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Growth Stimulation of Pines by Artificial Inoculation with Mycorrhizal Fungus, *Pisolithus tinctorius*.*1

Chang Duck Koo*2 Kyung Joon Lee*2 Kyong Bin Yim

菌根菌의 人工接種에 依한 소나무類의 生長促進*1

具昌德*2·李景俊*2·任慶彬

ABSTRACT

Two ectomycorrhizal fungi, *Pisolithus tinctorius* and *Thelephora terrestris*, were introduced from U.S.A. and inoculated to five pine species in Korea to evaluate the reported growth stimulation of host plants after inoculation. These fungi were grown as mycelial inoculum in large quantity and inoculated to the fumigated nursery soil just before seed sowing. At the end of the first growing season, *Pisolithus* stimulated the height growth of *Pinus densiflora, P. thunbergii, P. rigida,* and *P. rigida* x *taeda* by 55, 36, 69, and 37%, respectively, compared with control seedlings with no fumigation and no inoculation. When the growth stimulation was expressed with dry weight, *Pisolithus* increased dry weight of *P. densiflora* and *P. rigida* x *taeda* by 143% and 128%, respectively, over control seedlings. *Thelephora* failed to stimulate growth of inoculated plants. *Pinus koraiensis* did not respond to the inoculation during the first growing season. It is concluded that artificial inoculation of nursery pine trees with selected mycorrhizal fungi should be seriously considered to improve the quality of planting stocks and to stimulate early plant growth. The potential for use of *Pisolithus* in reforestation on adverse sites is also discussed.

Key words; ectomycorrhizae; Pisolithus tinctorius; Thelephora terrestris; mycelial inoculum.

要 約

美國에서 *Pisolithus tinctorious*(P. t.), *Thelephora terrestris*(T. t.)의 두 가지 外生菌根菌을 導入하여 韓國에 있는 5個 소나무樹種에 人工接種하여 그들의 生長促進效果 를 測定하였다. P. t.와 T. t.菌糸를 大量增殖培養하여, 播種 直前에 Methyl bromide로 薰蒸된 土壤에 接種하였다. 生長 첫해에, P. t.로 接種한 *Pinus densiflora, P. thunbergii, P. rigida, P. rigida*×*taeda*苗木은 無處理苗木보다 樹高生長이 各各 55%, 36%, 69%, 37% 增加되었다. *P. densiflora, P. rigida*×*taeda*苗木의 乾重量에서는 P. t. 接種苗가 無處理苗木보다 各各 143%, 128% 增加되었다. 그러나 *P. koraiensis*에서는 生長 첫해에 菌根菌接種效果가 나타나지 않았고 接種된 5個樹種에서 T. t. 接種效果는 없었다. 優秀한 菌根菌을 選拔하여 苗圃場에 人工接種할 경우 播種苗의 初期生長이 促進되고, 苗木의 品質向上을 圖謀할 수 있다고 結論지을 수 있다. 荒廢地造林時 P. t.菌의 利用에 關하여서도 討議하였다.

* 1.接受 2月18日 Received February 18, 1982.
* 2.林木育種研究所 Institute of Forest Genetics, Suweon, Korea.
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INTRODUCTION

It has been well documented that many agronomic plants as well as forest trees normally form mycorrhizae with soil fungi which bring in many benefits to the host plants. In particular, ectomycorrhizae are common features of many forest trees (Marks and Kozlowski, 1973) and have been shown to facilitate absorption of soil nutrients, supply trees with growth-regulating substances, deter root pathogens (Marx, 1972) decrease soil toxicity, and increase host resistance to drought (Dixon *et al.*, 1980) and high soil temperature (Marx and Bryan, 1971).

Artificial inoculation of host plants with certain mycorrhizal fungi has shown that the plant growth can be stimulated remarkably in conifers (Marx *et al.*, 1978; Marx *et al.*, 1976), in broad-leaved trees (Kormanik *et al.*, 1977; 1981), and in agronomic plants (Owusu-Bennoah and Mosse, 1979; Bagyaraj and Manjunath, 1980), and also in nitrogen-fixing plants (Islam and Ayanaba, 1981). Particularly in forestry, two super strains of ectomycorrhizal fungi developed by Dr. Marx and his colleagues in U.S.A. have been proved to stimulate growth and enhance survival of conifer tree species. The two super strains, *Pisolithus tinctorius* and *Thelephora terrestris*, are particularly beneficial to the trees planted under harshy conditions, such as eroded sites, spoiled mine areas, and reclamation sites (Berry and Marx, 1980).

In Korea there are very few papers on mycorrhizae. The authors located at the Institute of Forest Genetics initiated in 1980 a new research on ectomycorrhizae in woody species and published papers on ectomycorrhizal distribution in Korea (Lee *et al.*, 1981a), relationship between soil fertility and mycorrhizal formation (Lee *et al.*, 1981b), mycorrhizal defense against soil pathogens (Lee *et al.*, 1981c) and will publish shortly papers on identification of ectomycorrhizal fungi in pine stands. Readers are recommended to refer to following books for general discussion on mycorrhizae: "Mycorrhizae" (Hacskaylo, 1971), "Ectomycorrhizae" (Marks and Kozlowski, 1973), "Endomycorrhizas" (Sanders *et al.*, 1975), and "Tropical Mycorrhiza Research" (Mikola, 1980). Lee *et al.* (1981b) briefly described three kinds of mycorrhizae in higher plants.

The objectives of this study were to test the superiority of the two introduced "super strains" mentioned above and to evaluate methods of mass culture and inoculations of these fungi to nursery trees.

MATERIALS AND METHODS

A) Mass Culture of Ectomycorrhizal fungi

Pisolithus tinctorius #250 and *Thelephora terrestris* #223 were introduced as mycelium in tube slants from Institute for Mycorrhizal Research and Development (USDA Forest Service) in Athens, Georgia, U.S.A. These two fungi are "Super Strains" due to excellent growth stimulation of host plants in adverse sites. These fungi were first cultured in sterile Petri dishes with MMN agar medium (Modified Melin-Norkrans' medium) at 25°C for about two weeks. The MMN medium contains following nutrients (Marx, 1969):

Ingredient	Concentration
$CaCl_2$	50mg
NaCl	25mg
KH_2PO_4	500mg
$(NH_4)_2HPO_4$	250mg
$MgSO_4 \cdot 7H_2O$	150mg
$FeCl_3$ (1%)	1.2ml
Thiamine HCl	0.1mg
Malt Extract	3g
Glucose	10g
Bacto-agar (optional)	15g
Distilled Water	to 1,000ml

The original paper of Marx (1969) wrongly listed sucrose for carbon source, and it should be replaced with glucose. After fungi were fully grown on the agar, the fungi were transferred to pre-autoclaved glass bottles(1→ℓ in capacity, used as Ringer's solution bottles in hospital) containing 770cc of vermiculite (grade 2 or particle size larger than 2mm), 30cc of peat moss and 400ml of MMN solution (no agar added). The mouth of the bottle was plugged with cotton and the bottles were left at room temperature (25°C if possible) for about 3 to 4 months until the fungal mycelium permeated all the vermiculite particles. A total of 120 glass bottles were cultured.

Just before inoculation of these fungi to nursery soil, the bottles were broken open and the

vermiculite-mycelium mixture was leached with running tap water for about 5 min. to remove excess nutrients which had not been used by the fungi. If the excess nutrients are not removed, soil microorganisms competitive with mycorrhizal fungi will rapidly utilize the nutrients and multiply in large number. After removing excess water from the vermiculite, the inoculum is ready to use and should be used within 48 hours to minimize set back in fungal growth.

B) Nursery Preparation and Fumigation

Fresh seeds of *Pinus densiflora, P. thunbergii, P. koraiensis, P. rigida,* and *P. rigida* x *taeda* were soaked in water for 5 days before sowing. For *P. koraiensis,* seeds were stratified for two months before sowing.

Four different treatments were recognized as randomized block design with four replicated blocks for each treatment: 1) Fumigation only (no inoculation), 2) Fumigation plus inoculation with *Pisolithus tinctorius* (Pt), 3) Fumigation plus inoculation with *Thelephora terrestris (Tt).4) Control (no fumigation, no inoculation).*

Fig. 1. First year growth of *Pinus densiflora* (front row, D) and *P. rigida* x *taeda* (back row, RT) seedlings inoculated with *Pisolithus tinctorius* (Pt). CO: Uninoculated seedlings, III: Third replication (Block).

Nursery bed was prepared for microplots described by Marx and Bryan (1975). These plots were encircled with wood panels and each plot was 90cm x 90cm, and 25cm deep (Fig. 1). The adjacent microplots were placed 1.1m apart in four directions. The entire area of nursery bed was covered with an air-tight canvas tent and fumigated with methyl bromide (50g/m² surface) for 66 hours. For control plots where no fumigation was applied, the top soil in 30cm depth within the plots was removed before the fumigation and was put back after fumigation.

C) Inoculation and Care of the Nursery

Three days after the fumigation during which residual toxic fume escaped completely, 1-ℓ inoculum and 5-ℓ fumigated pine bark (sieved with 1cm wire net) added to each plot and mixed thoroughly with top soil. In inoculation plots live inoculum of Pt or Tt was used, while both fumigation plots and control plots received same amount (1-ℓ) of autoclaved vermiculite-mycelium mixture. The addition of autoclaved inoculum to both fumigation and control plots was intended to minimize the possible effect of vermiculite-mycelium mixture on the subsequent change in various soil properties. In this way four different treatments were considered to impose same modification to the soil environment except the effect of live mycorrhizal fungi.

Seeds of five pine species (three native and two introduced) were sown on the following day in rows and mulched with rice straw, and bird net was installed. No fertilizer or no biocides were used during the experimental period. Mycorrhizal development was frequently examined while the seedlings were thinned to final number of 200 trees per plot. At the end of the first growing season in mid-October, some of the seedlings were harvested to determine height, root-collar diameter, ovendry weight, top-root ratio, and percent of mycorrhiza formation.

RESULTS

A) Mycorrhizal Development

Formation of mycorrhizae was first noticed about a month after inoculation or two weeks after seed germination in control and inoculated plots. Fumigated plots were free of mycorrhizae until late June when roots near top soil started to form mycorrhizae, indicating the contamination of fumigated plots with fungi. Two months after seed germination, inoculated seedlings had large number of well formed mycorrhizae compared with fumigated or uninoculated seedlings, even though there was no

difference in height between inoculated and uninoculated seedlings. Three months after inoculation, fruiting bodies of *Pisolithus tinctorius* (Pt) started to appear on the inoculated lated plots, and two weeks later those of *Thelephora terrestris* (Tt) were observed epiphytically around the base of host plant stems. Fruiting bodies of Pt were also occasionally observed in fumigated plots, which suggested that autoclaving of Pt in glass bottles failed to kill ali the mycelia. However, the degree of contamination appeared to be minor.

Mycorrhizae formed by Pt were golden brown in color and had mostly bifurcate and repeated bifurcate root tips. The length of Pt infected short roots was relatively long and zigzag compared with naturally formed mycorrhizal root tips. Mycorrhizae formed by Tt were milky white (with slightly pale brown mixed in it) in color and the shape was similar to Pt-mycorrhizae, with relatively long zigzag and bifurcate, repeated bifurcate mycorrhizal tips. Coralloid tips were rarely observed in Pt and Tt. Mycorrhizae formed by natural populations of fungi in control plots were quite different in shape from those by Pt or Tt. Natural mycorrhizae were mostly light brown in color with occasional white tips. The infected short roots was much shorter in length and straight, and particularly coralloid tips were abundant.

In general mycorrhizal formation by Pt was much better than by Tt in all the species. In *Pinus rigida* x *taeda* plots where we counted mycorrhizal frequency, the percent of mycorrhizal root tips was 80-95% in Pt plots. On the other hand, seedlings inoculated with Tt had 40-60% of infected root tips. Natural mycorrhizal formation in control plots was quite successful, resulting in about 80% or more frequency.

It should be mentioned that Pt mycorrhizal frequency in blocks I and II was much lower than in blocks III and IV in all the species tested. Consequently seedling growth in blocks I and II were poorer than in blocks III and IV as discussed below.

B) Growth Stimulation

Three months after the inoculation, seedlings inoculated with Pt grew faster and looked more greenish than either control (uninoculated) or fumigated plot seedlings. The height growth of four pine species at the end of the first growing season (measured in October) is shown in Table 1 through Table 4. The height growth of *P. koraiensis* is not reported here because of inherent limited shoot growth of this pine during the first growing season. *Pisolithus* stimulated height growth of four pine species compared with uninoculated control. The amount of growth stimulation by Pt was 55%, 36%, 69%, and 37% compared with control for *P. densiflora*, *P. thunbergii*, *P. rigida*, and *P. rigida* x *taeda*, respectively, which was statistically significant at either 5 or 1% level except for *P. thunbergii* (Fig. 2).

Table 1. Height growth of *Pinus densiflora* seedlings inoculated with *Pisolithus tinctorius* (Pt) and *Thelephora terrestris* (Tt) at the end of the first growing season. Nursery soils were fumigated on Apr. 13-16 and inoculated on Apr. 21. Seeds were sown on Apr. 22, and seedling height was measured on Oct. 10. Seeds were sown in microplots (90cm x 90cm) made of wood panels.

Unit: cm

Treatment	Block				Mean*
	I	II	III	IV	
Fumigation	7.1	5.8	7.2	6.2	6.6[a]
Control	5.6	5.7	6.0	5.1	5.6[a]
Fumig. + Pt	6.7	8.5	10.4	9.3	8.7[b]
Fumig. + Tt	5.3	4.4	6.6	5.0	5.3[a]

* "b" is significantly different from "a" at 1% level.

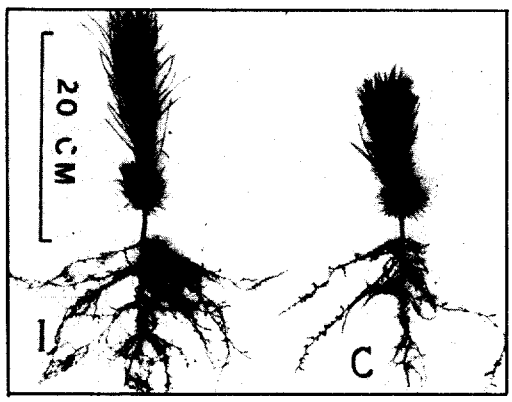

Fig. 2. *Development of more fine roots and more height growth in an inoculated seedling (I) of Pinus rigida* x *taeda* than in an un-inoculated seedling (C).

Table 2. Height growth of *Pinus thunbergii* seedlings inoculated with *Pisolithus tinctorius* (Pt) and *Thelephora terrestris* (Tt) at the end of the first growing season.

Unit: cm

Treatment	Block				Mean*
	I	II	III	IV	
Fumigation	6.5	5.6	6.2	6.6	6.2[a]
Control	5.7	5.3	6.2	5.5	5.7[a]
Fumig. + Pt	5.7	6.1	8.8	10.2	7.7[a]
Fumig. + Tt	6.2	6.1	4.4	5.2	5.5[a]

* These 4 means are not significantly different from each other at 5% level.

As shown in Tables 1 through 4, growth stimulation by Pt in blocks I and II were less conspicuous than in blocks III and IV. Percent of Pt mycorrhizal formation in blocks I and II was much lower than in blocks I and II. This poor growth in blocks I and II in all four species considerably lowered the percent of average growth stimulation by Pt. In fact, *P. rigida* seedlings inoculated with Pt in block III (Table 3) was more than two times bigger than control seedlings (17.0 *versus* 7.3).

Table 3. Height growth of *Pinus rigida* seedlings inoculated with *Pisolithus tinctorius* (Pt) and *Thelephora terrestris* (Tt) at the end of the first growing season.

Unit: cm

Treatment	Block				Mean*
	I	II	III	IV	
Fumigation	7.9	10.9	8.0	7.8	8.6[a]
Control	7.0	8.6	7.3	8.1	7.8[a]
Fumig. + Pt	8.6	16.8	17.0	9.9	13.1[b]
Fumig. + Tt	7.4	11.3	11.0	7.4	9.3[a]

* "b" is significantly different from "a" at 5% level.

Table 4. Height growth of *Pinus rigida* x *taeda* seedlings inoculated with *Pisolithus tinctorius* (Pt) and *Thelephora terrestris* (Tt) at the end of the first growing season.

Unit: cm

Treatment	Block				Mean*
	I	II	III	IV	
Fumigation	11.7	13.1	13.4	12.2	12.6[a]
Control	11.5	11.1	11.6	13.0	11.8[a]
Fumig. + Pt	13.1	13.3	18.8	19.7	16.2[b]
Fumig. + Tt	9.8	13.9	10.4	13.6	11.9[a]

* "b" is significantly different from "a" at 5% level.

Thelephora terrestris (Tt) failed to stimulate height growth in all the pine species except in *P. rigida* where about 20% of growth stimulation was observed with Tt treatment. However, it was not significantly different from control at 5% level.

Tables 5 and 6 show dry weight and T/R ratio

Table 5. Ovendry weight (D.W.) and top-root ratio (T/R) of *Pinus densiflora* seedlings inoculated with *Pisolithus tinctorius* (Pt) and *Thelephora terrestris* (Tt) at the end of the first growing season.

Unit of Dry Weight (D.W.): g

Treatment	Block								Mean*	
	I		II		III		IV			
	D.W.	T/R	D.W.	T/R	D.W.	T/R	D.W.	T/R	D.W.	T/R
Fumigation	0.77	1.20	0.33	1.35	1.10	1.56	0.60	1.14	0.80[a]	1.31[c]
Control	0.54	1.08	0.61	1.18	0.78	1.11	0.37	1.18	0.58[a]	1.14[c]
Fumig. + Pt	0.56	1.43	1.43	1.55	2.25	1.92	1.37	1.91	1.41[b]	1.70[d]
Fumig. + Tt	0.51	1.22	0.38	1.00	0.67	1.39	0.58	1.07	0.54[a]	1.17[c]

* "b" is significantly different from "a" and "d" is from "c" at 5% level.

Table 6. Ovendry weight (D.W.) and top-root ratio (T/R) of *pinus rigida* x *taeda* seedling inoculated with *Pisolithus tinctorius* (Pt) and *Thelephora terrestris* (Tt) at the end of the first growing season.

Unit of Dry Weight (D.W.): g

Treatment	Block								Mean*	
	I		II		III		IV			
	D.W.	T/R	D.W.	T/R	D.W.	T/R	D.W.	T/R	D.W.	T/R
Fumigation	1.15	1.88	1.78	2.71	1.95	2.82	1.63	2.26	1.63^a	2.42^c
Control	1.18	2.28	0.87	2.35	1.27	2.56	1.23	2.42	1.14^a	2.40^c
Fumig. + Pt	1.68	2.50	2.33	2.19	3.60	2.64	2.75	2.72	2.60^b	2.51^c
Fumig. + Tt	0.74	2.89	1.68	2.65	1.44	2.13	1.34	1.91	1.30^a	2.40^c

* "b" is significantly different from "a" at 5% level, and T/R is not different from each other.

(top-root ratio) of *P. densiflora* and *P. rigida* x *taeda*. The stimulation by Pt of seedling growth in terms of dry weight was more conspicuous than the stimulation expressed by height growth. Inoculation with Pt increased dry weight by 143% over control treatment in *Pinus densiflora* (1.41g *versus* 0.58g) and by 128% in *P. rigida* x *taeda* (2.60g *versus* 1.14g). The effect of Tt treatment was not seen in these tables. The T/R ratio in *P. densiflora* was significantly larger in Pt treatment than in other three treatments, while T/R ratio of *P. rigida* x *taeda* was not significantly changed by these treatments.

DISCUSSION

It was demonstrated in this experiment that seedling growth in the nursery was stimulated by manipulation of symbiosis between plant roots and soil fungi through artificial inoculation of plants with selected mycorrhizal fungi. The amount of growth stimulation by *Pisolithus tinctorius* (Pt) was up to 143% over uninoculated treatments (in dry weight basis). The same fungus tested in U.S.A. and other parts of the world showed similar stimulation in several pine species. For example, Pt stimulated the growth of *Pinus taeda* (dry matter production) by about two times (Marx and Bryan, 1975), fresh weight of same pine by 57% (Marx and Artman, 1978), and the growth of *P. virginiana* and *P. strobus* by 100% (Marx *et al.*, 1976).

By contrast with the remarkable growth stimulation by Pt, *Thelephora terrestris* (Tt) failed to stimulate seedling growth in this experiment due to the fact that Tt formed mycorrhizal roots in much less degree (40-60%) than Pt (80-95%). The poor performance of Tt was also observed in certain cases (Marx, 1980). Marx and Artman (1978) postulated that Pt which ecologically adapted to soils of low fertility was "probably more efficient than Tt in maximizing nutrient absorption from soil". In the present experiment, no fertilizer was applied during the experimental period and this fact could have enhanced the effect of Pt on seedling growth. However, Pt was also shown to stimulate plant growth in well-fertilized nursery soil (Marx, 1980).

Pisolithus tinctorius fungus has been reported to occur in Korea (Anonymous, 1978). The authors found large population of this fungus on sandy soil in Chilbo Mountain in Gyeonggi Province where soil erosion has been a serious problem for reforestation. The Pt strain #250 used in this experiment was originally isolated from eroded sites with sandy soil in U.S.A. When the above Pt (super strain) was cultured on the artificial medium (MMN), it grew much faster than Pt isolated from Chilbo Mt. in Korea, indicating that the Pt introduced from U.S.A. might be better source of inoculum than Korean Pt. The morphological characteristics of Korean Pt will be published later.

The irregular response of Pt among blocks, that is the stimulatory effect in block III and IV but less effect in the block I and II, may be explained by the difference in soil properties among blocks. It was found that blocks I and II contained more clay than

blocks III and IV. This resulted in poor drainage in blocks I and II and possibly poor aeration. However, the control seedlings in blocks I and II were as big as those in blocks III and IV. These two facts suggested that the effect of Pt was maximized when it was inoculated to well drained soil with high content of sand. This aspect of Pt needs further study.

The remarkable growth stimulation by artificial inoculation of pine seedlings with mycorrhizal fungi as shown in this experiment has opened up new horizon and will give enormous impact on Korean foresters who are very much concerned about reforestation on poor sites. Tree breeding is one of the ways to find best species which can adapt to this unfavorable condition. Another way of increasing survival and growth of trees on adverse sites is "tailoring" (Marx, 1980) of planting stocks through manipulation of symbiosis between plant roots and soil fungi as demonstrated in the present experiment.

Mycorrhizal research is expected to bring in fruitful results and benefits to not only forestry but also agronomy in near future. Endomycorrhizal research using agronomic crop plants has been very active in many parts of the world and is expected to develop new varieties of crops which require less amount of fertrilizer or can be grown in less fertile soils. Artificial inoculation with endomycorrhizal fungi have been reported to stimulate growth of many crop plants, such as cowpea (Islam and Ayanaba, 1981), cotton and finger millet (Bagyaraj and Manjunath, 1980; Pugh *et al.*, 1981), barley, lucerne and onion (Owusu-Bennoah and Mosse, 1979), white clover and ryegrass (Powell, 1979) and citrus (Timmer and Leyden, 1978). The authors suggest that research on endomycorrhiza of crop plants should be initiated to investigate the possibility of increase in crop yield by artificial inoculation of plants with selected mycorrhizal fungi.

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