論　文

優良遺伝形質を持つ*Eucalyptus camaldulensis*における リグニンの二三の特徴

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Some characteristics of lignin in elite genetic-based *Eucalyptus camaldulensis*

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タイ国東部において，*Eucalyptus camaldulensis*の組織培養により作られた苗木のプランテーションから，生育の良い5クローンを選び，とくにリグニンの特徴について調べ，以下の結果を得た．（1）5クローン間でクラーソンリグニン量を比較した．エタノールートルエン，1%NaOH抽出木粉に対する割合では，クラーソンリグニンは29.2～32.3%の範囲に分布した．5クローン間では，クローン4が最大値，クローン2が最小値を示した．（2）リグニン構成単位の特徴を知るために，アルカリ性ニトロベンゼン酸化を行った．その結果，シリングアルデヒドとバニリンの比率（S/V比）は,2.4～2.7の範囲を示した．クローン3が最大値を示した．（3）紫外線顕微分光測光法により，木繊維の二次壁中層のリグニンの吸光特性を調べた．吸光度は277～278nmで最大値を示した．また5クローン間ではクローン3が最大値を示した．このことは，木繊維二次壁中層のリグニン組成において，クローン間で顕著な差が認められないことを示している．また5クローン間ではクローン3が最大の吸光度を示し，木繊維二次壁のリグニン濃度がクローン3では最も高いことが示された．

以上の結果から，同一樹種のクローン間で，リグニンの性質に差異のあることが判明した．

キーワード：*Eucalyptus camaldulensis*，組織培養，クラーソンリグニン，S/V比，紫外線顕微分光測光法

Five superior clones of *Eucalyptus camaldulensis* from *in vitro* propagation or tissue culture at the plantation site in the eastern part of Thailand were selected for lignin analyses. Results were summarized as follows: (1) From the comparison of Klason lignin contents among 5 clones, they ranged from 29.2 to 32.3% in ethanol-toluene and 1% NaOH extracted wood meal basis. Clone 4 showed maximum and clone 2 showed minimum. (2)Alkaline nitrobenzene oxidation was applied to evaluate the lignin building units. Syringaldehyde/ Vanillin molar ratios of lignin (S/V molar ratio) ranged from 2.4 to 2.7. Clone 3 showed the greatest value. (3) Ultraviolet absorption spectra of middle layer of secondary wall of fibers were measured. The maximum absorbances were between 277-278 nm. Clone 3 showed highest absorbance among 5 clones. This implied that lignin composition of middle layer of fiber secondary wall was not much different among clones. Based on this implication, it was considered that the lignin concentration of clone 3 showed maximum.

From the investigations of lignin mentioned above, it was concluded that lignin characteristics showed variations among clones in the species, *E. camaldulensis*.

Key words: *Eucalyptus camaldulensis*, tissue culture, Klason lignin, S/V molar ratio, Ultraviolet microscopic spectrophotometry

1. Introduction

In South-east of Asia deforestation has been a serious problem especially in the past two decades. Parallel with the increase of population shortage of resources has also been the matter to be resolved. To cope with those problems, plantation of fast growing tree species was started from 1980s (Nobuchi 2001).

In tropical rain forest area such as Malaysia and Indonesia, *Acacia mangium, Eucalyptus deglupta, Paraserianthes falcataria* and *Gmelina arborea* are the main plantation species. In the tropical seasonal forest area such as Thailand with long dry season, however, the growth of those species is restricted. In Thailand, therefore, *Eucalyptus camaldulensis* which can grow in the area with dry habitat has been selected as one of the potential

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plantation species.

Seedlings or young plants for plantation are generally obtained from seeds or from cuttings. In addition to those propagation, the group of Faculty of Forestry, Kasetsart University started to produce young plants or plantlets through *in vitro* culture technology or tissue culture from 1989. They could succeed to get young plants from tissue culture, succeeded in naturalization and planted them in fields (Siripatanadilok *et al.* 1992).

Young plants generated through vegetative propagation including both cuttings and tissue culture belongs to the category of clone plant. Through tissue culture we can prepare much more number of young plants in a short term than those from seeds or cuttings, which provides advantages for promoting plantation.

In Thailand young plants from tissue culture have been planted in fields and already harvested especially to make chips for pulping.

The one important target in *E. camaldulensis* plantation from tissue culture is to evaluate the quality of wood for each clone. It is because in the future activities *E. camaldulensis* is expected to be utilized as lumber wood in addition to pulping. Therefore, the selection of clones with high quality of wood is the key research point. Anatomical characteristics and another properties of wood which affect on wood quality are very much necessary.

In this report, therefore, superior clones which showed good growth rate with straight trunk were selected for the experiment. The height growth and diameter growth of those superior clones showed about 40-50% larger values than clones of inferior growth. Lignin characteristics of 5 clones selected from 20 clones were investigated.

The researches of lignin of *E. camaldulensis* are very much limited. Ona *et al.* (1995) reported within-tree variation of lignin content and S/G ratio. Watanabe *et al* (1997) investigated methodological problem in histochemical study of heterogeneity of lignin. Baba *et al.* (1996) also studied chemical and anatomical characteristics of *E. camaldulensis* for comparing normal wood with tension wood.

For the promotion of plantation of this species, the more detailed study of lignin is necessary. Particularly, characteristics of lignin compared among clones are not reported. In this report, therefore, as the first step of investigating lignin, Klason lignin contents, S/V molar ratios and Ultraviolet (UV) absorption from fiber secondary walls were investigated.

Anatomical characteristics as well as some factors affecting wood quality will be published in a separate paper.

2. Materials and methods

2. 1. Materials

Five superior clones of *E. camaldulensis* among 20 clones in the plantation site at Sra Keaw province in eastern part of Thailand were selected as sample trees. The trees were planted in 1992 and sample trees were felled in 1997. Tree age was, therefore, 5-year-old. The names of clones were T5, Kitti, S9, Y2 and K2, which were named in this report clone 1, 2, 3, 4 and 5, respectively.

Two disks of 5 cm in thickness were cut at the height of 50 cm from the ground level for each clone and provided for the experiments. For lignin analyses one disk for each clone was used and another disk was utilized for anatomical observation. The diameter of each disk ranged from 18 cm to 22 cm.

2. 2. Methods

2. 2. 1. Klason lignin

Wood blocks for lignin analyses were cut from the outer part of sapwood in each disk to avoid the effects of heartwood substances. The wood blocks were chipped and grounded to pass 40-mesh screen. Lignin content was determined by the Klason method (Dence 1992) and expressed as percentage of extractive-free oven dry wood meal.

2. 2. 2. S/V molar ratio

In this report S/V molar ratios (hereafter referred to as S/V ratios) were obtained by alkaline nitrobenzene oxidation method. For the alkaline nitrobenzene oxidation, wood meal was extracted with an ethanol: toluene (1: 2) solution and 1% NaOH. Approximately 30-40 mg specimens were treated with 1.5 ml of 2N NaOH and 0.15 ml of nitrobenzene in stainless steel tubing reactors at 170 ℃ for 3 hours. The alkaline solution was diluted, extracted with ethyl acetate to remove nitrobenzene and neutral reaction products, and acidified to pH 2-3 with 2N hydrochloric acid. Acetovanilone then was added as an internal standard. This then was extracted with ethyl acetate to obtain the aldehydes. After the ethyl acetate

solution was concentrated, the products were acetylated with an acetic anhydride: pyridine (1: 1, v/v) solution. Vanillin acetate and Syringaldehyde acetate were determined quantitatively by gas chromatography (GC) on a fused-silica capillary column (Shimadzu, HR-1, 0.25 mm × 30 m). Then the molar ratio of Syringaldehyde (S)/ Vanillin (V) (S/V molar ratio) was calculated.

In addition to S/V molar ratio, total amount of the yield of S plus V is also considered to be important factor affecting pulping. The percentage of the weight of S plus V to Klason lignin was calculated.

2. 2. 3. Ultraviolet microscopic spectrophotometry

For UV microscopic spectrophotometry, the constant thickness of sections are the key factor to compare the data from different sections precisely, In this experiment new trial to get sections from 5 samples (clones) was devised to avoid errors coming from investigating different sections which might cause different thickness even the thickness was set in a fixed value in the cutting process using a rotary microtome.

A 150 μm thick radial longitudinal section (4 × 8 mm) was cut from each clone. Total five sections from 5 clones were embedded together in Epoxy resin as shown in Fig. 1. When they were embedded sections were arranged in order of the clone 1 to 5.

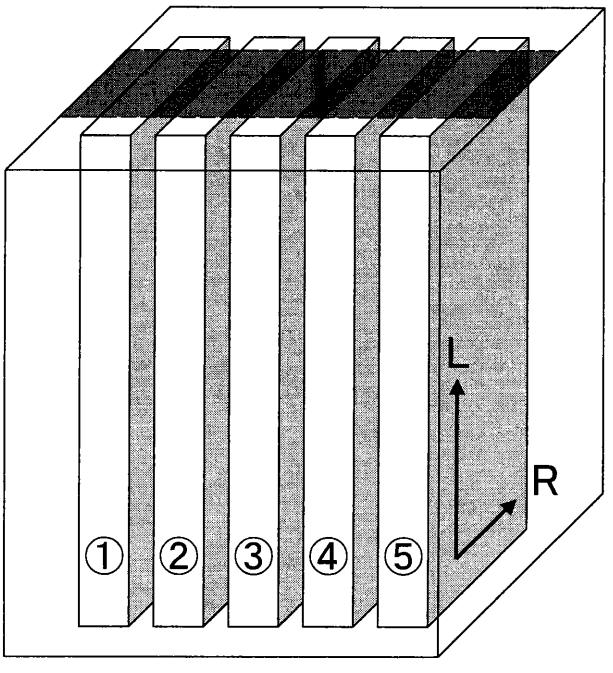

Fig. 1 An illustration showing a thin (1 μm) transverse section cut from an Epoxy embedded block in which 5 radial longitudinal sections are included. Numbers indicate clone No, L: longitudinal, R. radial.

One micrometer thick sections were cut using a rotary microtome equipped with a diamond knife. Sections were mounted on a quartz slide, immersed in glycerin, covered with a quartz coverslip. In one section 5 of transverse sections of each clone were included, Therefore, errors which might come out from measuring different sections would be minimized or negligible.

After observing and photographing 5 sections at 280 nm, 10 wood fibers were selected in each clone for UV microscopic spectrophotometry. UV absorption was surveyed in the range from 250 nm to 320 nm by 1 nm steps for spot diameter of 2 μm and a band width of an illuminating monochrometer (5 nm) using a microscopic spectrophotmeter (Carl Zeiss UMSP-80). Results were expressed as a mean value of 10 spots and smothing treatment was adopted.

3. Results and discussion

3. 1. Outline of 5 clones

Photograph 1 shows a part of the plantation site at Sra Keaw Province. In this picture, left row indicates trees generated from *in vitro* propagation and right one from seedlings. Both groups were in the same plantation age. It is clear that genetic-based trees have homogeneous tree shape with straight trunk.

Photograph 2 indicates light micrographs of 5 sections of each clone which corresponded to the part where samples for lignin analyses were cut. In Photo. 2 it is observed that vessel diameter, vessel density and the arrangement of pores are different among clones. The cell wall thickness of some clones shows thinner than those of other clones.

The detailed anatomical characteristics will be published in a separate paper. Some characteristics of lignin among 5 clones were exclusively reported in this paper.

3. 2. Klason lignin

Percentages of Klason lignin are summarized in Table 1 together with the data of ethanol-toluene extractives and 1% NaOH extractives. Klason lignin of original wood base are also listed in Table 1 (Klason lignin*2).

In Table 1 clone 4 showed maximum amount of lignin and clone 2 minimum. The difference between maximum and minimum was 3.1% in extracted wood basis and 2.5%

Photo. 1 A field site of *Eucalyptus camaldulensis* at Sra Keaw Province in the eastern part of Thailand.

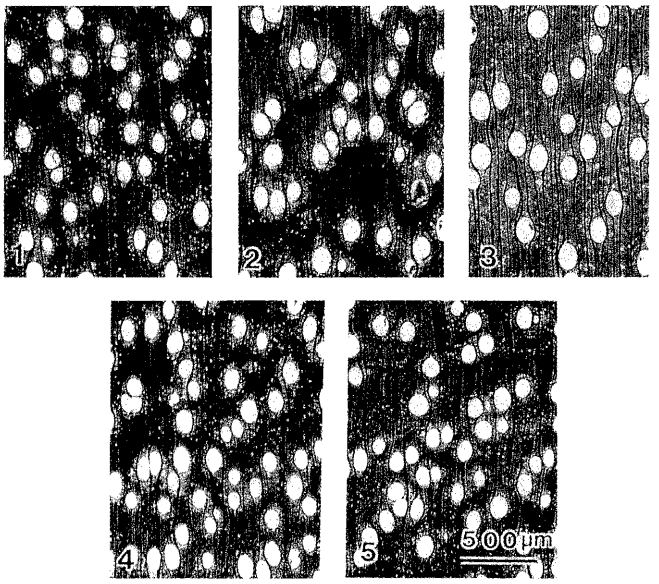

Photo. 2 Light micrographs of transverse sections of 5 clones (safranin staining).

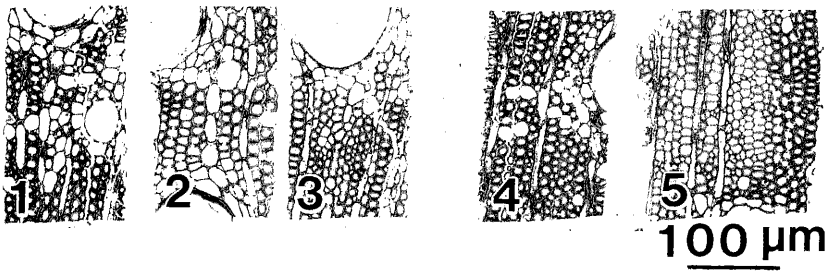

Photo. 3 Light micrographs of a thin section including transverse sections of 5 clones.

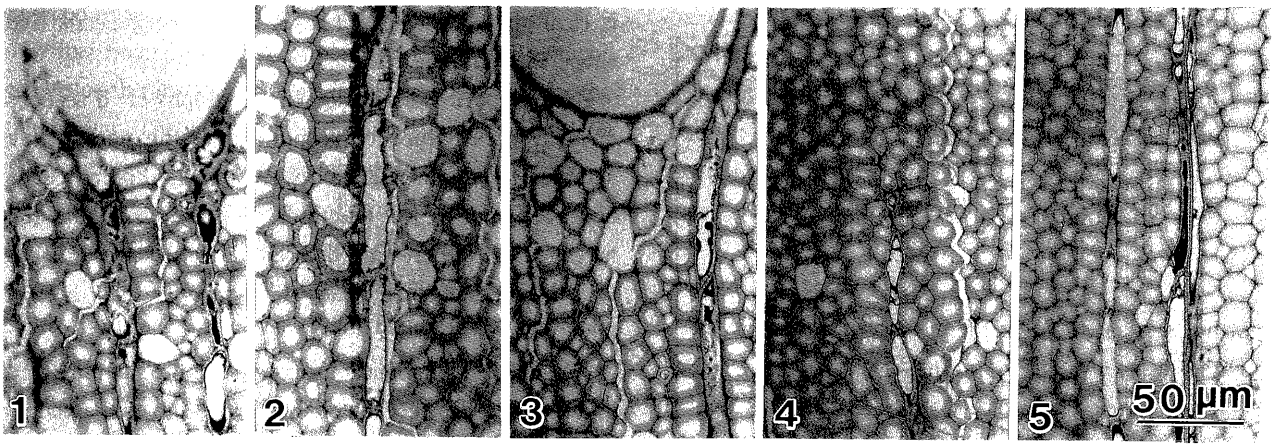

Photo. 4 Ultraviolet micrographs of thin sections of 5 clones photographed at 280 nm.

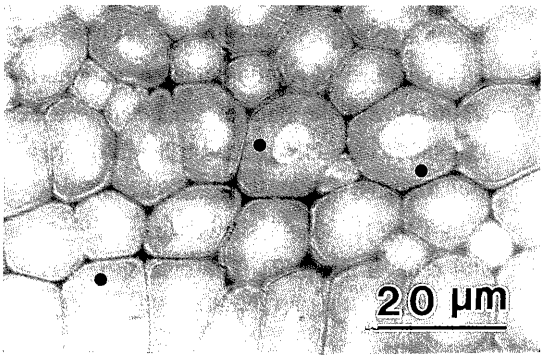

Photo. 5 An ultraviolet micrograph photographed at 280 nm. Circles on S_2 layer indicate 2 μm spots.

in original wood base.

The amount of Klason lignin of present study was compared with those reported by Ona *et al* (1995). Present data showed almost equivalent or slightly higher percentage than their report.

Pereira *et al.* (1984) and Garland *et al.* (1986)reported that lignin contents in *E. camaldulensis* did not show much difference in radial and longitudinal positions in a trunk. The samples of present experiment was cut from the similar height from the ground level and from the outer part of sapwood. The comparison of data among 5 clones, therefore, was considered to be reasonable. It was concluded that Klason lignin of 5 clones ranged from 24.9 to 27.4% in dry original wood basis.

3. 3. S/V molar ratio

S/V ratio is the important factor to particularize lignin building units. In application view point, the sample with large S/V ratio has advantage in pulping process because it demands lesser amount of alkali for pulping (Collins *et al* 2000) and it is delignified faster (Chang and Sarkanen 1973, Collins *et al.* 2000) than those having lower S/V ratio.

It is reported that S/V ratio showed variation among species (Yoshinaga *et al.* 2000). For example, *Aesculus turbinata* showed 2.20, 2.18 and 2.24 for three samples and *Betula grossa* 3.14 and 3.24.

Ona *et al.* (1995) investigated radial and longitudinal variations of S/G ratios (corresponding to S/V ratios in this report) in a trunk. They investigated each two trees of *E. camaldulensis* and *E. deglupta* reporting that former species showed 1.73-1.99, 1.39 - 2.03 and the latter showed 2.78 - 3.25, 3.43 - 4.12. In their method thioacidolysis was adopted to avoid the effect of phenolic substances other than lignin to evaluate S/G ratios. From their results large difference was considered to exist in the same genus *Eucalyptus*.

Within species, for example, S/V ratios showed in the range from 1.4 to 2.4 in *Quercus mongolica* in which water conducting unites such as vessels, vessel tracheids indicated smaller S/V ratios of lignin whereas the mechanical supporting tissues such as wood fibers indicated greater S/V ratios (Yoshinaga *et al.* 1993).

In the present research S/V ratios among clones in the same species were investigated. S/V ratios obtained by alkaline nitorbenzene oxidation are listed in Table 1. Among 5 clones, clone 3 had slightly higher value and clones 1 and 5 showed slightly lower values.

In the evaluation of S/V ratio in *Eucalyptus* polyphenols extracted by alkaline should be considered (Watanabe *et al.* 1997). In the present experiment S/V ratio was determined using wood meal after extractions. Therefore, it was considered that S/V ratios of present experiments represented the data of lignin. For the comparison of present data with those reported by Ona *et al.* (1995), differences in methodology in analyzing lignin should be pointed out. The direct comparison of present data with their data was considered not to be proper.

In addition to S/V ratio, total yield of S plus V was also calculated. Results are listed in Table 1. For pulping, the yield of S plus V would be another important factor in addition to S/V ratio because the yield reflects the degree of condensation in lignin. Higher value of the yield suggests

Table 1 Chemical analyses of lignin.

Clone	Ethanol-toluene (%)	1% NaOH (%)	Klason lignin*1 (%)	Klason lignin*2 (%)	S/V ratio	(S + V) yield (%)
1	0.85	15.0	31.6	26.6	2.4	46.1
2	0.96	14.0	29.2	24.9	2.5	52.8
3	0.74	16.2	31.4	26.1	2.7	46.8
4	1.25	14.2	32.3	27.4	2.5	53.3
5	1.29	15.0	32.2	27.2	2.3	48.3

*1 Extracted wood basis
*2 Original wood basis

that lignin is less condensed and that lignin would be more easily solubilized duriong pulping. . The yield of S plus V ranged from 46 to 53%. These data were higher than those reported by Collins *et al* (1990) for *E. deglupta* (35.9%) and *E. tereticornis* (30.1%).

3. 4. UV microscopic spectrophotometry

Photograph 3 shows a 1 μm thick section in which 5 transverse sections of each clone are included. Photograph 4 shows the transverse sections of 5 clones observed under UV microscope.

Ten wood fibers which were not adjacent to vessel elements and were supposed to be cut at the central part of a cell were selected. In microscopic spectrophotometry S_2 layer of each fiber in an area of 2 μm spot was measured (Photo. 5).

After calculating average of 10 spots and smothing treatment by moving average method, UV spectra of 5 clones are indicated in Fig. 2. Absorption maximum (λ max), absorbance at aborption maximum and A_{273}/A_{280} ratios are listed in Table 2.

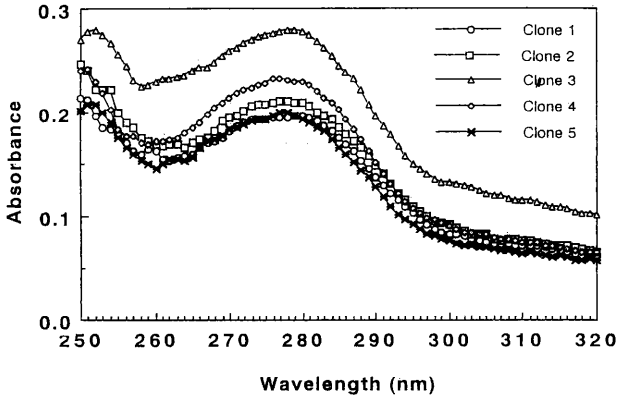

Fig. 2 UV absorption spectra of 5 clones.

Table 2 Data related to Ultraviolet microscopic spectrophotometry.

Clone	λ max	Absorbance	A_{273}/A_{280}
1	278 (3.0)	0.198 (0.05)	0.989 (0.06)
2	277 (1.2)	0.210 (0.04)	0.985 (0.02)
3	278 (1.5)	0.279 (0.03)	0.975 (0.01)
4	277 (1.1)	0.233 (0.03)	0.982 (0.03)
5	277 (0.5)	0.199 (0.03)	0.995 (0.03)

Numbers in parentheses are the standard deviation

When compare the UV absorbance and Klason lignin, clones with greater Klason lignin content showed not always higher UV absorbance. One of the reasons of this difference was considered to be the differnces in methodology. That is, the absorbance was measured in a 2 μm circle area of S_2 layer of wood fibers. Klason lignin was, however, analyzed from wood which included vessels and parenchyma cells in addition to wood fibers. As Yoshinaga *et al.* (1993) reported, the water conducting tissues indicated smaller S/V ratios of lignin than those of the mechanically supporting tissues. In Table 2, if there would not be large differences in S/G ratio of fibers among clones, it would be considered that lignin content of S_2 layer of fiber of Clone 3 would be slightly higher. In the future researches, we will clarify lignin characteristics in relation to anatomical features.

From the investigations of factors related to lignin, some variations were found out among clones. The sample trees used in the present study were different clones from *in vitro* propagation, tissue culture. Each clone, therefore, is considered to be controlled by genetic factors. It was concluded that lignin showed variations among clones.

In the present study some preliminary investigations of lignin were exclusively reported. Another aspects such as anatomical characteristics, specific gravity, minute organization of fibers such as cell wall thickness, microfibril angels have been investigated parallel with lignin. We will finally evaluate characteristics of 5 clones including all of the aspects investigated.

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