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Clonal Propagation of *Quercus acutissima* Carruth by Somatic Embryogenesis from Embryonic Axes

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Embryonic axes of Quercus *acutissima* were used as explants to initiate somatic embryogenesis *in vitro*. The addition of 1 mg/*l* BAP and 1 mg/*l* GA stimulated secondary somatic embryo production. Mature secondary somatic embryos developed normal shoots when transferred to WPM medium containing 0.1 or 0.5 mg/*l* BAP. Root formation occurred upon transfer of these shoots to medium supplemented with 0.5 or 1 mg/*l* IBA. These plantlets possessed the normal chromosome number of 2n = 24.

INTRODUCTION

The Fagaceae trees such as *Castanea* spp. and *Quercus* spp. have been difficult to culture *in vitro* (Cai *et* al. 1987), possibly due to tannin production (Nonaka et al., 1984 ; Ishimaru *et al.*, 1987a, b). However, some success has been achieved in the tissue culture propagation of *Castanea sativa* Mill (Vieitez *et al.*, 1978 ; Vieitez *et al.*, 1980a, b ; Rodriguez *et al.*, 1982 ; Vieitez *et al.*, 1982 ; Chevre *et al.*, 1983 ; Sanjose *et al.*, 1984 ; Qi-guang *et al.*, 1986) and Q. *rubra* (Chalupa, 1979). Moreover, attempts have been made to use tissue culture for clonal multiplication and for maintaining the resources of bed logs of *Lentinus edodes* Singer (Shiitake mushroom) have been investigated on Q. *acutissima* Carruth (Lee *et al.*, 1985 ; Ide and Yamamoto., 1986). Haraguchi (1987) reported the induction of Q. *acutissima* somatic embryo from cotyledon tissue, but regeneration of normal plants from these embryos has not been achieved. Juvenile explants generally respond better in tissue cultures than do mature-tissue explants of woody species. This communication reports a clonal propagation of Q. *acutissima* by somatic embryogenesis from embryonic axes.

MATERIALS AND METHODS

Seeds of Q. *acutissima* collected from an 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

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Abbreviations

*IAA, indole-3-acetic acid ; IBA, indole-3-butyric acid ; NAA, a-naphthaleneacetic acid ; 2, 4-D, 2, 4-dichlorophenoxyacetic acid ; BAP, 6-benzyl-aminopurine ; GA, gibberellic acid A,.

sterilized water. The cotyledons containing embryonic axes were aseptically dissected (4 mm cubes). The segments were aseptically dissected out from the cotyledon. The basal medium consisted of half-strength Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) or WPM 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 (WPM) ; gerlite, 3,000. Media were supplemented with auxins (2,4-D, NAA, IAA and IBA), BAP or GA in various concentrations and combinations (see Tables). Culture tubes, containing 30 ml of medium, were adjusted to pH 5.5 before autoclaving. Cultures were incubated in 16 hr light from cool white fluorescent tubes (2000 lux) at a temperature of 25± 1°C. Other culture conditions were as previously described (Shoyama ***et al.,*** 1983). Embryonic axes were cultured on 1/2-strength MS medium containing GA and BAP (1 mg/l each) for 11 weeks. Obtained shoots were transferred to WPM medium supplemented with BAP. Somatic embryos produced during embryonic axes culture were subcultured on 1/2-strength MS medium supplemented with GA and BAP (Table 1) for 6 weeks. Matured secondary somatic embryos were transferred to WPM medium supplemented with BAP and GA (Table 2), and cultured for 9 weeks. Regenerated shoots were cultured on 1/2-strength WPM medium containing IBA for 4 weeks. Root tips were collected from regenerated plants, pretreated with 2 mM 8-hydroxyquinoline at 12°C for 12hr in 1N HCl, fixed and stained with Feulgen. Chromosomes were counted in 10 cells from each plant.

RESULTS AND DISCUSSION

Cultured intact embryonic axes developed into plantlets with swollen roots, being similar to the result of *Castanea sativa* (Vieitez 1980a). The regenerated shoots were transferred to WPM medium containing BAP, resulting in multiple shoot formation. Higher levels of BAP produced higher shoot numbers, however, BAP higher than 0.5 mg/l suppressed shoot bud elongation (Fig. 1). WPM medium containing 0.1 mg/l BAP was best for stimulating multiple shoot formation (Fig. 1) and was used for routine shoot production.

Somatic embryogenesis occassionally occurred during the 11-week embryonic axe's culture on the 1/2-strength MS medium supplemented with GA and BAP (1 mg/l each). Somatic embryo at globular, heart-shaped and matured stages were observed (Fig. 2A). Supplements of BAP alone or with GA in 1/2-strength MS medium were investigated for proliferation of secondary somatic embryo (Table 1). The addition of BAP and GA (1 mg/l each) mostly stimulated the production of secondary somatic embryos. In this case 100% of secondary somatic embryo propagation occurred. On the other hand, the addition of IAA, NAA or 2,4-D to the medium stimulated callus and tissue mass production as shown in Fig. 2B. These tissues were light green, either solid or friable. Somatic embryogenesis or adventitious shoot formation did not occur in this tissue, although the subculture to fresh medium stimulated callus production. In order to determine the effect of basal medium for proliferation of secondary somatic embryo WPM medium supplemented with GA and BAP (1 mg/l each) was investigated. The favourable embryo propagation, however, was not obtained. From these results, the 1/2-strength MS medium containing GA and BAP (1 mg/l each) was

Fig. 1. Multiple shoot formation of *Quercus acutissima* from cultured embryonic axes on WPM medium supplemented with 1 mg/*l* BAP (right) and 0.1 mg/*l* BAP (left).

Table 1. Effects of hormone treatments on somatic embryo production*

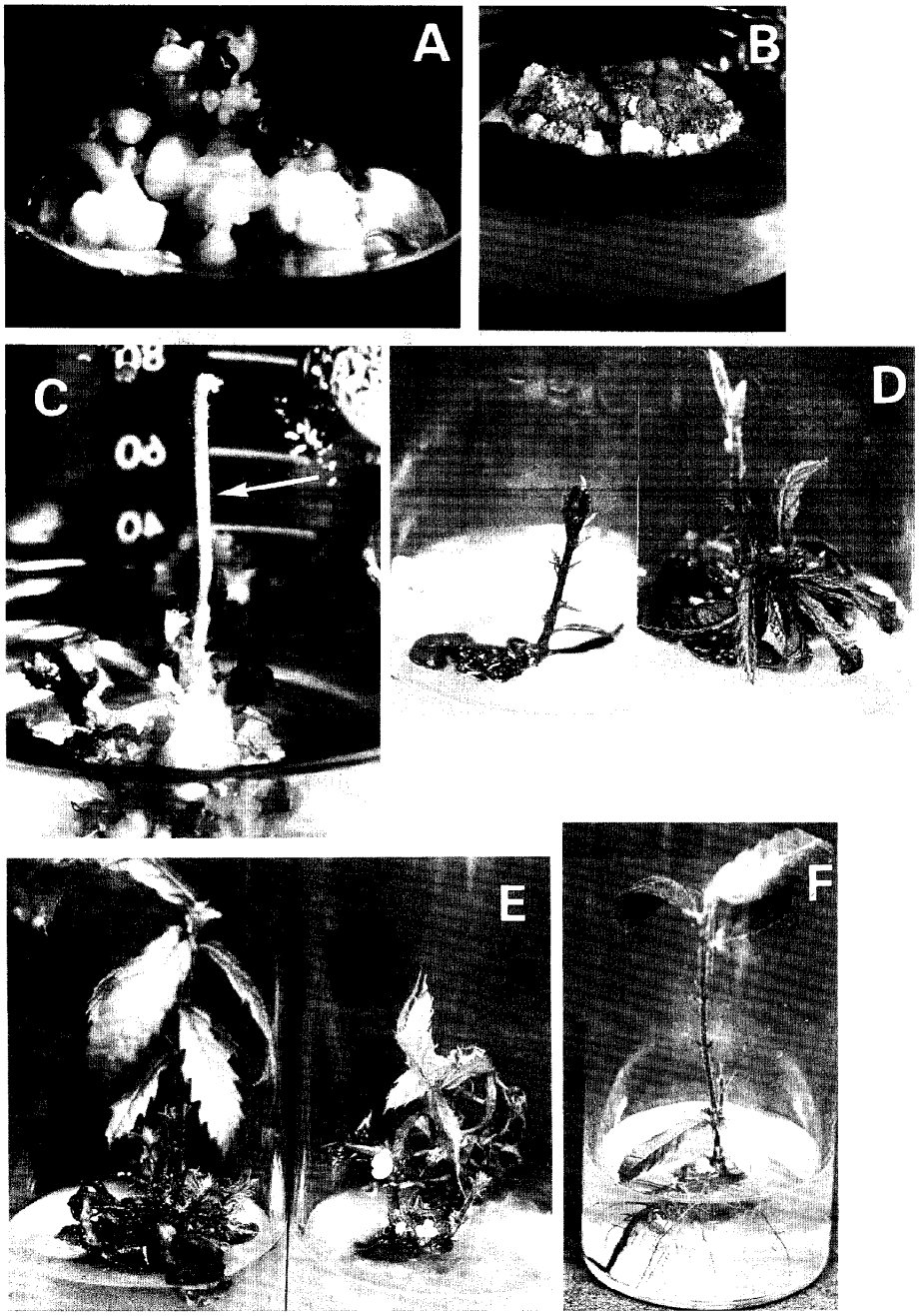
hormone (mg/l) BAP	GA	culture (%) producing somatic embryos	fresh weight (g) per culture	comments
1	0	40	0.39	heart-shaped embryo abnormal shoot formation
5	0	56	0.34	tissue mass formation abnormal shoot formation
10	0	17	0.12	tissue mass formation abnormal shoot formation
1	1	100	0.50	heart-shaped embryo
5	1	83	0.27	globular and heart-shaped embryo
10	1	25	0.25	globular and heart-shaped embryo

* Immature embryo weight approximately 30 mg. Eight to ten immature embryos in clusters were investigated in each treatment.

Table 2. Effects of hormone treatments on somatic embryo germination and shoot multiplication.

hormone (mg/l) BAP	GA	Cultures forming shoot* (%)	number shoot per culture	ave. shoot height (cm)
0.1	0	66.7	3.8	0.9
0.5	0	46.7	3.0	1.1
1	0	16.7	1	0.5
1	0.5	0		
1	1	0	–	–

* Based on samples of 24 mature embryos.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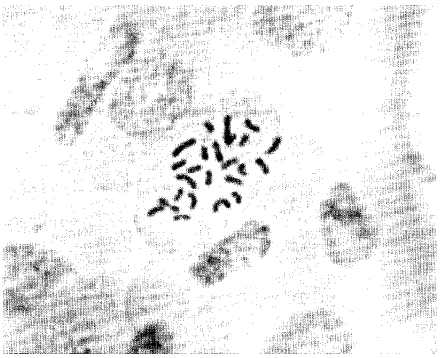

Fig. 3. Chromosomes of *Quercus* ***acutissima*** regenerated from somatic embryo (2n= 24).

routinely used as the propagation condition of secondary somatic embryo and this propagation ability was maintained for at least ‘2 years (12 generations).

The addition of GA and cytokinin to the medium is known to promote shoot regeneration from somatic embryos (Chang *et al., 1980;* Shoyama *et al.,* 1987 ; Hatano *et al., 1987).* As indicated in Table 1, since l/Z-strength MS medium containing BAP and GA stimulated secondary somatic embryo propagation without shoot regeneration, the effects of BAP alone or with GA using WPM medium were investigated (Table 2). The addition of 0.1 mg/*l* BAP mostly favoured shoot regeneration from mature secondary somatic embryos resulting in multiple shoot complex as shown in Fig. 2C and D. The experiments with supplement of GA in the presence of BAP did not affect shoot regeneration. However, when the regenerated shoots were transferred to WPM medium containing GA and BAP, multiple shoot formation was occurred as indicated in Fig. 2E.

In the final stage of this study, root formation was investigated using 1/2-strength WPM medium supplemented with 0.5 or 1 mg/*l* IBA. After 4 weeks root formation occurred (Fig. 2F) similarly as reported for chestnut (Vieitez *et al.,* 1982). Plants obtained by this process had the normal chromosome number of 2n=24 (Toda, 1965) as indicated in Fig. 3, and did not show any phenotypic abnormalities. Studies on the transplantation of regenerated plants to soil have been planned.

This is the first success of micropropagation of *Q. acutissima* by somatic embryogenesis. This procedure could be utilized to facilitate large scale planting of selected trees.

Fig. 2. Embryogenesis and regeneration of *Quercus* ***acutissima*** from embryonic axes.
(A) Somatic embryogenesis during primary embryonic axe’s culture.
(B) Callus formation from somatic embryos on 1/2-strength MS medium supplemented with 1 mg/*l* 2, 4-D.
(C) Germination of mature secondary somatic embryo on WPM medium supplemented with 0.1 mg/*l* BAP. Allow shows cotyledon.
(D) Shoot development following germination.
(E) Multiple shoot formation on WPM medium containing 1 mg/*l* BAP and 0.5 mg/1 GA (left) and 1 mg/*l* BAP and 1 mg/*l* GA (right).
(F) Root formation on 1/2-strength WPM medium containing 0.5 mg/*l* IBA.

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