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Studies on Micropropagation of *Quercus acu tissima* Carruth

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In order to determine the optimal condition for propagation of *Quercus* ***acutissima,*** shoot propagation, root formation and somatic embryo propagation have been tested using several elite clones. Concerning shoot propagation the concentration of BAP supplemented was effective. The kind and concentration of auxins added affect root formation. The concentration of sucrose is important for embryo propagation. However, it becomes evident that these sensitivities are dependent on individual clones.

INTRODUCTION

About **450** *Quercus* spp. have been distributed in the world and these species are crops of major importance to horticultural and forest industry such as for timber, tan bark or cork. *Q. acutissima* Carruth has a typical importance in Japan for maintaining the resources of bed logs of *Lentinus edodes* Singer (Shiitake mushroom). However, since the mass propagation of *Quercus* spp. has not been established yet except seedling propagation, a large amount of acorns of Q. *acutissima* (approximately 10 tons) has been imported from Korea. Therefore, approximately 700 elite trees of Q. *acutissima* which had the superior growth and mushroom-production abilities were selected in Japan and the propagation project by tissue culture started from about 8 years ago.

The clonal propagation of select strain is a useful method of accelerating the breeding of this tree. However, the Fagaceae trees such as *Castanea* spp. and *Quercus* spp. have been difficult to culture *in vitro* (Cai *et al. 1987,* McCown and McCown 1987), possibly due to a higher tannin contents such as the typical tannins, acutissimin A and B together with various other tannins which were isolated from the bark of Q. *acutissima* (Nonaka *et al., 1984;* Ishimaru *et al.,* 1987a,b). However, some success has been achieved in the tissue culture propagation of Q. *acutissima* Carruth (Lee et *al.,* 1985; Ide and Yamamoto, 1986; Haraguchi, 1987). We have also established the clonal propagation method by somatic embryogenesis using embryonic axes (Sasaki *et al.,* 1988a) and meristem shoot tip (Sasaki and Shoyama, 1988b; Shoyama *et al., 1992,* Sasaki and Shoyama, 1993).

We wish here to report the effect of clone on shoot propagation, root formation of regenerated plants and propagation of somatic embryos in Q. *acutissima* tissue culture.

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MATERIALS AND METHODS

The greenwoods were collected form 2 -year-old Q. *acutissima* (originated from seedlings or grafting) of elite trees grown in green house and dissected into explants having axillary bud (approximately 20 mm length) and washed with tap water, sterilized with 70% alcohol for 3 min, with 2% NaOCl for 10 min, and then with 70% alcohol for 3 min and finally washed twice thoroughly with sterilized water. The basal medium consisted of half-strength Murashige and Skoog (MS) salt (Murashige and Skoog, 1962) or woody plant (WP) medium (Lloyd and McCown, 1980), both of which contained (in mg/l); myoinositol, 100; nicotinic acid, 0.5; pyridoxine HCl, 0.5; thiamine HCl, 0.1; glycine, 2.0; sucrose, 30,000 (MS) or 20,000 (WP); gerlite, 3,000. Media were supplemented with auxins (NAA, IAA and IBA) or BAP in various concentrations and combinations (see Tables). Culture tubes, containing 30 ml of medium, were adjusted to pH 5,8 before autoclaving. Cultures were incubated in 16 hr light from cool white fluorescent tubes (4,000 lux) at a temperature of 25 ± 1 "C.

Seeds of Q. ***acutissimu*** collected from the elite trees in the open-pollinated orchard (Hita city in Ooita prefecture) were washed with tap water and then sterilized with 3% NaOCl for 10 min, then with 70% alcohol for 30 sec and finally washed twice thoroughly with sterilized water. The cotyledons containing embryonic axes aseptically dissected (4 mm cubes). The segments were aseptically dissected out form the cotyledon. Embryonic axes were cultured on half-strength MS medium containing GA and BAP (1 mg/l each) for 6 weeks to form immature secondary somatic embryos (Sasaki et ***al. 1988).*** Somatic embryos produced were subcultured on WP medium containing 1.0 mg/l BAP and varying concentrations of sucrose for 10 weeks.

All the measured values were calculated by variance analyses of one- way layouts, and the statistical significances of differences were determined by Duncan's new multiple range test.

RESULTS AND DISCUSSION

Effects on shoot propagation and shoot elongation

In order to propagate the elite tree, the effects of temperature, sucrose and BAP concentrations were investigated using the selected clones as indicated in Tables 1 to 3. First of all, the effects of temperature on shoot multiplication rate and shoot elongation were investigated by using the axillary buds of greenwood of 2 clones as explant. Table 1 shows that the higher temperature clearly accelerates the shoot propagation and shoot elongation except that of the Innai No. 2 clone at 25°C.

Table 2 indicates the effects of sucrose concentration. Shoot number in the strain of 18-3 increased dose-dependently, but the addition of 20 g/l sucrose mostly stimulated shoot propagation in the case of the strain 20-6. Althought the sucrose consentration dose-dependently responded to the shoot elongation in the 20-6 clone, the optimal shoot elongation occurred by the addition of 20 g/l sucrose in the 18-3 clone.

In the previous paper, the effects of BAP and a new cytokinin, TG - 19 on shoot elongation and propagation were reported using a single clone as preliminary results (Sasaki ***et al. 1993).*** The addition of BAP promoted shoot propagation, but suppressed

Table 1. Influences of temperature on shoot multiplication and shoot elongation in *Quercus acutissima* culture.

Clone	Temp. (°C)	Multiple shoot forming culture	Av. shoot No. per culture	Avshoot height per culture (mm)
Bungo-takada No.1	20	10/10	2.30 ±0.90a	7.1±4.7a
	25	5/ 8	3.40 ±1.36a	8.2±5.5a
	30	6/ 8	8.17±2.54b	8.8±6.6a
Innnai No.2	20	8/13	2.13 ±1.45a	6.5±3.5a
	25	4/ 9	3.75±1.64a	15.3±8.5c
	30	9/11	4.67±3.30a	10.5±6.2b

Culture conditions: WP medium containing 1 mg/l BAP, 25°C, 16 hr light, 5 weeks.
In the same column, numbers followed by same letter are not significantly different at the 5% level of confidence using Duncan's new multiple range test.

Table 2. Influences of sucrose concentrations on shoot multiplication rate and shoot elongation in Quercus ***acutissima*** culture.

Clone	Conc. (g/l)	Multiple shoot forming culture	Av. shoot No. per culture	Av. shoot height (mm)
18-3	10	4/ 5	3.00±0.71a	6.2 ±4.4a
	20	8/ 9	3.38 ±1.87a	8.0±6.4a
	30	7/ 8	4.14±1.46a	5.5 ±4.7a
20-6	10	6/ 7	4.00±2.39a	11.8±10.6a
	20	7/ 7	7.71 ±2.75a	13.2±11.8a
	30	11/11	5.27±2.90a	14.7±10.1a

Culture conditions: WP medium supplemented with 1 mg/l BAP, 25°C, 16 hr light, 5 weeks.
In the same column, numbers followed by same letter are not significantly different at the 5% level of confidence using Duncan's new multiple range test.

Table 3. Influences of BAP concentration on shoot multiplication rate and shoot elongation in Quercus ***actissima*** culture.

BAP (mg/l)	44-1 Avshoot No.	44-1 Av.Shoot height (mm)	Innai No.2 Avshoot No.	Innai No.2 Av.Shoot height (mm)
0.1	3.80 ±1.47a	14.0±10.2b	2.20±0.98a	4.2 ±2.2b
0.5	4.80±1.83a	6.6±5.3a	3.33±0.75ab	4.0±2.4b
1.0	3.50±2.06a	8.6±5.0a	3.17±0.37ab	3.3 ±1.2ab
2.0	6.33±1.25b	5.3 ±2.9a	4.25 ±0.83b	2.5 ±0.8a

Culture conditions: WP medium, 25°C, 16hr light, 6 weeks.
In the same column, numbers followed by same letter are not significantly different at the 5% level of confidence using Duncan's new multiple range test.

shoot elongation in spite of TG-19 supplement (Sasaki et *al.*, 1993). Table 3 shows the effect of BAP concentration on shoot propagation and shoot elongation. Shoot propagation is roughly proportional to the BAP concentration in the both clone. On the other hand, the shoot elongation is inversely proportional to BAP concentration. The higher levels of BAP produced higher shoot number. However, BAP higher than 0.5 mg /l suppressed shoot bud elongation to be similar primordia shape suggesting that the most favorable BAP concentration is 0.5 mg/l. From above three experiments, it is clear the sensitivity for shoot propagation and/or shoot elongation varied by individual clones.

Effects on root formation

It is well-known that the most important factor for root formation is the kind and the concentration of auxins in many plants. The effects of auxins for rooting of Q. *acutissima* have been tested by many groups (Ide and Yamamoto, 1980; Lee et al., 1985; Nakazawa and Toda, 1987; Sato, 1991). In the previous paper, we have demonstrated the favorable condition for root formation was the half strength WP medium supplemented with 0.5 mg/l IBA (Sasaki *et al.*, 1988). In this investigation the effects of auxins beside with sucrose concentrations and culture temperature have been carried out using different strains.

Table 4 shows variations of auxins on two elite clones regarding root forming ratio, root number and root length. The supplement of 0.1 mg/l IBA mostly stimulated root formation (81.8%), and the addition of NAA 0.1 mg/l was 66.7% of root formation when strain I was tested. Root number was best in hormone free medium, and root length was the best in medium supplemented with 0.1 mg/l NAA. On the other hand, clone II was insensitive against auxins in general. Supplements of two different concentration of IBA was indicated in Table 5. The good result for root formation using clone I was obtained by the supplement of 0.1 mg/l IBA rather than that of 1 mg /l IBA. No significant differences on root formation ratio was shown on clone II. Root

Table 4. Influences of auxins on root formation of propagated shoot in *Quercus* ***acutissima*** culture.

Clone	Auxin (mg/l)	Root forming shoot	Av. root No. per shoot	Av. root length per shoot (mm)
I	IAA (0.1)	6/12	5.33 ±3.35b	66.7±13.7a
	IBA (0.1)	9/11	1.89±0.74a	58.2±30.0a
	NAA (0.1)	8/12	4.88 ±2.98b	85.4±19.3a
	HF*	6/12	7.67 ±3.49b	53.0±25.0a
II	IAA (0.1)	4/15	5.75±2.26a	47.0±12.8a
	IBA (0.1)	5/15	4.80±2.20a	75.4 ±28.7a
	NAA (0.1)	5/14	4.20±2.03a	69.4±24.9a
	HF*	3/16	2.33±1.25a	43.0±6.9a

Culture condition:1/2WP medium, 25°C, 16 hr light, 8 weeks.
* Hormone free medium.
In the same column, numbers followed by same letter are not significantly different at the 5% level of confidence using Duncan's new multiple range test.

Table 5. Influences of IBA concentration on root formation of propagated shoots in Quercus ***actissima*** culture.

Clone	IBA conc. (mg/l)	Root forming shoot	Root No. per shoot	Root length per shoot (mm)
I	0.1	16/18	6.31±4.01a	57.4 ±20.0a
	1.0	8/20	5.38±3.50a	41.4±19.3a
II	0.1	7/18	1.71±0.45a	60.3±23.0b
	1.0	5/16	2.17±1.07a	26.8±10.2a

Culture condition:1/2WP medium, 25°C, 16 hr light, 8 weeks.
In the same column, numbers followed by same letter are not significantly different at the 5% level of confidence using Duncan's new multiple range test.

formation and its growth was also inhibited on clone II compared with clone I.

Table 6 shows the effects of sucrose concentration on root formation ratio and root growth using two clones. The addition of sucrose dose -dependently promoted root formation and root formation ratio, and reached to the maximum by addition of 10 g/l sucrose resulting in 80% of root formation occurred, and thereafter turned to inhibition. Same tendency was observed on clone II although sensitivities against sucrose concentrations were different between 2 clones.

Table 6. Effects of sucrose concentration on root formation in Quercus ***acutissima*** culture.

Clone	Sucrose conc. (g/l)	Root forming culture	Av.root No. per culture	Av.root length per culture (mm)
I	2	10/22	1.70 ±0.82a	40.0±21.3a
	5	14/23	2.07±1.33a	45.4 ±26.2a
	10	16/20	2.31 ±1.49a	43.8±22.5a
	20	19/25	2.00±0.88a	48.2 ±23.6a
II	2	3/ 9	1.67±0.58a	20.7±5.1a
	5	7/11	1.71±0.95a	43.6 ±20.6b
	10	6/10	2.00±1.26a	61.7±35.4b
	20	4/10	1.75±0.96a	52.5 ±23.3b

Culture condition:1/2WP medium, 25°C, 16 hr light, 8 weeks.
In the same column, numbers followed by same letter are not significantly different at the 5% level of confidence using Duncan's new multiple range test.

Effects on embryo propagation

We have already established the optimal conditions for propagation of somatic embryos and regeneration from propagated embryos (Sasaki et ***al.*** 1988). However, since it was evident that the influence of clone used for culture was larger in shoot propagation, the ability of somatic embryo propagation was also tested using two clones.

The effect of sucrose concentrations for the propagation of secondary somatic

embryo was investigated as shown in Table 7. In the case of clone A, the weight of embryo cultured for 10 weeks was dose dependent with respect to the sucrose concentrations. However, the supplement of 80 g/l sucrose inhibited the propagation of embryo in clone B. Moreover, the addition of 80 g/l sucrose promoted propagation of irregular shaped embryos in clone A. On the other hand, the addition of 10 g/l sucrose stimulated regeneration of embryos as indicated in Fig. 1. From these results, the optimal concentration of sucrose is determined as 40 g/l for both clones.

Table 7. Effects of sucrose concentration on somatic embryo propagation in Quercus ***acutissima*** culture.

Clone	N*	Sucrose conc. (g/l)	Fr.wt. embryo (g/culture)	propagation rate(%) **
A	11	10	0.480 ±0.233a	100
	11	20	1.124 ±0.629b	234
	11	40	1.586 ±0.953b	330
	11	80	1.645 ± 1.011b	343
B	8	10	0.362 ±0.422a	100
	8	20	1.095 ±0.566b	302
	8	40	1.848 ±0.579c	510
	8	80	1.635 ± 1.196c	452

*· culture number
**· culculated from fresh weight of embryo cultured on medium supplemented with 10 g/l sucrose
Culture condition: WP medium, 25°C, 16 hr light, 8 weeks.
In the same column, numbers followed by same letter are not significantly different at the 5% level of confidence using Duncan's new multiple range test.

Fig. 1. Effects of sucrose concentration on somatic embryo propagation in ***Quercus acutissima*** culture.
From left hand, 10g/l, 20g/l, 40g/l and 80g/l of sucrose. Regeneration from embryos was observed on medium supplemented 10g/l sucrose.

Three groups pointed out the sensitivity for root formation of *Q. acutissima* varies by individual clones (Ide and Yamamoto, 1988; Ito, 1989; Sato, 1991). In this

investigation, we found that variations concerning shoot propagation, shoot elongation ability, root formation, root growth and somatic embryo propagation ability clearly appeared depending on clones used for culture. Since these phenomena are known in the field experiments, the dominant trees have been selected and asexual propagated in Japan as previously described. We have already confirmed the regenerated plants from somatic embryo and multiple shoot system had the normal chromosome number of $2n=24$ (Sasaki et ***al. 1988).*** Therefore, selection of elite clone is most important for utilization of tissue culture technique to facilitate large scale of Q. *acutissima* planting. We have already propagated the selected elite trees ***in vitro,*** planted them as indicated in Figure 2, and determined these characteristics. These results will be presented elsewhere.

Fig. 2. Two-year-old Quercus *acutissima* propagated *in vitro.*

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