25° C to observe the fungi formation. The promising pure mycelial cultures were grown in different substrates. Marx (1980) recommended the substrate which contains vermiculite, peat moss and nutrient solution. In this study three different substrates were tried; (1) vermiculite-sphagnum moss and nutrient solution, adjust to pH 4.5; (2) vermiculite-pine needles and nutrient solution; and (3) vermiculite-sorghum grains and nutrient solution. The objective is to prepare the pure mycelial cultures using the locally available materials as a substrate.

The first experiment was performed in growth chamber using sand and clay soil at 1:1 by volume as a growing media, autoclaved at 15 lbs/inch^2 for 20 minutes, using the plastic cups as containers. *Pinus kesiya* seeds were washed with 0.1% $HgCl_2$ and soaked in pure water for 3 hours before 5 Seeds will be seeded in cach container. After 4 weeks, inoculated with the mycelial suspension prepared by using 2 ml of culture from the mixture of the mycelial and the slant agar in the test tube which was 3 weeks old (after isolation) in distilled water by pouring the suspension at the root collar of the seedling and cover with sterile coarse sands before raising in the growth chamber at 25°C under daylight fluorescence lamp. The controls were made without fungi inoculation. This experiment consists of 4 replication for each isolate.

After 10 weeks, representative seedlings were removed for mycorrhizal infection observation. The remained seedlings were observed for their performances up to 10 and 11 months.

The second experiment was conducted in the glass house. Seedlings of *Pinus kesiya* were grown in the sterile sand media in the small wooden box. After 3 weeks, the seedlings were transplanted into clay pots filled with methyl bromide fumigated soil + sand at 2:1 by volume. The mycelial culture in the vermiculite –sorghum substrate was placed immediately beneath the root system at about one tea spoon. Two isolates number 17 and 33 were used. Twenty five seedlings were inoculated by each fungi isolate. Uninoculated seedlings served as controls. Survival and height growth were measured. Mycorrhizal infection was observed on representative seedlings after 3 months.

The third experiment was carried out under the blue plastic net in the open. Two kinds of growing needia were used; methyl bromide fumigated forest soil and the mixed soil (clay soil + rice husk charcoal + sand at 2:1:1 by volume). Three *Pinus kesiya* seeds were sown in each pot. After seeding the surface was covered with sterile coarse sand. The numbers of pot using forest soil and mixed soil were 79 and 71 respectively. Two teaspoons of inoculum of isolate numbers 15 and 16 were spread around the root system. Fifteen pots were treated by each fungi isolate and also the controls. After one month, survival was checked and only one seedling was left in each pot. Fertilizer 15:15:15 at 1.5 g per seedling was applied on one–half of each treatment. In addition, 6 pots were inoculated with the mixture of isolate 15 and 16 for observation.

The fourth experimnt was carried out in the glass house. Two top soils from 2 natural forests from Nam Prom, Chaiyapoom province with pH 4.5, O.M. 6.74 %, P 17 ppm, K 86 ppm, Ca 157 ppm, and Mg 73 ppm and from Sakaerat, Nakhon Ratchasima province with pH 6.4, O.M. 2.05 %, P 36 ppm, K 46 ppm, Ca 790 ppm, and Mg 310 ppm, were used as growing media after methyl bromide

fumigation. The 20 cm diameter clay pots were filled with 1.5 kgs of soil before seeded with 5 sterile *Pinus kesiya* seeds will be sown after the soil media were inoculated with leach inoculum of isolates 13 and 17. Tap water in the nursery was adjusted to pH 6.0 by ortho-phosphoric acid 85% in the big jars before watering once a day in the morning. After one month, survivals were checked and culling was made to leave only one seedling per pot. Diameter at ground level and height were recorded every month up to 8 months. The controls were mixed with vermiculite and sphagnum moss before seeding to resemble the treatment. Fertilization with 15:15:15 at 1.5 g per seadling was applied at 2, 4 and 6 months on half of each treatment. Mycorrhizal infection was observed on 2 representative seedlings of each treatment. Biomass of shoot and root was also measured. The range of temperature was from 14° C in January to 43° C in April.

The fifth experiment was the field planting trial using the 33 seedlings under Namprom soil left from the fourth experiment. The planting area at Ratchaburi province is claimed as the adversed site with poor soil and low rainfall (700 mm per year) with high summer temperature (43° C in April). Planting was made in August 1983 with 11 PC, 12 PN-13, and 10 PN-17 seedlings in the same row of *Azadirechta indica*, After two months, survival and growth were measured (October 1983). Four months later (February 1984) the same measurements were made. Subsequent measurements were made every year up to 3 years. Observations were periodically made to investigate the sporocarp for species indentification.

RESULTS AND DISCUSSION

From 40 isolates, after 2 weeks isolates numbers 1, 7, 11, 14, 19 and 21 were excessive microbial contaminated and were discarded. All other isolates formed mycelial of varying colors; white, brown, yellow, black, blue or blends of

these colors. The mycelial cultures of these isolates were incubated at 25° C for further experiments.

The pure mycelial culture in different modified substrates according to the locally available materials showed that vermiculite–sphagnum moss adjust to pH 4.5 and moistened with Hagem nutrient solution was unfavourable, only isolate numbers 9 and 15 formed mycelium after growing at 25°C for 3 months. The pine needles mixture was totally failed. The vermiculite–sorghum grains (boiled for 10 minutes, drained water out) at 1 : 1 moistened with Hagem nutrient solution (pH 5), autoclaved at 15 lbs/inch^2 for 25 minutes, inoculated with mycelial culture from the test tubes and raised at 25°C for at least 3 weeks is most favorable.

Marx (1980) found that grain culture was not a good substrate in his study because he found from microscopic examination that the grain cultures were colonized by saprophytic fungi and bacteria as early as three weeks after soil inoculation. His result does not support the claim of Park (1971) that grain cultures of *C. graniforme* can be used to inoculate nursery soil.

The results of the inoculation experiments in different conditions are as followed :–

In the water suspension inoculation under growth chamber condition, isolates numbers 12, 17, 23, 26, 28, 30, 31, 33 and 37 showed promising growth and survival, while isolates numbers 2, 9, 10, 18, 25, 27, 29, 32, 35 and 38 showed fair results, all the rests were very poor. From the investigation of mycorrhizal infection, isolates numbers 16 and 33 seemed to form Hartig net on the roots while all the others were rather difficult to identify. The performace of the seedlings after 10 and 11 months varied but better than the controls, the roots

are folded in the plastic cups which are rather small to keep the plants for this long period. This experiment was not practical for large amount of seedling production.

In the second experiment, after three months, the treatments with isolates numbers 33 and 17 survived 32% and 24% respectively, while the controls survived only 16%. Seedling heights after 2 months are not quite different, isolate 17 had best average height of 14.1 cm. while isolate 33 was 12.6 cm. and the controls was 10.3 cm. The mycorrhizal infection is not clear but after 9 months the abservation was made again and found that the inoculated seedlings are very healthy and found many hyphal strands around the roots. The controls are still alive but are not as healthy as the inoculatedones

In the third experiment, after 3 weeks, germination in the forest soil treatment was 67.08% and in mixed soil was only 43.19%. After one month survival and height were recorded as shown in Table 1.

Table 1. Survival and Height After One Month.

Treatment	Forest soil			Mixed soil		
	Initial seedlings	Survival %	Mean Ht. cm.	Initial seedlings	Survival %	Mean Ht. cm.
Isolate 15	45	93.33	7.01	30	83.33	7.40
Isolate 16	54	94.44	7.05	33	96.97	7.14
Isolates 15+16	6	100	7.38	—	—	—
Control	48	95.83	8.01	19	94.74	7.05

After one month only one seedling was left in each pot and fertilization trial was conducted to observe the effect of fertilizer on mycorrhizal inoculation. Measurements were made every month. The results are shown in Tables 2 and 3.

Table 2. Average Height of *Pinus kesiya* After 1, 2, 3, 4, 5 and 6 Months

Age (Mon)	Ave Ht. (cm) in Mixed Soil						Ave. Ht. (cm) in Forest Soil							
	Isolate 15		Isolate 16		Control		Isolate 15		Isolate 16		Control		Isolate 15+16	
	Fert.	No.Fert.	Fert.	No.Fert.	Fert.	No.Fert	Fert.	No.Fert.	Fert.	No.Fert.	Fert.	No.Fert.	Fert.	No.Fert.
1	7.39	7.41	6.51	7.77	6.51	7.68	7.15	6.85	6.81	7.29	8.11	7.9	7.4	7.35
2	9.29	9.53	9.37	10.67	8.72	9.11	9.28	8.53	8.91	8.68	10.19	9.66	8.95	8.95
3	12.83	11.73	10.75	13.07	10.20	10.14	10.83	8.62	9.83	9.27	12.52	9.05	9.75	9.40
4	14.78	13.08	11.86	13.84	13.50	10.88	12.49	8.71	10.68	9.71	12.87	9.59	12.20	9.85
5	18.53	15.77	17.70	18.25	18.55	18.70	17.80	9.45	12.29	10.29	15.30	10.59	13.30	9.90
6	20.20	16.53	21.10	19.05	20.65	20.20	19.85	9.50	15.04	10.29	18.20	10.77	15.20	9.90

Table 3. Survival Percentages After 2, 3, 4, 5 and 6 Months of Inoculation

Age (Mon)	Survival in Mixed Soil						Survival in Forest Soil							
	Isolate 15		Isolate 16		Control		Isolate 15		Isolate 16		Control		Isolate 15+06	
	Fert.	No.Fert.	Fert.	No.Fert.	Fert.	No.Fert.	Fert.	No.Fert.	Fert.	No.Fert.	Fert.	No.Fert.	Fert.	No.Fert.
2	100	100	85.71	100	71.43	100	100	100	100	100	100	100	100	100
3	57.10	100	85.71	100	42.86	83.33	100	100	100	91.67	100	100	100	100
4	57.14	85.71	71.43	100	42.86	66.67	83.33	100	83.33	83.33	100	100	50	100
5	42.86	85.71	28.57	57.14	28.57	16.67	66.67	90.91	83.33	66.67	100	88.89	50	100
6	42.86	85.71	28.57	57.14	28.57	16.67	66.67	90.91	58.33	58.33	77.88	77.78	50	100

From Table 2, it is quite evident that fertilizer help promoting height, but there is no different between the inoculated and the control treatment. In Table 3, fertilization shows negative effect on survival, most of the unfertilized seedlings are better survived than the fertilized ones. The control seedlings are still survived up to six months. The forest soil gave better survival than the mixed soil. The mixture of islolate no. 15 and 16 without fertilizer gave highest survival. The fungi isolate no. 15 gave better survival than no. 16.

Since only small amount of pure mycelial inocula (1–2 teaspoons) were inoculated, the mycorrhizal root development is rather slow, it is suggested that more amount of pure inoculum be applied as was recommended by Marx (1980). He suggested to use 2.8 e/m^2 of leached inoculum to broadcast into the surface of nursery soil and immediately mixed thoroughly with hand tools into the upper 10 to 12 cm of soil. The 1.08 litres per m^2 of soil surface rate was the least amount that could effectively be used for maximum mycorrhizal development in one of his experiment in the nursery. He would recommend this rate for purposes of experimentation in properly fumigated nursery soils. The inoculum mixing ratio of 1 : 8 was also recommended for pot culture experiment. So higher rate of inoculum should be tried in our future experiment to find the appropriate rate for tropical condition in Thailand.

The results of the fourth experiment showed great variation in germination percentages ; the KC (control in Sakaerat soil), KN–13 (Sakaerat soil + inoculum no. 13), and KN–17 (Sakaerat soil + inoculum no. 17) has 17.3%, 40% and 50% germination respectively, while the PC (control in Namprom soil), PN–13

(Namprom soil + inoculum no. 13), and PN−17 (Namprom soil + inoculum No. 17) had 45.33%, 51.67% and 71.67% germination respectively. This may indicate that Namprom soil is more suitable for seed germination than Sakaerat soil and mycorrhiza inoculum no. 17 offered better result in both soil. For the seedling survival, the KC maintained at 100% up to 3 months and decreased to 93% in the fourth and fifth months and down to 60% in the sixth month, 33% in the seventh month and 0% in the eighth; the KN−13 survived 100% up to the fifth month and decreased to 60%, 50% and 30% in ths sixth, seventh and eighth month respectively; the KN−17 survived 100% up to the third month and then decreased to 90%, 90%, 60%, 30% and 30% in the subsequent months; while the PC, PN−13, and PN−17 treatments survived 100% up to the eighth months. This showed that Namprom soil and Namprom soil + inoculum nos. 13 and 17 are suitable for seed germination and survival than Sakaerat soil. The fertilization did not showed any difference in all treatments up to 5 months. The height growth was recorded up to 8 months and average height was used as a basis of comparison between treatments. KC treatment gave poorest and all of the seedlings died in the eighth month, while the KN−13 and KN−17 gave similar results but the seedlings were stunted and look unhealthy. The PC which was the control gave reasonable results but most seedlings had branches, while the PN−13 and PN−17 showed very high average height up to 49.5 cm and 48.5 cm without branches and healthy; the results are shown in Table 4.

The investigation on mycorrhiza infection was made after 5 months, 2 seedlings from each treatment were pulled out and checked the percentage of

infection at the roots with the aid of magnify glass. It was shown that KC had no infection, while KN–13 and KN–17 had about 1–2% dichotomy shape on the root hairs. The PC also showed no infection, while the PN–13 and PN–17 had about 1–5% dichotomy shape on the root hairs. It was observed that the inoculated seedlings looks more healthy than the controls.

Table 4. Average Height and Performance of Seedlings at Different Ages.

Treatment	Average ht. (cm)/age (mo.)								Performance
	1	2	3	4	5	6	7	8	
KC	3.47	6.06	9.2	11.9	12.3	17.6	14.5	—	all died
KN–13	4.2	6.9	11.1	15.2	18.9	25.1	27.4	20.0*	stunted, unhealthy
KN–17	4.7	7.7	11.6	14.4	16.3	18.1	19.5	19.5*	stunted, unhealthy
PC	4.6	9.3	16.5	23.7	30.2	33.6	37.6	41.0	branching, healthy
PN–13	4.8	10.0	18.9	28.5	38.2	42.8	47.6	49.5	straight, healthy
PN–17	5.3	9.6	17.7	26.3	33.6	38.8	44.8	48.5	straight, healthy

Notes : K = Sakaerat soil, P = Namprom soil

C = Control, N–13 = inoculum no. 13

N–17 = inoculum no. 17

*decreased average height due to death seedlings

The results of the fifth experiment in the field showed that after two months of planting 7 trees out of 33 died, they were all the controls (PC seedlings) and 4 months after the first measurement mortality rate was still the same (21.2%). One year later the number of survived trees was only 15 out of 33, which means survival is 45.45% and all the controls were died. But no fruiting body is ever present, because the climatic condition is never suitable, no rain in that area for

more than seven months. In September 1985, there was only 6 trees (18.18%) left in the plot with 1.0 to 1.5 m high and there was no evidence of fruiting body, so the author cannot identify the species of inoculum numbers 13 and 17 which were used in this experiment. Further observation will be made eventhough the project was terminated.

Mycorrhizal fungus species is usually identified from the fruiting bodies or mushrooms. One example is the formation of a puffball of *Pisolithus tinctorius* at certain times of the year. Nuhamara and Hadi (1981) identified the species by isolating from mycorrhizal short roots of a tree and from the fruiting bodies suspected to be associated with the mycorrhizal short roots. When the characteristics of the pure cultures isolated both from the mycorrhizal short root and from the fruiting body were similar, the fungus isolated was identified based on the characteristics of the fruiting body. This method is very interesting because we used to try to isolate from the peridioles of the fruiting body of many mycorrhizal fungi and totally failed. There should be specific technique for this which is not explained in their report.

Royal Forest Department (1980) found that *Suillus subolivaceus, Pisolithus tinctorius* and *Scleroderma flavidum* are associated with *Pinus kesiya* in natural stand and plantation in Thailand. So our identification of different isolates used in the studies is still in doubt. It is guest that the mycorrhiza my be *suillus subolivaceus* but confirmation with fruiting bady is still needed.

The experiment on different soil media with inoculum numbers 13 and 17 using treated water with pH 6.0 showed that Namprom soil is better than Sakaerat soil in all treatments. This also showed that soil properties is important for seed germination, survival, growth, and mycorrhiza infection. Fertilizer did not show any effect on seedling development in the first eight months.

CONCLUSIONS

This study is attempted to isolate the ectomycorrhizal fungi from the mycorrhizal short roots of the locally grown tree species, Forty isolates were prepared from the mycorrhizal short roots of 4 commercial tree species ; *Pinus kesiya P. murkusii, Shorea obtusa* and *Dipterocarpus obtusifolius*. Pure mycelial cultures were tried in 3 different substrates and it was found that vermiculite–sorghum grain with Hagem nutrient solution was most appropriate for mass inocula preparation. Inoculation experiments were made under different growing media and different mycorrhizal isolates. It is evident that mycorrhiza inoculation has positive effect on seedling survival. Mycorrhiza isolate no. 15 gave better survival on *Pinus kesiya* seedlings than isolate no. 16. Seedlings inoculated with mycorrhiza isolate no. 13 and 17 gave significant height growth than the controls after 9 months, but survivals were not much different.

The field planting experiment showed that uninoculated seedlings eventually died after 4 months, while the seedlings inoculated with isolate no. 13 and 17 survived 45.45 % after 18 months and the height ranged from 40 to 79 cm. No fruiting body was presented. Observation was made again 19 months after, and found that survival was only 18.18 % and the remaining seedlings are about 1.0 to 1.5 m high. This study showed that mycorrhiza helped the seedlings to survive in an adversed soil and climatic conditions (very poor soil with long drought period). It is expected to identify the mycorrhiza species from the fruiting body which is not yet present until now.

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