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Chapter 15

RESTORATION OF PROPAGATION AND GENETIC BREEDING OF A CRITICALLY ENDANGERED TREE SPECIES, *ABIES BESHANZUENSIS*

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ABSTRACT

Abies beshanzuensis, Pinaceae, is a geographically distinct tree species in China, which had grown widely at middle to south coastal mountainous area in China during the Riss Ice Age (130–180kBP). However, the population of the species reduced drastically being due to climate change, natural disaster and human activities after the last ice age (Würm: 15–70kBP). There were only 7 wild individuals in 1963, while in 1998 only 3 individuals were discovered at about 1700 m elevation of Mt. Baishanzu in Qingyuan, Zhejiang province, China. *A. beshanzuensis* was approved as one of 12 critically endangered plant species in the world in 1987 by the Species Survival Commission (SSC) of the International Union for Conservation of Nature and Natural Resources (IUCN). Though the efforts to conserve the species by people and many scientists have been performed, it has been recognized that the species has lost natural reproductive ability due to the loss of genetic diversity. It is urgent subject in particular to develop techniques for restoration of propagation and genetic breeding based on the genetic diversity of remaining trees. We developed techniques to produce seeds and seedlings with restoration of high propagation by finding the most suitable tree species for grafting based on taxonomic and genetic information of neighborhood species, and promotion of efflorescence using the most suitable gibberellin.

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INTRODUCTION

Abies beshanzuensis M. H. Wu, Pinaceae, is a geographically distinct tree species in China, which had grown thickly at middle to south coastal mountainous area of Zhejiang, Jiangsu, Anhui and Fujian provinces in China on the Riss (the Illinoian or Saale: 125-200 kBP) glacial period [1]. However, the population of the species reduced drastically due to climate change, natural disaster and human activities after the last (the Würm or Wisconsin: 15-70 kBP) glacial period. There were only 7 wild individuals in 1963 [1], while in 1998 only 3 individuals were discovered at about 1700 m elevation of Mt. Baishanzu in Qingyuan, Zhejiang province, China [2,3] (Fig. 1). *A. beshanzuensis* was listed as one of 12 critically endangered plant species in the world in 1987 by Species Survival Commission (SSC) of International Union for Conservation of Nature and Natural Resources (IUCN) [4,5]. In 1992, *A. beshanzuensis* stamp was issued, and in 1999, *A. beshanzuensis* was also listed as the primary protection tree species by the State Council of China (Fig. 2) [2,6].

Figure 1 *A. beshanzuensis* growing at 1700 m elevation of Mt. Baishanzu

Figure 2 *A. beshanzuensis* in a stamp of Chinese post.

In the past years, researches involving hybridization between natural *A. beshanzuensis* species (1976), grafting *A. beshanzuensis* onto Japanese *Abies firma* as stock (1978-1990), cutting experiments with ABT (rooting hormones developed by Chinese Academy of Forestry) treatment during 1978-1980, as well as hybridization among grafts, have been conducted [3,6]. However, all these efforts were not successful. The reason of *A. beshanzuensis* becoming endangered and difficult to propagate would be due to the long-term interval of flowering, the flowering in the rain season, and/or weak vitality of the pollen-cones [7,8]. It has been recognized that *A. beshanzuensis* has lost natural regeneration ability due to the loss of genetic diversity.

The absence of natural regeneration of *A. beshanzuensis* would be due to insufficient supply and/or low quality of the seeds produced by mother trees. However, failure to produce seeds may result from limited pollination or resources, and there is little consensus regarding their relative importance in natural systems. Techniques to promote production of cone improvement of seed quality and seedlings with high reproduction ability by selecting the most suitable parent species for grafting based on taxonomic, genetic and chemical information of neighborhood species should be urgently developed.

This chapter, we review the researches on the community structure of *A. beshanzuensis*; rejuvenation by grafting *A. beshanzuensis* onto *A. firma*; lignin characteristics of *A. beshanzuensis*; promotions of adventitious rooting of *A. beshanzuensis* cuttings by rooting hormones such as RTN (rooting hormones developed by the University of Tokyo), ABT, α-naphthalene acetic acid (NAA), and indole-3-butyric acid (IBA); flowering promotion by application of gibberellin $A_{4/7}$ ($GA_{4/7}$) and progeny vitality of *A. beshanzuensis* as shown in Fig. 3.

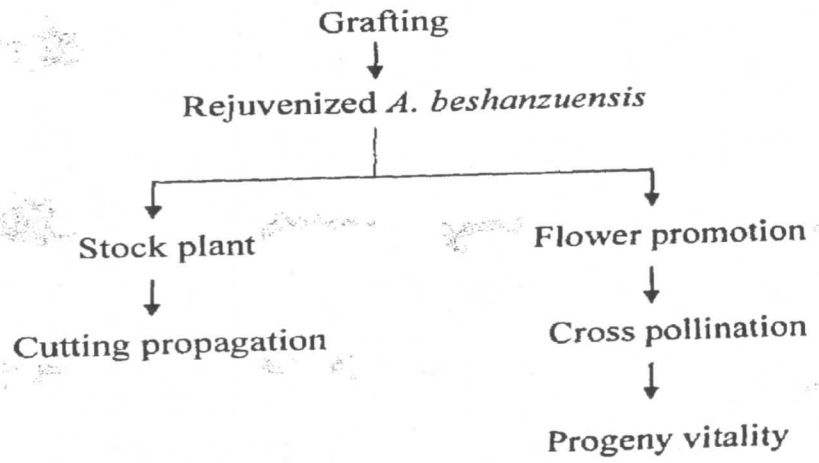

Figure 3 Scheme of the study

1. Forest Community Structure of *A. beshanzuensis*

A. beshanzuensis has gained considerable importance as one of 12 critically endangered plant species in the world [3,4]. *A. beshanzuensis* was found in 1963, while was published as a new species of Abies in 1976 [1]. *A. beshanzuensis* distributed in subtropical zone of Mt. Baishanzu in Qingyuan (119.3-119.6 E , 27.4-27.5 N), southern Zhejiang province, China, at altitudes about 1700 m (Fig. 4). The site belongs to the subtropical monsoon climate, where is abundant precipitation, and four distinct seasons. However, the site shows special alpine climate, namely the annual average temperature is 12.8°C, average annual precipitation is 2,342 mm, average annual relative humidity is 84%, extreme low temperature is -13.2°C, extreme high temperature 30.1°C, frost-free season is 187 day, because of high elevation [1,9]. Compare with the Qingyuan County locates at 350 m elevation, the site conditions of *A. beshanzuensis* forest plant communities are distinguished by lower temperature, higher rainfall, and longer frost day.

Figure 4. Location of *A. beshanzuensis* habitat, Mt. Baishanzu, Qingyuan, Zhejiang province, China (119.3-119.6 E , 27.4-27.5 N)

A. beshanzuesis commonly occurs in forestry together with evergreen *Cyclobalanopsis multinervis* and deciduous *Fagus lucicla*, which is a natural broadleaf mixed forest. About 8-9

species form the high canopy, and the high canopy divided into 3 sub-layers, the first sub-layer composed of *A. beshanzuensis* with 13 m height, the second dominant community of *C. multinervis* and *F. lucicla* with 8-12 m height, and the third one by young trees of the second layer with 4-8 m height [9,10]. The diversity of the community has 17-19 species and the diversity index of high canopy is higher than shrub and ground layer.

2. Rejuvenation by Grafting

Cuttings collected from natural *A. beshanzuensis* branches treating with rooting hormones (ABT) were not successful in 1978 [6]. The failure of root regeneration in cuttings might be due to the physiological age of plant. The physiological age of natural *A. beshanzuensis* branches was too old to regenerate adventitious roots in cuttings. Grafting is a method of asexual plant propagation where the tissues of stock are encouraged to fuse with scion. A shoot of a desired selected plant cultivar is grafted onto the stock plant. An easily rooted plant is used to provide regeneration of roots, which is called the stock, and the scion contains the desired genes to be duplicated in future production by the stock/scion plant. For taking place of successful grafting, the vascular cambium tissues of the rootstock and the scion plants must be placed in contact with each other to ensure the vascular connection between the two tissues. The physiologically young and vigorous *A. firma* was selected as a rootstock. Grafting of *A. beshanzuensis* onto *A. firma* was conducted to rejuvenation of *A. beshanzuensis* during 1978-1990, because the *A. beshanzuensis* was difficult to propagate by other methods, such as cuttings [7,8]. The grafts grew well and the average height of grafts was investigated in 2005 as shown in Fig. 5 [7,8,11].

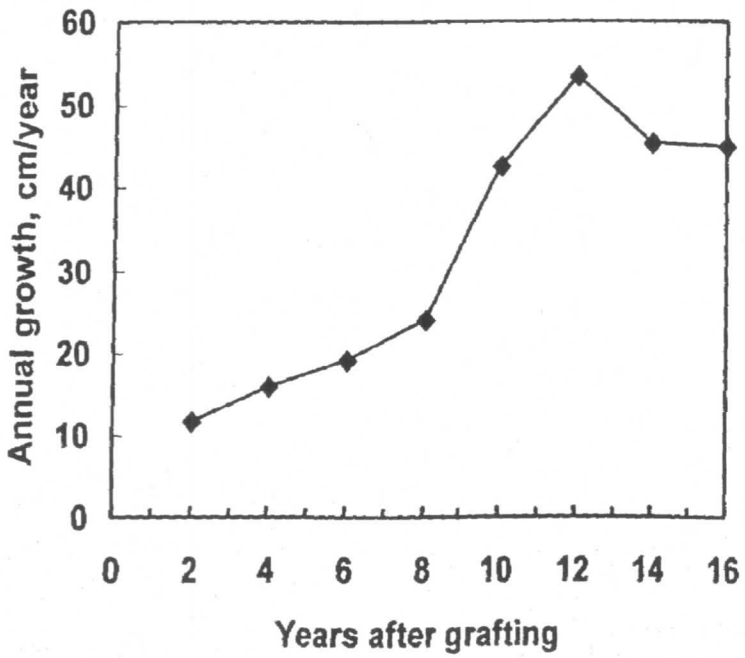

Figure 5. Average annual height growth of grafted trees of *A. beshanzuensis*.

3. LIGNIN CHARACTERISTICS OF *A. BESHANZUENSIS*

Lignin is one of major component of plant cell walls, distributes mainly throughout the secondary xylem. As integral cell wall components, functions of lignin are mechanical support of plants, long-distance water conduction, impenetrable barriers, and defense from biotic attacks [12]. Lignin contents and monomeric aromatic compositions as well as their intermolecular linkage patterns vary predictably among different species. Lignin is derived from the phenylpropanoid (sikimic acid) pathway [13]. Lignin macromolecule is formed by the dehydrogenative polymerization of three monolignols, coniferyl, sinapyl, and *p*-coumaryl alcohols, which differ only in the degree of methoxylation of the aromatic ring. Dehydrogenative polymerization gives varieties of substitution patterns and intermolecular linkages, mainly non-condensed ether linkage and condensed type C-C linkage [14].

Root development of cuttings taken from natural *A. beshanzuensis* was not successful, because of physiologically old of stock plant, while cuttings from grafts grew well [7]. These facts suggest that *A. beshanzuensis* rejuvenated by grafting and their branches recovered vigor and repropagation ability. It is well known that nutrients and plant hormones would be exchanged between stock and scion, while genetic basis of scion would not be affected by the stock. In other words, *A. forma* would not affect on the genetic basis of *A. beshanzuensis*. *A. forma* was selected as stock not only physiologically young and vigor, but also genetically the closest species of *A. beshanzuensis* and the similarity of lignin characteristics, which is one of the most important constitution of vascular system of gymnosperm plants, tracheids. The similarity in the genetical and chemical status of the stock would result good affinity between scion and stock.

Table 1. Klason lignin contents and yield of alkaline nitrobenzene oxidation and ozonation products.

	A. beshanzuensis	*A. firma*
Lignin content %		
KR	39.2	33.3
ASL	0.4	0.4
Total	39.2	33.7
Alkaline nitrobenzene oxidation products (mmol·(200g lignin)$^{-1}$)		
Total yield	264.9	280.6
H/G	0.067	0.091
Ozonation products (mmol·(200g lignin)$^{-1}$)		
E	77.5	85.1
T	67.8	75.1
Total yield	145.2	160.2
E/T	1.14	1.13

KR: Klason residue; ASL: acid soluble lignin;
H/G: H/V: molar ratio of *p*-hydroxybenzaldehyde to vanillin
E: *erytro* type of β-O-4 intermonomer linkage;
T: *threo* type of β-O-4 intermonomer linkage;
E/T: molar ratio of *erythro* type to *threo* type.

Lignin content of *A. beshanzuensis* was higher comparing with *A. firma* (Table 1) and other coniferous species (25-35% of cell walls). The high lignin content may be due to the adaptation of *A. beshanzuensis* to environmental stresses in surviving from the Riss glacial period. The composition of non-condensed linkage, total yield of alkaline nitrobenzene oxidation products, based on lignin of *A. beshanzuensis* was almost the same with that of *A. firma* (Table 1), and good agreement with gymnosperm wood lignins [15,16]. The lignin of both species was characterized with significantly high molar ratio of *p*-hydroxyphenyl nuclei to guaiacyl nuclei (H/G ratio) of alkaline nitrobenzene oxidation products. These findings agree with the results reported for other Abies species, *A. concolor* [17], *A. fraseri* [18], *A. balsamea* [19], *A. sibirica* [20] and *A. sachalinensis* [21].

Alkaline nitrobenzene oxidation of the Björkman lignins of both *A. beshanzuensis* and *A. firma*, which were isolated by neutral solvent extraction after finely ground extract free wood meal with Björkman's procedure [22], gave quite similar results with those of the extract-free samples (Table 2). The H/G ratio of Björkman lignin of moso bamboo, *Phyllostachys pubescens*, which is known to have *p*-hydroxyphenyl nuclei involved in both esterified and arylglycerol-β-aryl intermonomer linkages [23], gave considerably high value (Table 2). The presence of arylglycerol-β-aryl ether intermonomer linkages is essential to distinguish lignin from other polyphenols [24]. The acidolysis products of the Björkman lignins were identified by gas chromatography-mass spectroscopy (GC-MS) as trimethylsilyl derivatives. Although H/G ratio of *P. pubescens* was considerably high (Table 2), the relative intensities of *p*-hydroxyphenyl propanones of *P. pubescens* Björkman lignin were smaller than those of *A. beshanzuensis* and *A. firma* [25]. The molar ratio of *p*-hydroxyphenyl-type acidolysis products to the corresponding alkaline nitrobenzene oxidation products (H_{acid}/H_{nb}) was calculated and the results showed that the values of *A. beshanzuensis* and *A. firma* were higher than that of *P. pubescens* (Table 2). These results suggest that the most of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid in alkaline nitrobenzene oxidation products of *P. pubescens* would originate from *p*-coumaric acid esterified to lignin [26]. In contrast, lignin of *A. beshanzuensis* was characterized by the presence of *p*-hydroxyphenylglycerol-β-aryl ether intermonomer linkages with the same rate of *A. firma* lignin.

Table 2. Alkaline nitrobenzene oxidation products from Björkman lignin (mmol·(200g lignin)$^{-1}$)

	A. beshanzuensis	*A. firma*	*P. pubescens*
Total yield	263.5	260.1	325.1
H/V	0.066	0.086	0.206
S/V	-	-	1.56
H_{acid}/H_{nb}	0.18	0.19	0.12

H/V: molar ratio of *p*-hydroxybenzaldehyde to vanillin;
S/V: molar ratio of syringaldehyde to vanillin:
H_{acid}/H_{nb}: molar ratio of *p*-hydroxyphenyl-type acidolysis products to *p*-hydroxybenzaldehyde in alkaline nitrobenzene oxidation products.

The arylglycerol-β-aryl ether intermonomer linkage is an important characteristic of lignin relating with the definition [27,28], and can be either *erythro*- or *threo*-forms, and the ratio of *erythro*- to *threo*-forms of arylglycerol-β-aryl ether intermonomer linkage (*E*/*T* ratio).

The molar ratio of erythronic acid to threonic acids in ozonation products of the extract-free sample of *A. beshanzuensis*, which reflects the *E/T* ratio of arylglycerol-β-aryl ether intermonomer linkage, was exactly the same with that of *A. firma* (Table 1). The values were in the range of some gymnosperm wood lignins reported by Akiyama et al. [28]. The ^{1}H NMR spectra of acetylated Björkman lignins of both *A. beshanzuensis* and *A. firma* exhibited similar strength of signals at δ 6.01 and δ 6.06 ppm [25]. These results were in agreement with *E/T* ratios of *A. beshanzuensis* and *A. firma* found by ozonation (Table 1). The *p*-hydroxybenzoic acid, *p*-coumaric acid, and ferulic acid have been reported to enhance rooting of stem cuttings [29]. The high level of *p*-hydroxyphenylglycerol-β-aryl ether intermonomer linkages in *A. beshanzuensis* and *A. firma* may promote adventitious rooting of cuttings.

Wang et al. [30] investigated genetic phylogenies and divergence times in Pinaceae using three genomes, the chloroplast *matK* gene, the mitochondrial *nad5* gene, and low-copy nuclear gene encoding 4CL (4-coumarate CoA ligase), which is one of the genes concerned deeply in lignin biosynthesis [14]. They concluded that *A. firma* is genetically the closest species of *A. beshanzuensis* [30]. It was confirmed that structural feature of lignin of *A. beshanzuensis* was quite similar with that of *A. firma* [25]. All these results suggest that *A. firma* would be the best species as the rootstock for grafting *A. beshanzuensis*, and the similarity between *A. beshanzuensis* and *A. forma* could have good affinity between scion and stock. In addition, physiologically young and vigorous *A. forma* supply enough water, nutrients, minerals resulting rejuvenation of *A. beshanzuensis*.

4. Root Promotion of *A. beshanzuensis* Cuttings

Propagation through stem cuttings in woody plants is used to capture specific genetic combinations (phenotypes) and to provide superior cultivars for planting. The formation of adventitious roots of cuttings is affected by plant growth regulators, stock plant age, season of cutting collection and shoot position [29,31]. Especially, the formation of adventitious root in shoot cuttings was strongly dependent on aging of the donor tree [32,33]. Cutting propagation of *A. beshanzuensis* conducted in 1978 could not succeed. The reason of failure would be due to the physiological age of natural *A. beshanzuensis* branches, which was too old to regenerate adventitious root in cuttings [6]. In Feb. 2004, cutting experiment was conducted with cuttings taken from 2.5 m height, healthy, disease-free, the year's growth branches of grafts. Cuttings were treated with 0.02% RTN, ABT, NAA, or IBA solution, and adventitious roots were propagated in the Forest Nursery, Lishui (119°54′ W, 28°27′ N), Zhejiang province, China [7]. RNT and NAA exhibited the most outstanding promotion effects on the formation of adventitious roots (Fig. 6). The cuttings treated with RNT, NAA, ABT, IBA and control (the cuttings without any hormone treatment) had average rooting percentages of 79.2%, 70.8%, 62.5%, 58.3% and 54%, respectively (n=3) (Table 3). The highest rooting rate (79.2%) was observed in the cuttings treated with 0.02% RNT solution, which showed significantly differences with control and the treatment with IBA solution [7]. There was no significant difference among other treatment (Table 3). The cuttings treated with 0.02% RNT solution had 10 adventitious roots per cutting, and the average length of adventitious roots was 28 cm, which was significantly higher than those of control and other treatments (Table 4). Cuttings taken from natural grown *A. beshanzuensis* branches could not

form adventitious roots, while cuttings collected from grafts grew well, suggesting that the branch of *A. beshanzuensis* was rejuvenated by grafting and recovered vigor and repropagation ability [7].

The effects of cutting on adventitious rooting were examined by the $L_8(2)^7$ orthogonal experiment (Table 5). There were 12 cuttings in every treatments, and every variables took 2 levels, namely length of cutting (5 and 10 cm length), with or without top bud, leaf size (whole and half leaf), the way of base incision (basal and mallet ends of cutting), and the interaction of cutting length×top bud and cutting length×leaf size were investigated [7]. Among those 6 variables, cutting length and top bud were two important factors affecting on rooting rates of *A. beshanzuensis* cuttings (Table 6). Cuttings with top bud and 10 cm length cuttings showed better rooting rate than those of without top bud and 5 cm cuttings. The average rooting rates of 5 cm and 10 cm length cuttings were 5.5 and 8.5, and the longest adventitious roots were 14 cm and 25 cm, respectively (Table 7). Rooting rate of cuttings with top bud was higher than cuttings without top bud. There were no significant differences between whole and half leaf, or basal and mallet ends of cuttings.

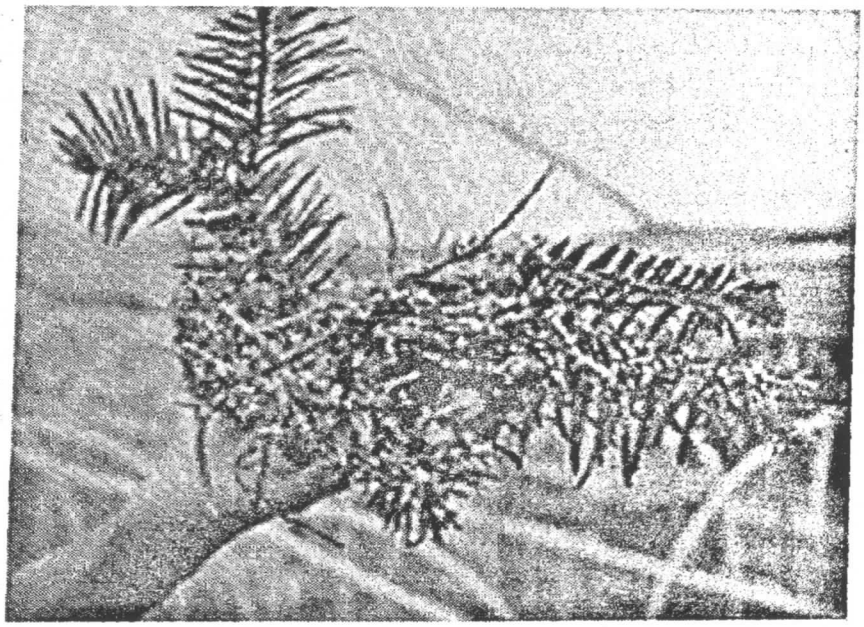

Figure 6. Adventitious roots of *A. beshanzuensis* of cuttings

Table 3. Effect of hormone treatment on rooting rate

Hormones	Rooting rate (%)	x_1- x_5	x_1- x_4	x_1- x_3	x_1- x_2
RTN	79.2 (x_1)	25.2*	20.9*	16.7	8.4
NAA	70.8 (x_2)	16.8	12.5	8.3	
ABT	62.5 (x_3)	8.5	4.2		
IBA	58.3 (x_4)	4.3			
Control	54.0 (x_5)				

note : * significant difference.

RTN: rooting hormones developed by the University of Tokyo, ABT: rooting hormones developed by Chinese Academy of Forestry, NAA: α-naphthalene acetic acid, IBA: indole-3-butyric acid, Control; without any hormones (n=3).

Table 4. Effect of hormone treatment on adventitious root development

Hormones	Adventitious root		The longest adventitious root (cm)	
	Average	Range	Average	Range
RTN	10.0	7.5-12.0	28.0	23.5-32.0
NAA	8.0	6.5-9.5	21.5	17.0-23.5
ABT	7.5	5.0-8.5	21.0	16.5-22.0
IBA	6.5	5.5-7.5	20.0	15.5-23.0
CK	6.5	5.5-7.0	21.0	16.0-22.0

Abbreviations: refer footnotes of Table 3. (n=3)

Table 5. Four cutting type of the $L_8(2)^7$ orthogonal experiment

Level	Length (A)	Top buds (B)	A×B	Leaf size (C)	A×C	Residual (*e*)	Incision (*D*)
1	5 cm	With	1	Whole	1	1	Mallet
2	10 cm	Without	2	Half	2	2	Basal

A×B and A×C: Interactions between factors A and B, and A and C, respectively.

Table 6. Analysis of variance of the $L_8(2)^7$ orthogonal experiment of rooting rate

Variables	Degree of freedom (df)	Sum of deviation square	Mean square	Mean square ratio	*F*-test
A	1	701.3	701.3	81.4*	$F_{0.05}$=18.5
B	1	705.0	705.0	81.9*	$F_{0.01}$=98.5
A×B	1	217.4	217.4	25.2*	
A×C	1	78.8	78.8	9.1*	
D	1	217.4	217.4	25.2*	
Residual (C、e)	2	17.2	8.6		

Note: *significant difference with 5% level.

Table 7. The results of adventitious roots of different cuttings

Cutting type		Number of roots		The longest root (cm)	
		Average number	Range	Average number	Range
Cutting length (cm)	10	8.5	6.5-11.0	25.0	21.5-30.5
	5	5.5	4.5-6.0	14.0	10.5-17.0
Top bud	With	10.0	7.5-13.5	26.5	21.5-31.5
	Without	4.5	4.0-6.0	12.0	10.0-15.5
Leaf size	Whole leaf	6.5	4.5-7.0	24.5	19.5-28.0
	Half leaf	7.5	6.5-8.5	23.0	20.0-26.5
Incision	Mallet	7.5	5.0-8.5	24.5	22.5-28.5
	Basal	7.0	5.5-8.5	23.5	18.5-25.5

The formation of adventitious root of *A. beshanzuensis* was investigated by Olympus BH2 Microscope (Olympus Co. Ltd., Tokyo). Callus growth was very slow at the beginning, only a tine of callus was observed until the early June. Callus formation could be observed near the cortex and vascular cambium of incision of the cuttings. During middle June to early July, the callus grew rapidly, and quickly covered the xylem. The hard cuttings of *A. beshanzuensis* had no latent root primordial. The root primordial was induced from a group of parenchyma cells originating from vascular cambium and phloem, and inner callus. Those parenchyma cells have thick cytoplasm, big nuclear and closely-arranged, which was different obviously with other cells. Root primordial differentiation into primordial roots, developed finally to adventitious roots. It was found that 80% of adventitious roots were originated from callus [7].

5. Promotion of Cone Production *A. beshanzuensis* by $GA_{4/7}$ Application

The successful promotion of flowering in a forest tree species by gibberellins (GA's) was first reported by Kato et al. [34] for a member of the Taxodiaceae family. However for the Pinaceae family conifers seemed to be unresponsive to GA's until 1973 when a mixture of less-polar GA_4 and GA_7 and occasionally also GA_9 were found to promote flowering in Douglas-fir seedlings and mature propagate [35]. The first positive results on Douglas-fir were quickly extended to a variety of other Pinaceae species. Now it is well known that gibberellin $A_{4/7}$ ($GA_{4/7}$) is effective in promoting pollen- and seed-cone productions, especially for the Pinaceae family [36,37]. Grafts of *A. beshanzuensis* were sprayed with $GA_{4/7}$ [8]. The two-way analysis of variance (n=3) showed highly significant (α=0.01) differences among variations for the $GA_{4/7}$ concentration and spraying timing, and significant (α=0.05) differences among variations for the interaction of $GA_{4/7}$ concentration × spraying timing (Table 8).

Table 8. Analysis of variance of $GA_{4/7}$ promoted pollen-cones

Variations	Degree of freedom (df)	Sum of deviation square	Mean square	Mean square ratio	*F*-test
Concentration (A)	2	63,494	31,747	11.18**	$F_{0.01(2,18)}$=6.01
Spraying time (B)	1	37,203	37,203	13.10**	$F_{0.01(1,18)}$=8.29
A×B	2	30,808	15,404	5.42**	$F_{0.05(2,18)}$=3.55
Residual (e)	18	51,120	2,840		
Sum (T)	23	182,625			

Note: **: significant difference with 1% level, *: significant difference with 5% level.

The highest pollen-cone, 93.1 per branch (150 cm^3), was recorded in the treatment with $GA_{4/7}$ concentration of 500 mg/L, followed by 250 mg/L, and the lowest was in the control (Table 9). It was suggested that high $GA_{4/7}$ concentration within the 0-500 mg/L affect strongly on the promotion of the pollen-cone production. The results of Q test showed

highly significant difference (α=0.01) between 500 mg/L concentration and control, and significant difference (α=0.05) between 250 mg/L concentration and control (Tables 9, 10). However, the difference between 500 mg/L and 250 mg/L concentration was not significant, suggesting that the effect of 250-500 mg/L concentration on the promotion of pollen cone production of *A. beshanzuensis* was clear (Tables 9, 10) [8].

Table 9. Multiple comparison of different concentration of $GA_{4/7}$ promoted pollen-cones

Concentration (mg/L)	Pollen-cones per branch (150 cm^3)	x_1-x_3	x_1-x_2
500	93.14 (x_1)	89.61**	51.95
250	51.19 (x_2)	47.66	
0	3.53 (x_3)		

$GA_{4/7}$ sprays were applied at different timings during the growing season to promote pollen-and seed-cone production of *A. beshanzuensis*. The average numbers of pollen-cone were 15.4 and 54.8 per branch (150cm^3) sprayed with $GA_{4/7}$ during May-June ("Early") and June-September ("Late"), respectively. Treatment in June-September significantly increased pollen-cone production. Interaction between 500 mg/L × Late spraying significantly increased pollen-cone production, the average number of pollen-cone reached 126.5/branch (150cm^3) (Table 10, Fig. 7). The result of *A. beshanzuensis* was different from that of eastern white pine [36]. For the eastern white pine, early $GA_{4/7}$ application by either spraying or injection during the period of rapid shoot elongation (May-June) promoted pollen-cone production, while the late applications (August-September) did not increase the production [36]. The late $GA_{4/7}$ spraying was more effective, and early treatment in *A. beshanzuensis* may be counting on environment at high altitude habitat of *A. beshanzuensis*. Results indicate that the best treatments for pollen- and seed-cone productions should be carried out in June-September with the concentration of 250-500 mg/L of $GA_{4/7}$ spraying. The $GA_{4/7}$ application could promote pollen-cone production. Although the pollen-cone production did not closely relate to increase of seed-cone, the application of $GA_{4/7}$ is important for genetic resource preservation. The highest seed-cone production was recorded at the $GA_{4/7}$ concentration of 250 mg/L, followed by 500 mg/L, and the control was the lowest (Tables 11, 12). The Q test results showed significant difference (α=0.05) between 250 mg/L concentration and control, while there was no significant difference among other concentrations. Similar to pollen-cone promotion by $GA_{4/7}$, the 250-500 mg/L concentration was also appropriate for seed-cone production (Tables 11, 12). Different from pollen-cone, the seed-cone influenced directly to the seed production. The effect of $GA_{4/7}$ application on promotion of seed-cone was not significant compare with pollen-cone production [35,36]. This result was in good agreement with previous papers [35,36]. Spraying of $GA_{4/7}$ increased the number of pollen-cone, and also seed-cones with some extent [37].

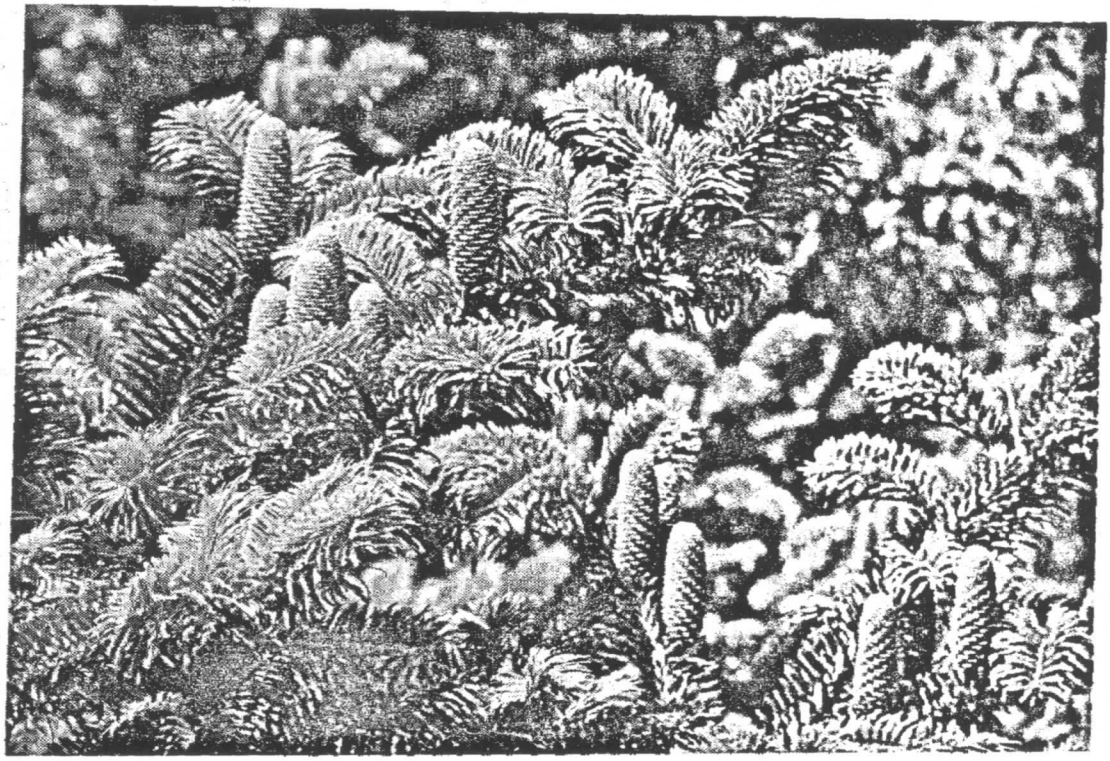

Figure 7 Pollen-cone production promoted by $GA_{4/7}$ spray.

Table 10. $GA_{4/7}$ promoted pollen-cone of concentration × spraying timing

Concentration × spraying timing	Pollen-cones per branch ($150cm^3$)	x_1-x_6	x_1-x_5	x_1-x_4	x_1-x_3	x_1-x_2
500 mg/L × Late	126.48(x_1)	123.33*	122.26*	106.68	101.01	69.58
250 mg/L × Late	56.90(x_2)	53.75	52.68	37.1	31.43	
500 mg/L × Early	25.47(x_3)	22.32	21.25	5.67		
250 mg/L × Early	19.80(x_4)	16.65	15.58			
0 mg/L × Late	4.22(x_5)	1.07				
0 mg/L × Early	3.15(x_6)					

Table 11. Analysis of variance of $GA_{4/7}$ promoted seed-cones

Variations	Degree of freedom (df)	Sum of deviation square	Mean square	Mean square ratio	*F*-test
Concentration (A)	2	36.4	18.2	5.17*	$F_{0.05}(1,18)=4.41$
Spraying time (B)	1	14.3	14.3	4.06	$F_{0.05}(2,18)=3.55$
A×B	2	22.58	11.3	3.21	
Residual (e)	18	63.36	3.52		
Sum (T)	24	161.03			

Table 12. Multiple comparison of different concentration of $GA_{4/7}$ promoted seed-cones

Concentration (mg/L)	Seed-cones per branch ($150cm^3$)	x_i-x_3	x_i-x_2
250	3.70 (x_1)	2.49*	1.65
500	2.05 (x_2)	1.84	
0	1.21 (x_3)		

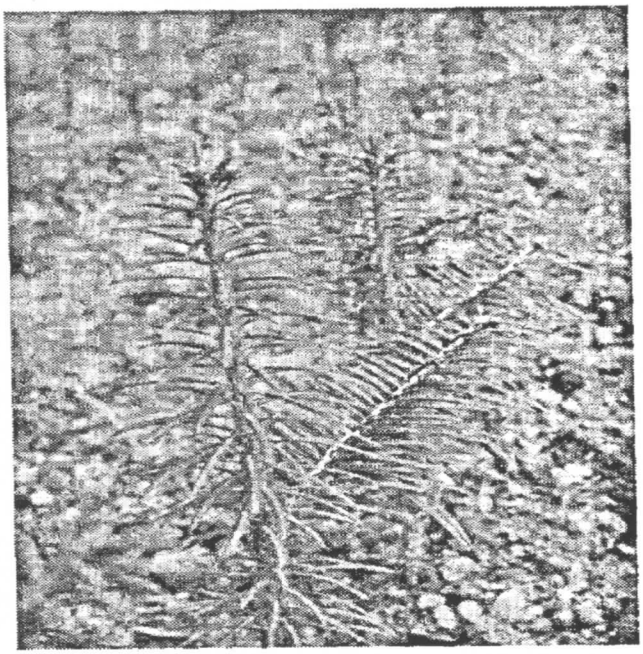

Figure 8. *A. beshanzuensis* recovering vigor and repropagation ability by grafting.

6. Progeny Hereditary Vitality of *A. beshanzuensis* by Cross-Pollination

Sexual reproduction is the basic method to conserve *A. beshanzuensis*. The level of genetic variation in population originated from cross-pollinated seedlings was higher than that in population of old trees and population of graft-originated, suggesting that cross-pollination increased genetic diversity of *A. beshanzuensis* [38-40]. The absence of seed vitality may be due to the lack of genetic diversity and low quality of the seeds produced by mother trees. Low quality of seeds may result from limited pollen- and seed-cones and self pollination.

It is important to note that the grafts of *A. beshanzuensis* were from a few natural *A. beshanzuensis* trees, the genetic diversity may not be improved. Therefore even the cross-pollination among pollen-cone and seed-cone produced by $GA_{4/7}$ application was performed, significant increase in the vitality of seeds would not be expected. The weight of 1,000 seeds increased from 39.3 g of natural pollination to 44.7 g of cross-pollination of *A. beshanzuensis* grafts [8], namely the quality of seeds from cross-pollination was obviously improved. In

addition, the vitality of seeds was increased by cross-pollination, especially the vitality of seeds was increase 24-28% by cross-pollination in the early May (Fig. 8).

CONCLUSIONS

1) The dominant community of *A. beshanzuensis* phytogroup is a natural broadleaf mixed forest, in which about 8-9 species form the high canopy divided into 3 sub-layers, and the first sub-layer composed of *A. beshanzuensis* with 13 m height.
2) *A. beshanzuensis* rejuvenated by grafting onto *A. firma* as a stock and the branch recovered vigor and repropagation ability.
3) Structural feature of lignin of *A. beshanzuensis* was investigated comparing with that of *A. firma* to be used as a stock for grafting. The lignin of both species was characterized with the presence of significant amount of *p*-hydroxyphenyl nuclei. Structural feature of lignin of *A. beshanzuensis* was quite similar with that of *A. firma*, suggesting that *A. firma* would be the best species as the mother tree for grafting.
4) The cuttings taken from grafts of *A. beshanzuensis* onto *A. firma* treated with 0.02% RNT had the highest average rooting rate of 79.2%, and had higher adventitious rooting formation. The root primordium and adventitious root developed during middle of June to early July.
5) The $GA_{4/7}$ application could promote seed- and pollen-cone production, and the concentration of 250-500 mg/L was appropriate for the production of seed- and pollen-cone. And late (June-September) $GA_{4/7}$ spraying increased significantly pollen-cone production. The effect of cross-pollination of *A. beshanzuensis* grafts on restoration of filial generation hereditary vitality of *A. beshanzuensis* was made clear.

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