韓國林學會誌 79(2) : 109-114. 1990.
Jour. Korean For. Soc. 79(2) : 109-114. 1990.

In Vitro Plantlet Regeneration from Axillary Buds of *Tilia amurensis* Mature Trees and Clonal Variation in Tissue Culturability[1]

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피나무 成熟木의 腋芽培養에 의한 幼植物體 再生과 組織培養能力에 있어서의 클론間 變異[1]

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ABSTRACT

The axillary buds of 15-year-old *Tilia amurensis* were cultured on Saito and Ide (IS), Murashige and Skoog (MS) media and woody plant medium (WPM) to establish an effective micropropagation method. Five levels of 6-benzylaminopurine (BAP) were tested. On IS medium and WPM addition of 1.0mg/l BAP enhanced shoot development and shoot elongation, whereas addition of 0.5mg/l BAP was effective on MS medium. A better results were obtained from WPM with 1.0mg/l BAP and MS with 0.5mg/l BAP. Developed shoots were subcultured on each basal media but with 0.2mg/l BAP. Multiple shoots were almost doubled in a month. Root formation could be enhanced at higher concentration of indole -3-butyric acid (IBA). Better rooting rate (83.3%) was achieved on a half-strength MS medium with 3.0 mg/l IBA. Regenerated plantlets were successfully transferred to soil. To investigate the clonal variation in shoot development and shoot elongation by axillary bud culturing, seven plus tree clones were tested. Clonal variation in tissue culturability among plus trees was recognized by the Duncan's multiple range test at the 5% level. Kang Won No. 12 showed the best response on WPM with 1.0mg/l BAP.

Key words : Tilia amurensis, in vitro *plantlet regeneration, clonal variation, tissue culturability*.

要 約

피나무의 組織培養에 依한 大量增殖 方法을 確立하기 위하여, 15年生 成熟木의 腋芽를 IS, MS 및 WPM培地에 培養하였다. 5水準의 BAP濃度가 檢定되어, IS와 WPM培地에서는 BAP 1.0mg/l 添加가 줄기 形成과 生長을 促進한 反面, MS培地에서는 BAP 0.5mg/l 添加가 效果的이었다. 最適條件은 BAP 1.0mg/l 添加의 WPM과 0.5mg/l 添加의 MS培地이었다. 形成된 줄기는 多莖誘導를 위하여 BAP 0.2 mg/l 添加 培地에 移植하여, 한달안에 約 2倍로 增殖되었다. 發根은 高濃度의 IBA에서 促進되어, IBA 3.0mg/l 添加의 1/2MS에서 83.3%의 發根率을 얻었다. 再分化된 幼植物體는 環境馴化될 수 있었다. 腋芽培養에 依한 줄기 形成과 生長面에서 클론間 變異를 調査하기 위하여 秀型木 7클론이 檢定되었다. 組織培養能力에 있어서 秀型木 클론間 變異는 Duncan의 多重檢定 5% 有意水準에서 有意性이 認定되었으며, 江原 12號가 BAP 1.0mg/l 添加 WPM에서 가장 좋은 反應을 나타냈다.

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INTRODUCTION

Tilia amurensis Rupr. is one of the most valuable hardwoods in Korea. Generally *T. amurensis* is propagated by seedlings. However, it is not easy to germinate its seeds due to impermeable hard pericarp and seed coat and immaturity of embryo. Therefore, vegetative propagation might be desirable. The clonal propagation of forest trees, which provides valuable methods for tree improvement programs, has been achieved using tissue culture techniques. In particular, plantlets regenerated from mature trees are of great value, since selected genotypes can be utilized within short interval after selection.

A few have been reported about *in vitro* propagation of *Tilia* species. *In vitro* propagation from juvenile seedlings and mature trees of *T. cordata* have been achieved (Chalupa, 1984; Youn *et al.*, 1988). And Youn *et al.* (1989) have reported that *T. amurensis* could be *in vitro* propagated from juvenile seedlings.

This report describes *in vitro* plantlet regeneration from axillary buds of 15-year-old trees and the clonal variation in tissue culturability among seven plus tree clones of 34- to 44-year-old *T. amurensis*.

MATERIALS AND METHODS

Experiment I

Five 15-year-old *Tilia amurensis* trees, growing within the Institute of Forest Genetics, Suwon, were used as the source of test materials. Axillary buds were taken in mid-May, 1989, from leafed branches which had elongated in the previous year. The buds were surface-sterilized by soaking in 70% ethanol for two minutes, and then in 2% sodium hypochlorite for seven minutes. And the buds were washed with sterile distilled-water three times. Then they were dried on sterilized filter paper on a clean bench and cut into about 10 to 15mm lengths. These buds were cultured in autoclaved 25 x 150mm test tubes containing 8ml of agar nutrient media.

Three basal media of Saito and Ide (IS) (1985), Murashige and Skoog (MS) (1962) media and woody plant medium (WPM) (Lloyd and McCown, 1980) were tested. All three media were modified by the same method as described in Youn *et al.* (1988, 1989). For developing shoots, all media contained 20g/l sucrose and were supplemented with five levels (0.2, 0.5, 1.0, 2.0, and 5.0mg/l) of BAP concentration. After 4 weeks of initial culture, explants were transferred to each basal media supplemented with 0.2mg/l BAP to induce multiple shoots. For rooting from shoots, MS medium and WPM with a half strength salts and Gresshoff and Doy (GD) (1972) medium with full strength salts were used. Rooting media contained 10g/l sucrose and three levels (0.03, 0.3, and 3.0mg/l) of IBA concentration. The media were adjusted to pH 5.8 before addition of agar (Difco Bactor, 0.8%) and autoclaved for twenty minutes at 120℃.

Cultures were maintained with a 16 hour light/8 hour dark cycle under white fluorescent-light at an intensity of about 5,000 lux and a temperature of 25℃.

Rooted plantlets were transferred to four different potting mixtures which were perlite, peat moss : perlite (1 : 1), perlite : vermiculite (1 : 1), and peat moss : perlite : vermiculite (1 : 1 : 1). Pots were placed in the greenhouse.

Experiment II

Seven 34- to 44-year-old plus tree clones were tested on the clonal variation in tissue culturability (Table 1). Axillary buds were taken early June, 1989, from leafed branches which had elongated in the previous year. The buds were surface-sterilized by soaking in 70% ethanol for three minutes, 3% hydrogen peroxide for fifteen minutes, and then 2% sodium hypochlorite for ten minutes. The preparation of explants was the same as the Experiment I.

WPM with 1.0mg/l BAP was used for culturing medium. After 3 weeks of initial culture, explants were cultured again on the same media for 3

Table 1. General description of *T. amurensis* plus tree clones.

Plus tree No.		Location	Age (years)	Height* (m)	DBH* (cm)
Kang Won	8	Pyong-chang, KangWon	34	16.5	25.5
	9	〃	43	20.0	28.0
	12	Hongcheon, KangWon	38	16.0	25.0
	13	〃	42	17.0	26.0
	14	〃	44	18.0	29.0
	15	〃	36	16.0	23.0
	16	〃	39	21.0	26.0

* Data were taken at that time of selection made from 1976 to 1979.

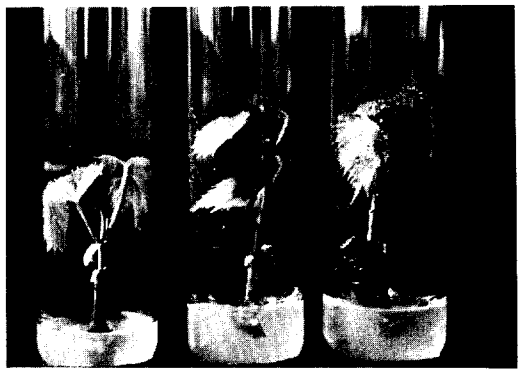

Fig. 1. Shoots developed from axillary buds of 15-year-old trees on WPM with 1.0mg/l BAP.

weeks. And then the shoot development rate and shoot length were measured. Each treatment was replicated three times with 30 explants. The shoot development rate and shoot length were analysed statistically by the Duncan's multiple range test at the 5% level.

RESULTS AND DISCUSSION

Shoot development and multiplication

Bud burst occurred within 2 or 3 weeks on all media and at all concentrations of BAP tested, and the shoots developed from axillary buds about 3 to 4cm of length after 4 weeks of culture (Fig. 1). Some differences of shoot development and shoot elongation among media were observed (Table 2). On IS media and WPM shoot development and shoot elongation were better on 1.0mg/l BAP than on higher and lower concentrations. Especially, shoot development and shoot elongation were suppressed on higher concentrations of BAP, while on MS media lower concentrations (0.2-0.5mg/l) of BAP were effective. The best results were obtained from WPM with 1.0mg/l BAP and MS medium with 0.5mg/l BAP. These results differ from those of *T. cordata* mature trees in which higher concentrations of BAP (1.0-2.0mg/l) enhanced shoot development and shoot elongation from axillary buds (Youn *et al.*, 1988). This may have resulted from differences of genetic character of species. WPM with 1.0mg/l BAP were selected for use in the further studies.

To induce shoot multiplications, nodal stems with a bud were cultured on basal media containing 0.2mg/l BAP after 4 weeks of culture. About two-fold increase in number of shoots were observed (Table 3). The best shoot multiplication and shoot elongation were obtained from WPM and IS medium, respectively. On MS medium shoot multiplication and shoot elongation were suppressed. The multiplication rates of mature tissues were low in comparison with those of juvenile seedlings of the same species which were

Table 2. Effect of interactions of media and BAP on shoot development and shoot elongation from axillary buds of 15-year-old *T. amurensis**.

Media	BAP(mg/l)				
	0.2	0.5	1.0	2.0	5.0
IS	57.1[a]/29.5[b]	78.6/36.4	82.8/37.2	59.3/30.0	39.3/26.2
MS	85.7 /38.6	89.3/41.2	78.6/35.6	55.6/31.2	28.6/27.4
WPM	66.7 /39.2	79.3/38.7	89.7/40.8	58.6/36.2	33.3/21.4

[a] Shoot development rate(%)

[b] Mean length of shoot/explant(mm)

* Data were taken from 27-30 explants per treatment after 4 weeks of culture.

Table 3. Multiple shoots formation on each basal media supplemented with 0.2mg/l BAP*.

Media	No. of nodal stems cultured	No. of shoots multiplicated	Multiplication rate	Mean length of shoot (mm)
IS	60	93	1.6	31.2
MS	60	78	1.3	19.4
WPM	60	107	1.8	29.7

* Data were taken after 4 weeks of subculture.

multiplicated average 2.6 fold and maximum 6 fold (Youn *et al.*, 1989). This may have resulted from differences of physiological conditions between juvenile and mature stages.

Rooting from shoots

Following the shoot multiplication, shoots were excised and placed on rooting media, MS medium and WPM with a half strength salts and GD medium with full strength salts containing 10g/l sucrose and three levels of IBA. The lower part of shoots swelled slightly and the roots were formed within 4 to 5 weeks and showed well developed root systems (Fig. 2). The rooting percentages after 6 weeks of culture varied with IBA concentrations used (Table 4). The best result was obtained from MS medium with 3.0mg/l IBA. Rooting were highly enhanced on higher concentration (3.0mg/l) in comparison with lower concentrations (0.03 and 0.3mg/l) of IBA regardless of the media. These results are same to those obtained with *T. cordata* mature trees (Youn *et al.*, 1988), and suggest that *in vitro* shoots derived from mature tissues of *Tilia* species can be rooted by higher concentration of IBA.

Table 4. Effect of media and IBA on rooting from shoots developed*.

Media	Concentration of IBA (mg/l)	No. of shoots cultured	No. of shoots rooted	Percentages of rooting
1/2 MS	0.03	29	0	0.0
	0.3	29	1	3.4
	3.0	30	25	83.3
1/2 WPM	0.03	29	0	0.0
	0.3	30	2	6.7
	3.0	29	21	72.4
GD	0.3	29	4	13.8

* Data were taken after 6 weeks of culture.

Transfer of plantlets and their establishment on the soil

The *in vitro* regenerated plantlets were taken out from the culture test tubes, washed thoroughly to remove the agar medium and transplanted to 15 x 8cm vinyl pots four non sterile, different potting mixtures. Potted plantlets were placed in the greenhouse and high humidity was maintained by covering the plantlets with transparent plates for 14 days, followed by watering with 0.1% Hyponex every three days.

Transplanted plantlets started shoot elongation (Fig. 3). After 4 weeks of planting, differences among the treatments in survival rate were not observed (Table 5). Best result (66.7%) was obtained from peat moss : perlite : vermiculite (1 : 1 : 1). After 4 weeks of planting, survived plantlets were subsequently transferred to larger pots.

Fig. 2. Rooting on 1/2MS medium with 3.0mg/l IBA.

Fig. 3. Acclimation of potted plantlets after 4 weeks of planting in the greenhouse.

Table 5. Survival rate and growth of plantlets in different potting mixtures after 4 weeks of planting in the greenhouse.

Potting mixtures*	No. of plantlets planted	No. of plants survived (%)	Mean length of shoot (cm)
PER	18	9(50.0)	6.2
PEAT : VER (1 : 1)	18	10(55.6)	5.6
PER : VER (1 : 1)	18	6(33.3)	7.5
PEAT : PER : VER(1 : 1 : 1)	18	12(66.7)	7.9
Total	72	37(51.3)	6.8

* PEAT : peat moss, PER : perlite, VER : vermiculite

Clonal variation in tissue culturability

The shoot development and shoot elongation from axillary buds *in vitro* was studied with seven plus tree clones on WPM supplemented with 1.0 mg/l BAP. Percentages of success in culture without fungi contamination were ranged from 71.7 to 88.9% depending on clones (Table 6). In spite of extensive treatments of surface-sterilization, more contamination rate was observed. Axillary buds began to break within 3 weeks, and then were subcultured to the same media to promote the shoot development and shoot elongation. After 6 weeks from initial culture, shoot development rate and shoot length were measured. The shoot development and shoot elongation varied with clones (Table 6). Significant differences of clonal variation in tissue culturability in the view of shoot development rate and shoot length were observed among seven plus tree clones. Plus trees Kang Won (K.W.) No. 12, 13, and 16 showed better shoot development rate than K.W. No. 8. And similar trend was observed in shoot elongation. The best shoot development rate and shoot length were obtained from K.W. No. 12.

Rutledge and Douglas (1988) found that capability for establishment of shoot cultures of *Populus* was strongly affected by genotype. Tricoli *et al.* (1985) reported the clonal differences in shoot development of *Prunus serotina* and Chalupa (1984) also found that shoot multiplication of *Tilia cordata* varied with clones. It is proved that tissue culturability varied with clones of *T. amurensis*.

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Table 6. Clonal variation in tissue culturability among *T. amurensis* plus trees in the view of shoot development and shoot elongation from axillary buds.

Plus tree No.	No. of explants cultured	No. of explants without contamination	No. of shoots developed (%)*	Mean length of shoot (mm)*
K.W. 8	90	73(83.1)	13(17.8)^{b**}	17.5^{bc}
9	90	67(74.4)	20(29.9)^{ab}	20.2^{abc}
12	90	76(84.4)	37(48.7)^{a}	24.8^{a}
13	90	64(71.1)	28(43.8)^{a}	21.6^{ab}
14	90	74(82.2)	24(32.4)^{ab}	15.6^{bc}
15	90	76(84.4)	15(19.7)^{b}	14.1^{c}
16	90	80(88.9)	31(38.8)^{a}	21.3^{ab}

* Mean of three replications with 20-27 explants per treatment after 6 weeks from initial culture.
**Letters are grouping by the Duncan's multiple range test at the 5% level.

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