

Investigation of Ectomycorrhizal Fungal Colonization in *Pinus thunbergii* Seedlings at a Plantation Area in Gangneung, using Morphotyping and Sequencing the rDNA Internal Transcribed Spacer Region

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Abstract : The status of ectomycorrhizal (ECM) fungal colonization in *Pinus thunbergii* seedlings was investigated 2 years after planting in an eastern coastal area of Korea. We established three 10×10 m plots at a *P. thunbergii* plantation in Gangneung and sampled lateral roots from 10 seedlings in each plot. ECMs were classified into morphological groups and the number of root tips of each morphotype was counted. In total, 8 ECM morphotypes were observed and fungal species that form each morphotype were identified by sequencing of the internal transcribed spacer (ITS) region of the nuclear rDNA. *Suillus granulatus* was the most abundant species (44.1-65.7% of relative abundance) in all plots, followed by *Tomentella ellisii* (14.0-37.8%) and unidentified fungus belonged to Atheliaceae (10.6-20.1%). These 3 fungal species accounted for almost all of the ECM abundance in each plot (94.9-99.8%). The remaining 5 fungal species were uncommon and rare. There was no clear difference in ECM fungal communities among plots. Community structure of ECM fungi in the young *P. thunbergii* plantation was simple and composed of fungal species that were also observed in mature coastal pine forests.

Key words : Ectomycorrhizal fungi, *Pinus thunbergii*, plantation, Seedling, *Suillus*

Introduction

Coastal forests carry out significant functions for human activity such as alleviating damage from salt and sand dispersion to inland areas and providing place for recreation (Konta, 2001). *Pinus thunbergii* Parl., which grows naturally in Korea, has excellent abilities to tolerate high salinity and drought stress and grows well in the coastal areas. Therefore, *P. thunbergii* has been used for afforestation on many coastal dunes (Lee *et al.*, 2005) and became one of the major constituents of the coastal forests in Korea (Choi, 1986; Park *et al.*, 2002). Recently, however, much coastal pine forests have been cut down and have not maintained properly due to the recent increase of the developments for tourism or military purposes. In recent years, several studies related to afforestation such as recovery and maintenance of coastal pine forest were started in Korea (Chun *et al.*, 2008).

Mycorrhizal associations are common in terrestrial

forest ecosystems and play a significant role in plant establishment including promoting nutrient and water uptake of host plants and also enhancement of tolerance towards stressful situations encountered by their hosts (Smith and Read, 1997). Therefore, the status of mycorrhizal colonization in planted seedlings is one of the important factors for their successful establishment. It is known that the effect of ectomycorrhizal (ECM) associations on host plant growth differs among different species of ECM fungi (Baxter and Dighton, 2001). Therefore, it is important to clarify what kinds of fungal species comprise a given community in plantation sites.

It is known that ECM fungi extensively colonize the fine roots of *P. thunbergii*. Several studies have been done to describe the ECM fungal community in *P. thunbergii* in coastal pine forests using sporocarps survey and/or molecular tools. Recorded various ECM fungal partners include basidiomycetes *Amanita*, *Laccaria*, *Lactarius*, *Inocybe*, *Pisolithus*, *Rhizopogon*, *Russula*, *Suillus*, *Tomentella* and ascomycetes *Cenococcum geophilum* Fr. (Taniguchi *et al.*, 2007; Matsuda *et al.*, 2009; Obase *et al.*, 2009a). However, there are no reports on the status of ECM fungal colonization

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in *P. thunbergii* plantation in coastal areas.

In this study, we investigated the status of ECM fungal colonization in *P. thunbergii* seedlings at a plantation area in Gangneung, Korea. ECMs were classified into morphological groups and the number of root tips of each morphotype was counted. Fungal species were identified from ECM root tips by sequencing the rDNA internal transcribed spacer (ITS) region.

Materials and Methods

1. Study site

For developing diversity-rich coastal forests that provide significant functions such as prevention of sand and salt dispersion, scenic values and recreation, a plantation was established in Gangneung, Korea (37° 23' N, 129° 13' E). Wood fences were constructed along the coast from a distance of about 40 m from shorelines for protecting seedlings against strong wind from sea and promoting of initial seedling growth. At an inland side from the fences, 3600 *P. thunbergii*, 2400 *Rosa rugosa* Thumb., 700 *Chionanthus retusus* Lindl. et Paxton, 900 *Koelreuteria paniculata* Laxm. and 700 *Vitex rotundifolia* L. seedlings were planted at interval of 1 m in 0.9 ha area in 2006. Seedling age of *P. thunbergii* was 6 when they were planted in the plantation site. A preliminary study revealed that only *P. thunbergii* seedlings grew well and other plants frequently died or showed symptoms of poor growth. Plant height and stem diameter (at the ground level) of *P. thunbergii* seedlings (377 individuals) were 136.7±21.1 and 2.6±0.6 cm in average ± standard deviation in 2008, respectively. The soil consisted entirely of maritime sand and shallow inland soils and has no litter and humus layers. In 2008, climatic data for Gangneung from the Korea Meteorological Administration indicated annual precipitation of 1343 mm, ranging between 0 and 330.6 mm (February and August, respectively) and annual temperature of 14.0°C, ranging between 1.1 and 26.7°C (January and July, respectively).

To understand ectomycorrhizal (ECM) fungal community in the plantation, we established three 10×10 m plots, parallel to fences and at intervals of c.a. 50 m. The plant community of this plantation includes herbaceous plants such as *Cassia nomame* (Sieb.) Honda, *Phragmites australis* (Cav.) Trin. ex Steud., *Artemisia* spp. and a fern, *Equisetum arvense* L. We sampled three soil cores (5 cm in diameter and 5 cm in depth) from each plot in September 2008 and determined soil water content, averaged 5.1 to 5.5% (w/w).

2. Sampling methods and observation of ECM colonization

We sampled lateral roots that were included in soil

blocks (within a radius of 30-40 cm from a stem and 15-20 cm in depth) from 10 seedlings in each plot. All samples were stored in plastic bags at 4°C until further analysis. Roots were separated from adhering soil by soaking and washing carefully with tap water using 0.5 mm sieve. All root segments were viewed under dissecting microscope after cutting into 5-10 cm segments. Viability of ECMs was interpreted based on color, texture of surface and degree of development of mantle layer. We excluded non-vital ECMs that looked shrunken, discolored and brittle to a piece. ECMs from each sample were classified into morphological groups based on such as ramification system, color, shape, texture, organization and abundance of emanating hyphae or cystidia, rhizomorphs and hyphal arrangement of mantle surface according to Agerer (1995) and Ingleby *et al.* (1990).

3. Identification of ECM fungal species

ECM fungal DNA was extracted from one ECM root tip of each morphotype using the DNeasy Plant Mini kit (QIAGEN, USA) according to the manufacturer's instructions. DNA amplification of the ITS regions, including the 5.8S rDNA, was performed on My Cycler thermal-cycler (BioRad, USA) by using Takara Ex Taq (TAKARA, Japan) with a specific primer for higher fungi ITS1-f (Gardes and Bruns, 1993) and basidiomycete ITS4b (Gardes and Bruns, 1993). We used following PCR amplification conditions; 94°C (3 min), followed by 30 cycles of 94°C (30 s), 50°C (30 s) and 72°C (2 min), and final hold at 72°C (10 min). We determined the quality and quantity of the PCR products by agarose gel electrophoresis using QA-Agarose TM (MP Biomedicals, USA).

Each PCR product arising from different ECM morphotypes was purified using the QIA quick PCR purification kit (QIAGEN, USA) according to the manufacturer's instructions, and sequenced with primer ITS1f and ITS4. Sequencing reactions were entrusted to Solgent Co., Ltd. (Daejeon, South Korea). Fungal sequences were compared with the GenBank database at the National Center for Biotechnology Information (NCBI: <http://www.ncbi.nlm.nih.gov>) and UNITE (<http://unite.ut.ee>), using the nucleotide-nucleotide basic local alignment search tool algorithm (BLAST program). Sequences were aligned with closely matched reference sequences and submitted to neighbor-joining analysis at NCBI for taxonomic interpretation at the species, genus or family level. Sequences were considered to be identified at the species level when more than 97% identities with reference sequences derived from sporocarps were obtained.

4. Estimation of mycorrhizal colonization

ECM abundance indicating the number of ECM root tips of each morphotype was recorded separately for

each sample. Relative ECM abundance was calculated as an average value of a ratio of abundance of a given morphotype to total ECM abundance present in a given sample. The frequencies of ECM colonization that indicated the number of samples that contained a given ECM morphotype were also recorded.

Species-accumulation curves were drawn by plotting the mean of the accumulated number of expected species in pooled samples after 50 randomizations without replacement using EstimateS program version 8.0 (Colwell, 2005) for estimating whether sampling effort was enough to describe ECM fungal flora in plots. Estimated measurement of ECM fungal species richness using bootstrap, Jackknife 1 and Jackknife 2 estimators and similarity of fungal flora using Morishita-Horn index between plots were calculated by using EstimateS program version 8.0 (Colwell, 2005). ECM fungal diversity (Shannon's and Simpson's index) was also estimated using EstimateS program version 8.0 (Colwell, 2005).

Results

1. Identification of ECM fungi

We observed 7228 ectomycorrhizal (ECM) root tips (range 1628-2923 root tips in a plot) and classified them into 8 groups by morphological characteristics (Table 1, Figure 1). Each morphotype was formed by different ECM fungal species (Table 1). We identified 4 (*Suillus*

granulatus (L.) Roussel, *Suillus luteus* (L.: Fr.) S.F. Gray, *Thelephora terrestris* Fr. and *Tomentella ellisii* (Sacc.) Jülich & Stalpers) and 3 fungal species (*Inocybe* sp., *Tomentella* sp. 1 and *Tomentella* sp. 2) to species and genus level by matching sequences with those of known sporocarps. One fungal species could not be matched with sequences of known sporocarps but matched with those of estimated fungal species in other reports; it could be identified to the family level (Atheliaceae).

2. Status of ectomycorrhizal colonization

All samples contained ECMs in almost all vital root tips. The number of fungal species observed in each plot ranged 4 to 6 (Table 2). Average species richness per sampling ranged 2.6-3.3 and did not differ among plots. Total estimated species richness by bootstrap, Jackknife 1 and Jackknife 2 estimators were 9.3, 10.9 and 12.8, respectively. The species accumulation curve for ECM fungi was ascending, indicating that the probability of detection of rare fungal species is still high as sample size increases (Figure 2). Three fungal species (*S. granulatus*, *T. ellisii* and unidentified species belonged to Atheliaceae) were common among plots. These three common species accounted for 94.9 to 99.8% of total abundance of ECMs in each plot. Similarity of species composition of ECM fungi between plots were 0.88 to 0.97 using the Morishita-Horn index.

Table 1. Morphological characteristics and possible identities of ectomycorrhizal (ECM) fungal species that formed each ECM type observed in the roots of *Pinus thunbergii* seedlings in a plantation in a coastal area in Gangneung, Korea.

Possible identity (Accession No. of the highest similarity)	Morphological characteristics of an ECM				
	Color	Shape	Texture	Mantle (outer layer)	Emanating hyphae
<i>Inocybe</i> sp. EU554696 (556/557; 99%)	Pale yellow to yellow	Unbranched to forked-branched	Smooth	Net synenchyma	Absent to infrequent
<i>Suillus granulatus</i> AB284447 (594/597; 99%)	White and partially brown	Monopodial-pinnate to dichotomous	Densely wooly	Net synenchyma	Abundant, rhizomorphs were infrequently observed
<i>Suillus luteus</i> DQ068969 (616/616; 100%)	Reddish brown	Dichotomous	Smooth and shiny	nd *	Infrequent
<i>Thelephora terrestris</i> FN393122 (619/625; 99%)	Pale brown	Unbranched to dichotomous	Smooth	Net synenchyma	Absent to infrequent
<i>Tomentella ellisii</i> FJ013069 (604/606; 99%)	Pale yellow to yellow	Unbranched to forked-branched	Loosely wooly	Net synenchyma	Absent to infrequent
<i>Tomentella</i> sp. 1 EF619788 (564/587; 96%)	Reddish brown	Unbranched to dichotomous	Smooth and shiny	Non interlocking irregular synen- chyma	Absent
<i>Tomentella</i> sp. 2 AB253522 (544/546; 99%)	White (very tip) to brown (older part)	Dichotomous	Smooth	Net synenchyma	Infrequent
Unidentified species belonged to Atheliaceae AB253524 (531/533; 99%)	Pale yellow to yellow	Unbranched to dichotomous	Cottony	Net synenchyma	Abundant

*nd; could not determined.

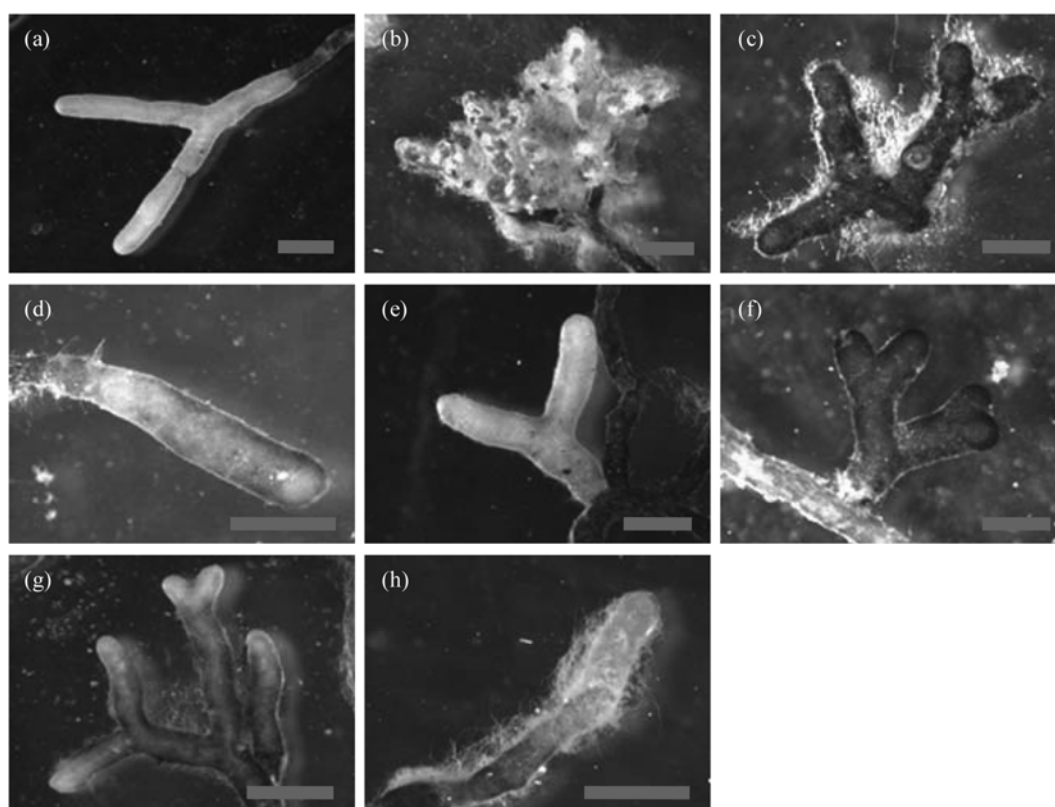


Figure 1. Appearance of ectomycorrhizas observed in the roots of *Pinus thunbergii* seedlings in a coastal plantation area in Gangneung, Korea; (a) *Inocybe* sp., (b) *Suillus granulatus*, (c) *Suillus luteus*, (d) *Thelephora terrestris*, (e) *Tomentella ellisii*, (f) *Tomentella* sp. 1, (g) *Tomentella* sp. 2, (h) unidentified species belonged to Atheliaceae. Bars indicated 1 mm.

Table 2 Relative abundance (RA) and frequencies (F) of each ectomycorrhizal (ECM) fungi in roots of *Pinus thunbergii* seedlings in a plantation in a coastal area in Gangneung, Korea.

ECM fungal species	Plot 1		Plot 2		Plot 3		Total	
	RA (%)	F (/10)	RA (%)	F (/10)	RA (%)	F (/10)	RA (%)	F (/30)
<i>Suillus granulatus</i>	44.1	9	65.7	8	58.5	10	54.3	27
<i>Tomentella ellisii</i>	37.8	10	14.0	9	25.8	10	28.0	29
Unidentified species belonged to Atheliaceae	16.4	10	20.1	8	10.6	9	15.1	27
<i>Tomentella</i> sp. 1					3.8	2	1.4	2
<i>Tomentella</i> sp. 2	1.5	2	0.2	1			0.7	3
<i>Thelephora terrestris</i>					1.3	1	0.5	1
<i>Inocybe</i> sp.	0.2	1					0.1	1
<i>Suillus luteus</i>	0.1	1					<0.1	1
Total No. ECM root tips observed	2923		1628		2677		7228	
Mean No. species per sampling*	3.3±0.5		2.6±0.7		3.2±0.4		3.0±0.6	
Observed species richness	6		4		5		8	
Mean estimated species richness								
Bootstrap	6.8		4.4		5.5		9.3	
Jackknife 1	7.8		4.9		5.9		10.9	
Jackknife 2	8.7		5.7		6.0		12.8	
Shannon's index	1.1		0.9		1.1		1.1	
Simpson's index	2.8		2.0		2.4		2.5	

*Averages and standard deviations were represented.

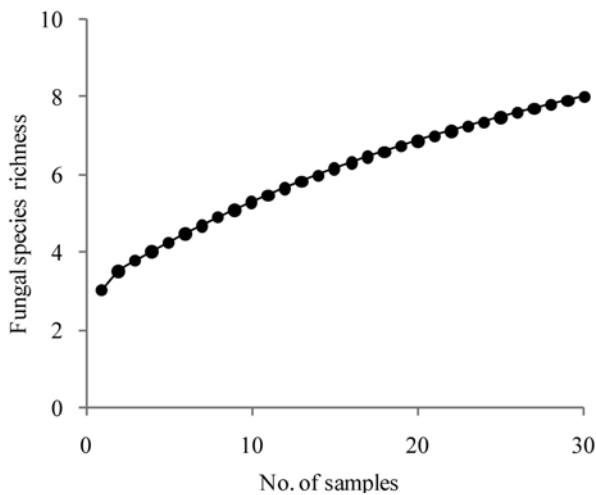


Figure 2. Species-accumulation curve for ectomycorrhizal fungi observed in roots of *Pinus thunbergii* seedlings in a plantation in a coastal area in Gangneung, Korea.

Suillus granulatus was the most abundant ECM fungal species observed in all the plots and accounted for 44.1 to 65.7% in relative abundance. *T. ellisii* and unidentified species belonged to Atheliaceae were the second or third most abundant fungal species and accounted for 14.0 to 37.8% and 10.6 to 20.1% in relative abundance, respectively. These 3 fungal species were observed in 27 to 29 out of 30 soil samples. The remaining 5 fungal species were uncommon and showed low relative abundance (less than 0.1 to 3.8%) and frequencies (1 to 3 out of 30 soil samples).

Discussion

We investigated abundances and frequencies of ectomycorrhizal (ECM) fungi colonized roots of *P. thunbergii* seedlings planted in an eastern coastal area and showed ECM fungal communities. However, it appeared that sampling efforts such as number of seedlings observed and replicated sampling locations was not enough to clarify ECM fungal community in *P. thunbergii* plantations; it means our results are relevant only to this particular locations and cannot be extrapolated to *P. thunbergii* plantations in general. Further investigations involving more intense sampling efforts are needed for obtaining general information about ECM fungal community in *P. thunbergii* plantations.

Our results showed a species-poor and simple ECM fungal community in the *P. thunbergii* plantation. No more than 3 ECM fungal species frequently colonized seedlings and occupied in the range of 10.6 to 65.7% in relative abundance, and the remaining fungal species were detected from the samples of small amount of root tips. Few dominant fungi and the majority of rare fungi in species richness is a common pattern as described in

previous surveys conducted in several forest ecosystems (Jonsson *et al.*, 1999; Matsuda and Hijii, 2004; Valentine *et al.*, 2004). *Suillus granulatus* was the most dominant in abundance and frequency in the plantation. This fungus is possibly one of the most widespread species of *Suillus* associated with pine species in Asia (Yamada and Katsuya, 1995; Sims *et al.*, 1997; Dunstan *et al.*, 1998), Europe (Pera and Alvarez, 1995; Manian *et al.*, 2001; Wiemken and Boller, 2006), America (Jacobson *et al.*, 1993; Kretzer *et al.*, 1996) and South Africa (Dunstan *et al.*, 1998). This fungus has also been observed in *P. thunbergii* coastal forests in Japan (Taniguchi *et al.*, 2007) and Korea (Obase *et al.*, 2009a). Sporocarps of this fungus often observed in mature and young pine stands. Cullings *et al.* (2003) showed dominance of this fungus in roots of young pine stands that had undergone stand-replacing fire and as a result, has very little resident litter and organic matter. Wiemken and Boller (2006) also showed dominance of this fungus in young pine trees established on edge of forests and grassland. Obase *et al.* (2009a) detected this fungus in roots of 0-10 year old *P. thunbergii* seedlings and also c.a. 50 year old mature trees in coastal pine forests. It appears that this fungus has characteristics as an early stage fungus and is able to colonize young *P. thunbergii* plantations.

Tomentella ellisii was the second dominant species. Recent reports showed that *Tomentella* spp. were common ECM symbionts in several boreal and temperate forests (Horton and Bruns, 1998; Taylor and Bruns, 1999), including *P. thunbergii* coastal forests (Obase *et al.*, 2009a). On the other hands, *T. ellisii* was also detected in seedlings established on disturbed areas under initial vegetation recovery. We have detected this fungus in *P. densiflora* seedlings established on exposed granite slopes and in surrounding forestlands (data not shown). Obase *et al.* (2007) detected *T. ellisii* in roots of *Salix*, *Populus* and *Betula* seedlings established on volcanic debris where organic matter was absent (Assigned name as Thelepholaceae 1). Obase *et al.* (2009b) showed that this fungus promote the growth of aspen seedlings on the volcanic debris.

The third dominant species was unidentified species belonged to Atheliaceae. This fungus has been detected mainly in roots of pine trees. Nara and Hogetsu (1996) isolated this fungus from roots of *P. densiflora* grown in a nursery and assigned name as unidentified species Tanashi 01. We also observed this fungus abundantly in roots of *P. densiflora* seedlings established on exposed granite slopes (data not shown). Taniguchi *et al.* (2007) and Kataoka *et al.* (2008) observed this fungus in roots of *P. thunbergii* seedlings in coastal pine forests. In view of these observations, the ECM fungal community in the plantation was composed of ECM fungal species that

were also observed in mature coastal pine forests. These fungi seem to have an excellent ability to immediately invade and colonize roots of seedlings growing in nurseries and plantation.

Obase *et al.* (2009a) investigated ECM fungal community in roots of 0-10 year old regenerating *P. thunbergii* seedlings and mature trees in coastal pine forests and showed that *Cenococcum geophilum* Fr. was the most dominant species both in seedlings and mature trees. Matsuda *et al.* (2009) investigated abundance of ECM fungal species in four stands of *P. thunbergii* coastal forests and showed that *C. geophilum* is common and the most probable dominant fungal species in coastal areas of Japan. We also observed ECMs of *C. geophilum* abundantly and isolated its strains from *P. thunbergii* coastal forests in Gangneung, Taean and Incheon, Korea (data not shown). So, it appears that *C. geophilum* is a major colonizer in *P. thunbergii* coastal forests in Korea. It is uncertain why *C. geophilum* was absent in the plantation, however our study revealed the possibility that some ECM fungal species, including even the most common ones in coastal areas, could not invade immediately after plantation establishment.

This study revealed that seedlings established in the plantation were associated with several naturally occurring ECM fungi. Cram *et al.* (1999) showed that naturally occurring ECM fungi could increase survival and growth of forest trees better than artificial inoculation. So, the survival and adaptation of *P. thunbergii* seedlings in a coastal area may be partly due to their symbiotic associations with these symbionts. However, it is possible that the status of ECM fungal colonization in out-planted seedlings is variable among stand locations because it is affected by environmental conditions such as soil properties, intensity of salt and/or drought stress and presence or absence of enough mycorrhizal propagules in the site or surrounding areas. Some effective indigenous ECM fungi like *C. geophilum* in coastal areas can not immediately invade and colonize planted seedlings possibly due to lack of dispersal ability. Therefore, it should be advantageous to inoculate site-adaptable and effective fungal strains on seedlings before planting in plantation site in coastal areas. Further investigations on the effects of naturally inhabiting ECM fungi on the establishment of *P. thunbergii* seedlings in coastal areas are needed for understanding what kinds of ECM fungal strains are effective for establishment of seedlings and applicable in reforestation program in coastal areas.

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