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Effects of *Pisolithus tinctorius* Ectomycorrhizal Inoculation on *in vitro* Rooting of Tissue-Cultured *Quercus acutissima* Carr. and of Cutting of *Pinus densiflora* Sieb. et Zucc.[1*]

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ABSTRACT

We examined the *in vitro* rooting and survival of tissue cultured plantlets of *Quercus acutissima* Carr. and *Pinus densiflora* Sieb. et Zucc. after addition of *Pisolithus tinctorius* (Pt) ectomycorrhizal fungus inoculum to the medium and effects of three levels of sucrose and phosphorus in culture media. Shoots of *Quercus acutissima* were obtained from winter buds of a 30-year old tree and cuttings of *Pinus densiflora* from germinated seed, and they were inoculated with Pt *in vitro*. In both species, Pt enhanced shoot length, survival, number of adventitious roots, root length, and rooting percentage. Survival in *Quercus acutissima* was increased from 75% in control to 100% in Pt inoculation. Pt inoculation increased the percentage of rooting from 20% to 70% in *Quercus acutissima* cuttings and from 63% to 100% in *Pinus densiflora* cuttings. It is concluded that mycorrhizal inoculation to tissue cultured *Quercus acutissima* Carr. and to *in vitro* cutting of *Pinus densiflora* Sieb. et Zucc. has practical application to improvement of poor root development and initial period of reduced shoot growth *in vitro*.

Key words : *Rooting of cuttings*, *Survival*, *Mycorrhizal inoculation*, *Tissue culture*, Pisolithus tinctorius, Quercus acutissima, Pinus densiflora.

要 約

본 연구는 모래밭버섯 菌根菌(*Pisolithus tinctorius*)의 人工接種이 30年生 상수리나무와 소나무 組織培養苗의 줄기생장과 發根에 미치는 影響을 調査하여, 組織培養을 통한 營養增殖에 菌根菌의 潛在的 利用可能性을 把握하고자 遂行하였다. 상수리나무의 芽培養은 겨울철에 休眠狀態에 있는 30年生 個體木에서 採取한 休眠枝의 冬芽를 利用하여 實施하였고, 소나무의 器內插木은 種子에서 發芽한 實生苗를 利用하였다. 全般的으로 상수리나무와 소나무 모두 모래밭버섯 菌根菌의 接種으로 不定根의 數, 길이, 發根率, 生存率 등이 向上되었다. 소나무는 生存率이 100%로 處理間에 差異를 보이지 않았지만, 상수리나무에서는 接種區가 100%인 反面, 非接種區는 75%로 나타났다. 發根率에 있어서도 소나

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무 器內插木에서는 發根率이 非接種區에서 63%에 그친 反面에, 接種區는 100%로 나타났고, 상수리나무는 非接種區가 20%로 매우 낮은 데 比하여 接種區가 70%로 有意的인 菌根菌 接種效果가 나타났다. 따라서 器內 插木과 組織培養에 菌根菌을 應用함으로서, 發根誘導와 뿌리 發達 및 줄기 生長을 促進시킬 수 있을 것으로 期待된다.

INTRODUCTION

The methods of vegetative propagation, such as the rooting of cuttings and tissue culture, have been widely used to propagate and multiply the genetically uniform individuals which have superior phenotypes(Campbell and Durzan, 1976). In the difficult-to-root plants, root initiation is mainly controlled by the physiological conditions of cuttings (Hong, 1969). It is generally recognized that the physiological process in the adventitious root initiation is controlled by basipetally translocated auxin at cutting base(Hartig and Larson, 1980). This stimulating effect of the endogenous auxin can be also augmented by applying additional exogenous auxins such as IAA(indole-3-acetic acid), IBA(indole-3-butyric acid), or NAA(α-naphthalene acetic acid) to the stem base. However, there are some problems with the propagation by cuttings. In general, rooting ability decreases rapidly, as the age of the ortet increases(Girouard, 1974).

Tissue culture techniques provide an alternative to cutting for propagation of forest trees(Mott, 1981), but have also some limitations for massive propagation. Buds and shoots induced from tissue culture often fail to root(Grönroos and Arnold, 1985), and plantlets regenerated from adventitious buds experience an initial period of reduced growth under field conditions, compared with seedlings(Grönroos *et al.*, 1993). The main differences between plantlets and seedlings apparently could be related to a poorly developed root system, which contribute insufficiently to nutrient uptake, such as nitrogen and phosphorus(Mckeand and Allen, 1984). Probably, this may lead to increased mortality of plantlets, especially during the hardening process. It is another serious limiting factor in vegetative propagation.

Mycorrhizae, either naturally occurring or artificially introduced, improve the growth and development of host plants(Navratil and Rochon, 1981). Many workers reported that mycorrhizal fungi produced extracellular growth regulators(Slankis, 1973; Allen *et al.*, 1980), and mycorrhizal inoculation was applied to rooting of cuttings by several investigators(Linderman and Call, 1977; Nelson, 1987). Navratil and Rochon(1981) suggested that hormones produced by mycorrhizal fungi stimulated the processes of root formation, root and shoot elongation.

In this study, we hypothesized that *in vitro* mycorrhizal inoculation to the culture media could lead to stimulation of root formation through mycorrhizal production of plant hormones. Thus, objectives of this study were to determine the effects of mycorrhizal fungus, *Pisolithus tinctorius*(Pt), on enhancement of *in vitro* rootings of tissue-cultured shoots of *Quercus acutissima* Carr. and *Pinus densiflora* Sieb. et Zucc. and to understand the effect of sucrose and phosphorus level in the culture medium on efficiency of mycorrhizae in root initiation.

MATERIALS AND METHODS

Plant materials and *in vitro* bud culture of *Quercus acutissima*

In order to reduce the genetic variation commonly observed among the individual trees in *Quercus acutissima* Carr., we selected a 30-year-old tree growing in the Arboretum of Seoul National University located in Suwon, Kyonggi-do, Korea. Dormant branches were taken in January, 1991. They were surface sterilized by 7.5% sodium hypochlorite solution(w/v), washed for 2 minutes in 70% ethanol, and finally rinsed three times with sterile water(Grönroos and Arnold, 1985). They were incubated for 2 months under continuous light(6,000 lux) at 26℃ in a growth chamber. Fully open buds were gently removed on the branches with a forcep. They were surface sterilized and placed on shoot induction medium in a jar. The shoot induction medium used here was WPM(Woody Plant

Medium ; Lloyd and McCown, 1980) containing growth regulators, NAA and BAP(6-benzyl aminopurine) at the concentrations of 0.01mg/l and 0.5mg/l, repectively. The expanding shoots from the buds were excised about 4cm in length and subcultured at 3 to 4 weeks of interval until use.

Isolation and culture of Pt mycelium

Fruiting bodies of Pt(*Pisolithus tinctorius*) ectomycorrhizal fungus were collected from *Cedrus deodora* stands located in Yaeum-dong, Woolsan, Kyongsangnam-do, Korea in July, 1990. Fungal tissue was axenically isolated from fruiting bodies and placed on 1.5% MMN agar medium(Modified Melin and Norkran's ; Marx and Bryan, 1975) in petri-dishes. They were cultured at 28℃ in a incubator for 3 to 4 weeks.

Rooting experiment with *Quercus acutissima* plantlets

Rooting media of *Quercus acutissima* consisted of a mixture of perlite : vermiculite : peatmoss in the ratio of 1 : 1 : 1(v/v/v) and also contained WPM liquid medium with the different concentration of sucrose(2%, 1%, and 0%) and phosphorus(170mg/l, 85mg/l, and 17mg/l). Excised shoots of *Quercus acutissima* were asepitically placed on the rooting medium in a jar. The length of shoots was approximately 2cm. After 5 days of incubation, only the uncontaminated shoots were used in this experiment. Pt inoculum was prepared by cutting out an agar plug(1cm^2 in surface area) with Pt mycelia and transferring to a jar with *Quercus acutissima* shoots. They were cultured under continuous light(6,000 lux) at 26℃ during the day and 22℃ at night for 3 months in a tissue culture room. Shoots were observed for the root and shoot development in response to the different concentrations of sucrose and phosphorus with and without Pt inoculation.

***In vitro* cutting of *Pinus densiflora* seedlings**

Seeds of Korean red pine, *Pinus densiflora* Sieb. et Zucc.(Kangwon 10) collected from a seed orchard were obtained from the Forestry Genetic Research Institute located in Suwon, Kyonggi-do, Korea. They were surface sterilized, washed for 2 minutes in 70% ethanol, and rinsed 10 times with sterile water. Seeds were germinated in a jar, half filled with vermiculite with WPM liquid medium. At the same time, large test tubes(20cm long) filled with 80 cc of vermiculite and 30cc of peatmoss moistened with WPM medium and 0.2ppm IBA were autoclaved, inoculated with Pt, and incubated for 4 weeks until mycelium reached the bottom of tube. To prepare cuttings, roots of 4-week-old seedlings in above mentioned jars were severed, and cuttings were placed in the Pt-inoculated test tubes with and without sucrose. They were rooted under continuous light(6,000 lux) at 26℃ during the day and 22℃ at night in a tissue culture room.

Measurements

The plantlets were carefully removed from the medium after three months of incubation and recorded for survival and the number and length of adventitious main and lateral roots.

The roots of *Pinus densiflora* were fixed with FAA (Formalin-Acetic Acid-Alcohol) and stained with Acid Fuchsin solution(Ko and Lee, 1990). Mycorrhizal infection was examined by observing the Hartig net and fungal mantles under a microscope after cross-sectioning the roots with a hand microtome. Percent infection was determined by the numLer of infected roots per unit number of root.

Experimental design and statistics

A completely randomized design with 20 plantlets or cuttings per treatment were used. The treatments consisted of following : (1)Effect of three levels of sucrose concentrations(2%, 1%, and 0%) in the medium, (2)Three levels of phosphorus(170mg/l, 85 mg/l, and 17mg/l), and (3)Effect of Pt inoculation for *Quercus acutissima*. For *Pinus densiflora*, only effect of Pt inoculation was tested. A test tube had one plantlet or cutting and randomly arranged in a tissue culture room and a incubator. All analyses were performed using the general linear model (GLM) procedure in the Statistical Analysis System (SAS Institute, 1986). This statistical model accounts for differences among plantlets or seedlings within species. The effect of treatments were detected by Duncan's new multiple range test at 5% level of significance. This test controls the type I comparisonwise error rate, but not the experimentwise error rate.

RESULTS AND DISCUSSION

Shoot growth and survival of *Quercus acutissima* plantlets

Tables 1 and 2 show effects of mycorrhizal inoculation on shoot growth of *Quercus acutissima* plantlets produced by bud culture. Length of shoots was significantly greater in the Pt(*Pisolithus tinctorius*) treatments than in the control. For example, shoot growth was 1.9 times($P<0.05$) greater in the Pt treatment than in the control, when 2% sucrose was employed (Table 1). When it was considered that basal concentrations of sucrose in WPM medium are 2%(Lloyd and McCown, 1980), effects of the addition of Pt inoculum into culture medium were obvious. Shoot growth relative to initial length had 40% and 100% greater in the treatment of 85mg/l and 170 mg/l of phosphorus with Pt inoculum, respectively, compared to those in control treated with 170mg/l phosphorus only(Table 2). In general, mycorrhizal inoculation had little effects on plant growth under the rich condition with phosphorus nutrition(Lee *et al.*, 1983). However, phosphorus nutrition below basal concentration in WPM medium reduced the effects of Pt inoculation on shoot growth.

Survival of plantlets during *in vitro* culture was significantly high in the treatment of Pt inoculum (Table 1). The lower level of sucrose resulted in smaller percentages of survival. The decrease in survival was accompanied by necrosis of leaves and caused by increasing mortality due to the lack of sucrose in the culture medium during the culture. This result was similar to that of Moon *et al.* (1987), who observed that the lower level of sucrose resulted in more necrosis of tissue in bud culture of *Quercus acutissima*.

In spite of increasing mortality following lower nutrition of sucrose, Pt inoculation enhanced survival of plantlets during *in vitro* culture. For example, the percentage of survival showed 25% more at 2% sucrose treatment with Pt inoculum than that in control treated with 2% sucrose but without Pt. Furthermore, at 1% sucrose treatment, Pt inocula-

Table 1. Effects of sucrose concentration in culture medium and Pt(*Pisolithus tinctorius*) mycorrhizal inoculation on shoot growth of *Quercus acutissima* plantlets produced by bud culture.

Treatment		Initial shoot length (cm)	Final shoot length (cm)	Increase in shoot length (%)	Survival rate (%)
Mycorrhizal inoculation	Sucrose concentration in medium(%)				
Inoculated	2.0	2.2	3.3	50.0a*	100
	1.0	2.1	3.0	42.4a	90
	0.0	2.4	2.7	12.5c	55
Un-inoculated	2.0	1.9	2.4	26.3b	75

* The same letters in a column are not significantly different at 5% level by Duncan's new multiple range test.

Table 2. Effects of phosphorus concentration in WPM* medium and mycorrhizal *Pisolithus tinctorius* inoculation on shoot growth of *Quercus acutissima* plantlets produced by bud culture.

Treatment		Initial shoot length (cm)	Final shoot length (cm)	Increase in shoot length (%)	Survival rate (%)
Mycorrhizal inoculation	Phosphorus concentration in medium(mg/l)				
Inoculated	170	2.1	3.1	47.6a**	100
	85	2.2	2.9	31.8a	100
	17	2.0	2.5	25.0b	100
Un-inoculated	170	1.9	2.4	26.3b	100

* WPM contains 170mg/l of phosphorus.

** The same letters in a column are not significantly different at 5% level by Duncan's new multiple range test.

tion increased the percentage of survival by 15% more than that of control.

Carbohydrates are essential for the mycelial growth of mycorrhizal fungi in pure culture(Marx, 1975) and for plantlets *in vitro* culture. In this experiment, sucrose was only source of carbohydrates for plantlets and Pt fungus. Competition for limiting nutrient, sucrose, between plantlets and Pt fungus was expected under such condition. Nevertheless, Pt inoculation enhanced shoot growth, survival of shoots and decreased the mortality of plantlets.

Rooting response of *Quercus acutissima* plantlets

In rooting experiment of *Quercus acutissima* plantlets, GD medium containing 0.2ppm IBA was used as basal rooting medium. And there were no limitation of nutrition levels for any nutrients. Plantlets were the same ones used in the above two experiments.

The results of Pt inoculation on the rooting response of plantlets in *Quercus acutissima* are shown in Table 3. The addition of Pt inoculum enhanced the shoot growth. Shoot length in Pt treatment had approximately 2.5 times greater than control treatment without Pt inoculum. And also, Pt inoculation enhanced the percentage of survival. The treatment with Pt inoculum showed 100% of survival, but only 80% in control.

Significant differences in number of lateral roots and total length of adventitious roots did exist between the treatments(Table 3). The addition of Pt mycelium to the rooting medium increased both the number and length of adventitious roots by about two times compared with the control. This enhancement of root development of inoculated plantlets was consistent with the observation of Kim and Lee (1990), who demonstrated that Pt inoculation to rooting medium enhanced the root development of cuttings in *Quercus acutissima* Carr. The percentages of rooting of plantlets were also significantly higher at the treatment with Pt inoculum(70%) than that of the control(20%). These results suggested that Pt inoculation to rooting medium in tissue culture *in vitro* promoted rooting of plantlets, when 0.2 ppm IBA were applied to the rooting medium. In addition, Pt inoculation to growth medium stimulated the shoot and root development of *Quercus acutissima* plantlets. These were supported by the observation that the adventitious roots of plantlets with Pt mycelium were highly infected by Pt fungi and had the fungal mantles and the Hartig nets.

Rooting response of *in vitro* cuttings in *Pinus densiflora*

This experiment was attempted to understand that inoculation with Pt was applicable to *in vitro* cutting of difficult-to-root species. In this experiment, cuttings were derived from germinated seedlings of *Pinus densiflora*.

Addition of Pt inoculum to the rooting medium increased the development of adventitious roots *in vitro* cutting of *Pinus densiflora*(Table 4). In order

Table 3. Effects of mycorrhizal(*Pisolithus tinctorius*) inoculation on rooting of tissue cultured *Quercus acutissima* shoots and plantlets under hardening process.

Type of plant material	Mycorrhizal treatment	Initial shoot length (cm)	Final shoot length (cm)	Increase in shoot length (%)	Number of roots formed	Total length of roots (cm)	Rooting (%)	Survival (%)
Unrooted shoots on GD medium	Un-inoculated	1.9a	2.3b	21.1b	1.2b	2.9b	20	80
	Inoculated	2.0a	3.0a	50.0a	2.a8	6.2a	70	100
Rooted plantlets under hardening process	Un-inoculated	3.3a	4.9a	48.5a	2.5b	Not measured	100	100
	Inoculated	3.2a	4.8a	50.0a	3.4a	Not measured	100	100

* The same letters in a column at the same type of plant material are not significantly different at 5% level by Duncan's new multiple range test.

Table 4. Effects of sucrose addition to the medium and mycorrhizal (*Pisolithus tinctorius*) inoculation on *in vitro* rooting and survival of germinated *Pinus densiflora* seedlings whose roots were previously severed.

Growth characteristics	Treatments			
	Sucrose omitted		Sucrose added	
	Uninoculated	Inoculated	Uninoculated	Inoculated
Seedling height (cm)	4.9a*	5.1a	3.9b	4.8a
Number of adventitious roots	1.2a	1.3a	1.4a	1.4a
Length of adventitious roots (cm)	2.8c	5.5b	4.9bc	11.5a
Number of fine roots	0.0b	1.9b	0.6b	9.8a
Rooting (%)	62	88	63	100
Hyphal growth	None	LHG	None	EHG
Mycorrhizal formation (%)	0.0	0.0	0.0	3.0

* The same letters in a row are not significantly different at 5% level by Duncan's new multiple range test.

LHG : Limited hyphal growth on medium surface only.

EHG : Extensive hyphal growth down to the medium.

to evaluate the effects of Pt inoculation on root development, the mycelial growth of Pt fungus was regulated by adding 2% sucrose (standard) or omitting sucrose (control treatment) in this experiment. The length of adventitious roots (LAR) was doubled by Pt inoculation regardless of sucrose addition, while number of adventitious roots was not affected by Pt treatment. Sucrose addition stimulated formation of fine roots when Pt was inoculated. Statistical significances between the treatments with and without Pt inoculation for each LAR and number of fine roots (NFR) did exist at 5% level. The effect of sucrose addition on root development was observed in this experiment. Sucrose addition without Pt inoculum increased both LAR and NFR. This result was consistent with the results reported by Greenwood and Berlyn (1973). They postulated that sucrose was essential for the development of roots *in vitro*.

Unlikely to LAR and NFR, the percentages of rooting were not different between the two sucrose treatments in the absence of Pt inoculation (Table 4). Leroux (1973) reported that sucrose had most efficient effects among other carbohydrates in stimulating root formation, especially in dark grown stem cuttings. In our experiment, cuttings were grown under the relatively low light intensity (about 6,000 lux). However, in rooting reponse of *Pinus densiflora* cuttings *in vitro*, there was no difference between the two levels of sucrose. This result was similar to that of Moon *et al.* (1987), who reported that percentages of rooting of cuttings of *Quercus acutissima in vitro* were not affected by the level of sucrose.

Contrast to the rooting in the absence of Pt inoculum, Pt addition increased the percentage of rooting (Table 4). The percentage of rooting was enhanced by 37% in the presence of Pt. Futhermore, Pt inoculation in the absence of sucrose enhanced the rooting by 25% than in the treatment with sucrose. Probably, this may be resulted from the exudates from Pt mycelia in rooting medium, where mycorrhizal infection were not observed (in 1% sucrose treatment) or extremely low (3% in 2% sucrose treatment). But, the higher level of sucrose resulted in the greater mycelial growth. Therefore, above results may be related to the possibility that poor occupation of mycelia in substrates influences the activation and concentration of exudates from mycorrhizal fungi. Kim (1990) reported that the same ectomycorrhizal fungus, Pt, exudes growth regulators (IAA and GA_3) in rooting medium. And also, it is likely that enhancement of rooting response by Pt inoculation may be related to another unknown compounds produced by Pt fungus or synergistic effects, such as both the hormonal liberation and enzymatic activation during the mycelial growth and mycorrhizal formation in culture medium (Smith and Gianinazzi-Pearson, 1988).

Before our experiments, we considered the possi-

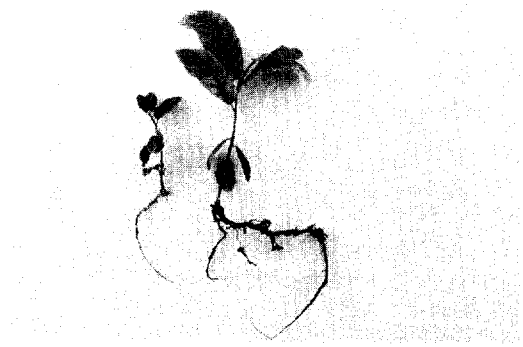

Fig. 1. Effects of ectomycorrhizal *Pisolithus tinctorius* inoculation on *in vitro* rooting of tissue-cultured shoots of *Quercus acutissima*. (Left : No-inoculation, Right : Inoculation)

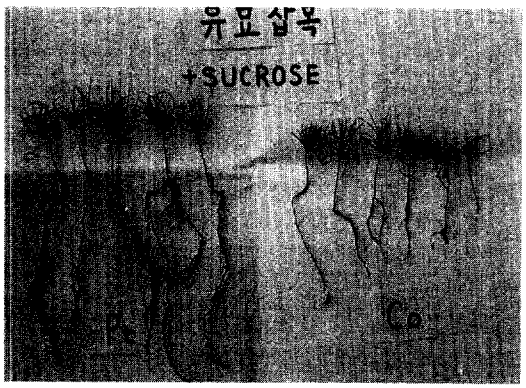

Fig. 2. Effects of ectomycorrhizal *Pisolithus tinctorius* inoculation on adventitious root formation of *in vitro* germinated *Pinus densiflora* cuttings.
(Pt : Inoculation, Co : No-inoculation)

bility that the effects of Pt inoculation on rooting might not be apparent, due to reliance of Pt to sucrose for its energy, rather than to host plants. However, our experiments suggested additional considerations that mycorrhizal application to tissue culture and cutting *in vitro* requires sufficient nutrition of sucrose and phosphorus. Sucrose level was critical to mycelial growth of mycorrhizal fungi *in vitro*. Survival percentages of plantlets were related to the degree of mycelial occupation in substrates and the effect of Pt addition was proportionally reduced by lower level of sucrose.

On the other hand, the effects of Pt inoculation on shoot growth of tissue culture plantlets in *Quercus acutissima* were dependent on the level of phosphorus, rather than that of sucrose. Levels of phosphorus below basal concentration in WPM medium hide the mycorrhizal effects. Thus, more studies are necessary for mycorrhizal application to tissue culture and cutting *in vitro*. Studies on the optimal levels of sucrose and phosphorus for mycelial growth of symbiotic mycorrhizal fungi, survival of plantlets, and determination of mycorrhizal effects in tissue culture of *Quercus acutissima* are recommended. In addition, rooting response to mycorrhizal inoculation following the different levels of sucrose and phosphorus treatment may provide good information for mycorrhizal application to tissue culture and cutting *in vitro* for mass propagation. Futher studies related to hormonal liberation and enzymatic activation during mycelial growth and mycorrhizal formation in rooting medium are needed to confirm the promotion of rooting by Pt itself.

CONCLUSION

Main objective of this study was to determine the effect of *Pisolithus tinctorius* inoculation on shoot growth and rooting of plantlets cultured *in vitro* in *Quercus acutissima* Carr. and *Pinus densiflora* Sieb. et Zucc. The observed enhancement of shoot growth and root development by the addition of mycorrhizal inoculum was strongly evident. There were some variations between the species and among the level of sucrose in each species. However, addition of Pt inoculum to rooting medium enhanced the shoot growth, especially in *Quercus acutissima* plantlets. And also, Pt inoculation stimulated rooting response of tissue culture plantlets in *Quercus acutissima* and of cuttings in *Pinus densiflora in vitro*. These results suggested that the Pt addition to the rooting medium in the tissue culture enhanced initiation of adventitious roots and stimulated the growth of roots, regardless of successful mycorrhizal formation. Thus, it is concluded that mycorrhizal inoculation to rooting medium in tissue culture and *in vitro* cutting of *Quercus acutissima* Carr. and *Pinus densiflora* Sieb. et Zucc. has potential benefits to improvement of poorly developed root system and initial period of reduced growth of plantlets produced from *in vitro*

tissue culture. Mycorrhizal application to *in vitro* tissue culture and cutting is recommendable and also more study is needed.

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