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Multiple Shoot Induction from Seeds of Japanese Chestnut (*Castanea crenata Sieb. et* Zucc) and Successive Shoot Multiplication *In Vitro*

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ABSTRACT

Micro plant propagation of chestnut plants through reproductive organ culture was examined with seeds. (1) Multiple shoot buds were induced from chestnut seeds by BAP *in vitro* but not by kinetin and 2iP. Multiple shoots formed from cotyledonary nodes, an embryonic part of a seedling. Sometimes axillary buds of main shoots proliferated to grow to multiple shoots. (2) Excised single shoots from multiple shoots of initial seed cultures were continuously multiplied by using combinations of BAP (1 μ M) and IBA (0.025 μ M). Axillary buds of excised single shoots proliferated to be multiple shoots continuously. Three of four shoots were harvested every four weeks for further culture. One can calculate that more than 1,600,000 shoots could be obtained from a single shoot within a year using the present technique.

INTRODUCTION

Regeneration of plants *in vitro* can be achieved via organized and unorganized systems (Biondi and Thorpe, 1982; Hisajima *et al,* 1986). Organized systems include meristems, shoot tips, nodes, stem segments, dormant buds, hypocotyls (vegetative organ/tissue), seeds and mature/immature embryos and unfertilized ovaries (reproductive organs), and other differentiated cells. Aberrant plants are common among regenerated plants via unorganized systems but not common among those via organized systems. At present the idea is widely accepted that plant propagation via organized system is adequate for mass propagation of industrial crops and that plant regeneration through an unorganized system is useful for establishment of new variant lines in breeding programs. Mass propagation via reproductive organs *in vitro* is thought to be useful in both mass clonal propagation of desired hybrid lines and genotypes (Fischer and Zimmer, 1987) and in breeding programs combining conventional cross breeding and *in vitro* cross breeding such as *in vitro* fertilization (Hisajima *et al,* 1986) However, limited reports deal with micropropagation of plants through reproductive organs namely seeds and embryos.

To date tissue culture technique is expected to be useful in mass propagation and breeding programs of woody species (Biondi and Thorpe, 1982). As propagation of chestnut plant by conventional methods is difficult (Vieitez, 1974), tissue culture approaches are in progress. (Vieitez *et al,* 1978, Vieitez and Vieitez, 1980, Hisajima,

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1981, Hisajima, 1982, Rodorigvez, 1982, Vieitez and Vieitez 1983, Chevre *et al,* 1983, Vieitez and Ballester 1983, Sanjose *et al,* 1984, Rodoriquer, 1982 and Qi - Guan *et al,* 1986). So far multiple shoot induction from organized systems and plant regeneration from organized systems have been reported. However, successive shoot multiplications have not been reported and therefore the entire mass micropropagation technique is not complete.

The authors have been interested in both mass propagation and breeding of different plants *in vitro* through reproductive organs, organized systems. (Hisajima *et al*,1986, Hisajima, 1981, Hisajima, 1982 a, Hisajima 1982 b, Hisajima and Church 1982 Hisajima 1982 c, Hisajima 1985, Hisajima *et al*,1987, Hisajima and Arai, 1987, Hisajima *et al,* 1989, Tanaka *et al,* 1988). In this communication multiple shoot induction from seeds and successive shoot multiplication are described.

MATERIALS AND METHODS

Chestnut *(Castanea crenata* Sieb. et Zucc) seeds were purchased from local market.

After the seedcoat was removed, seeds were immersed in 70% ethanol for 1 min. Successively the seeds were sterilized in 5% hydrogen peroxide solution for 10 min. The seeds were rinsed in sterile deionized water four times. The seeds were horizontally planted in 50 ml of modified Murashige and Skoog's medium (Hisajima, 1982 c) containing plant growth regulators, white organic nutrient 3% sucrose, and 0.6% agar. The initial pH of the medium was adjusted to 5.7 with 0.1 N NaOH. Modified Murashige and Skookg's medium containing (mg/liter) : 1.650 NH_4NO_3 , 1.900 KNO_3, 440 $CaCl_2 \cdot H_2O$, 370 $MgSO_4 \cdot 7H_20$, 170 KH_2PO_4, 12.4 H_3BO_3, 33.8 $MnSO_4 H_2O$, 21.0 $ZnSO_4 \cdot H_2O$, 1.7 KI, 0.5 $Na_2MoO_4 \cdot 2H_2O$, 0.05 $CuSO_4 \cdot 5H_2O$, 0.05 $CoCl_2 \cdot 6H_2O$, 15 $FeSO_4 \cdot 7H_2O$, 18, Na_2EDTA, 500 Inositol and 5 Thiamine HCL. Twelve seeds per treatment were used. The cultures were maintained at 27°C under 18 hr illumination of 3,000 lux provided by cool white fluorescent tubes. The same medium as used in seed culture was employed to multiply excised single shoots except for hormonal constituents. The same environmental conditions were employed throughout the experiment.

RESULTS AND DISCUSSION

1. Multiple shoot induction from seeds

Multiple shoot buds have been induced from various dicotyledonous plant seeds by cytokinin alone in the experiments of this series. (Hisajima *et al*,1986, Hisajima, 1982 b, Hisajima and Church 1982, Hisajima, 1985, Hisajima *et al*,1987, Hisajima and Arai, 1987, Hisajima *et al*,1989). Effect on N^6 -benzyl aminopurine (BAP), kinetin and N^6 (2 - isopentenyl) adenine (2iP) at 0, 0.5, 2.5, 5 and 50 μ M were examined on multiple shoot induction from chestnut seeds. The seeds were cultured for four weeks and the number of shoots longer and shorter than 5 mm in length was separately counted (Table 1) Kinetin and 2iP did not induce multiple shoot buds at any of the concentrations used. Multiple shoot buds were induced in only the highest concentration range of BAP (25 - 50 μ M)(Table 1 and Figure 1-a). Many small buds shorter than 1 mm in length were observed in the culture at BAP 50 μ M.

Multiple shoots were observed from nodal regions (embryonic part of chestnut seedlings). The development of a pair of lateral shoots from cotyledonary nodes were occasionally observed in a lower concentration range in pea, cucumber (Hisajima *et al,* 1989) and peanut, while no pair of lateral shoots were observed from cotyledonary nodes of chestnut seedlings. On the other hand a longer culture in the same medium stimulated proliferation and growth of axillary buds from each shoot of the multiple

Table 1 Effect of BAP on multiple shoot induction from seedlings.

	BAP (M)					
	0.0	9.5	2.5	5.0	25.0	50.0
average shoot number/seed						
> 5 mm	1.0	1.0	1.0	1.0	0.0	0.0
1 mm < < 5mm	0.0	0.0	0.0	0.0	3.8	5.0*
Rate of multiple shoot formation (%)	0.0	0.0	0.0	0.0	100	100

Seeds were cultured for 4 weeks and the results were recorded.

* with many buds shorter than 1 mm.

shoots. When poorly - developed multiple shoot buds induced in the medium containing high concentration of BAP were transferred to a lower BAP medium such as 0 or 0.5 M, growth was highly stimulated. When seeds were cultured to the medium containing 500 μ M BAP for four weeks and transferred to the medium containing 0.5 μ M BAP, more than 4 shoots of 2 cm in length were developed from a seedling in the initial 8 weeks in culture. The shoots of 2.5 cm in length were used for the following shoot multiplication experiment. In a preliminary experiment, multiple shoots were also induced from chestnut embryos excised from raw seeds by BAP alone.

2. Successive shoot multiplication

To establish a shoot multiplication cycle in dicotyledonous plants, continuous multiple shoot induction from single shoots is usually attempted by the combination of cytokinin and auxin Hisajima *et al.* 1986, Hisajima, 1982 b, Hisajima and Church, 1982, Hisajima, 1982 c, Hisajima, 1985, Hisajima *et al.* 1987, Hisajima and Arai 1987, Hisajima *et al.* (1989). In the above experiment, axillary buds were observed to grow from multiple shoots of seed cultures. Therefore, stimulation of axillary shoot bud growth was examined. BAP (1 μ M) and indole-butyric acid (IBA)(0.025 μ M) seemed to be effective hormonal combination in general among the species in the previous experiments to multiply shoots successively. Taking account of the above results, excised single shoots longer than 2.5 cm in length were cultured in the medium containing BAP 0.5 μ M and IBA 0.025 μ M (Figure 1-b). In this condition axillary shoot buds grew well and sometimes axillary buds were observed on single shoots of multiple shoots in the cultures (Figure 1 - c). It was observed that the axillary buds on the single shoots proliferated to be multiple shoots. Every four weeks, shoots were excised and inoculated in the fresh medium. This process continued successively (single shoots → multiple shoots → single shoots →) (Figures 1-b, 1-c). Three or four shoots were continuously harvested from a culture every four weeks. One can calucalate that $3^{13} - 4^{13}$ = 1,594,323 - 67, 108, 864 shoots could be obtained from a single shoot in a year.

3. General consideration

To date deviant plants in morphological traits have not been observed at any stage of the present experiment. Further investigation should however, be carried out on the production side when industrial application is considered.

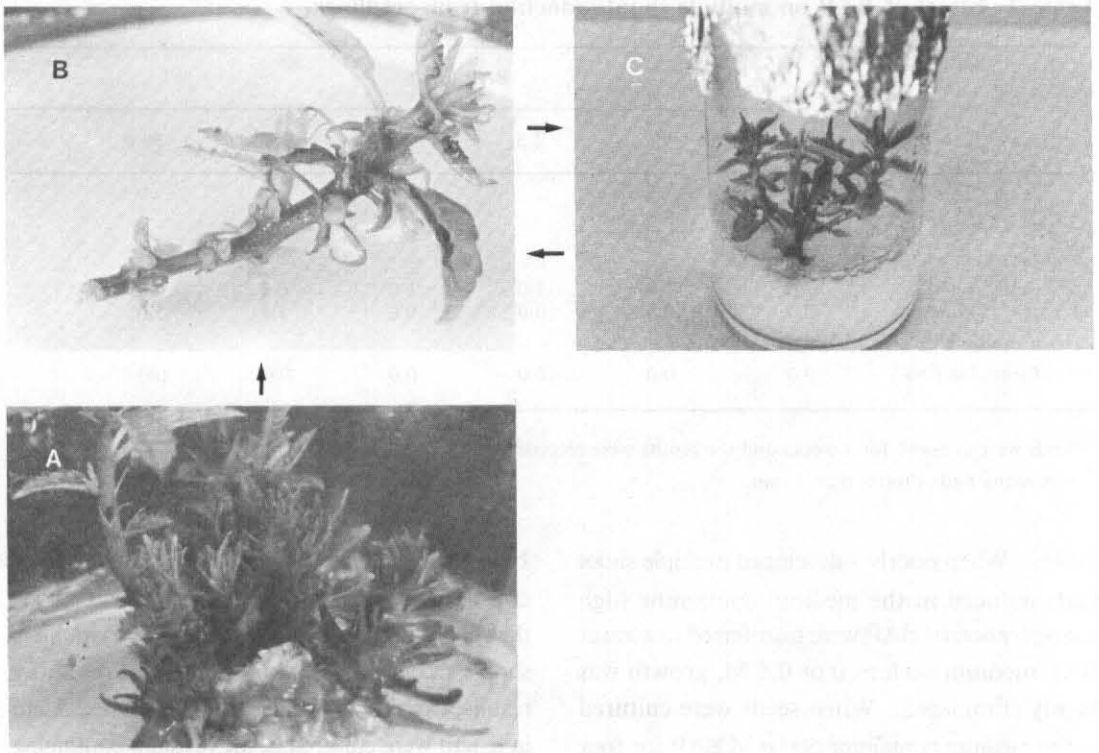


Figure 1. *In vitro* **shoot multiplication through seed culture.**

a. Multiple shoots from chestnut seed culture. Seeds were cultured in the medium containing BAP 50 μ M for 33 days and subculture in the medium containing BAP 1 μ M for 23 days.

b. Single shoots excised from multiple shoots of seed culture.

c. Multiple shoots induced from excised single shoots. Shoots of about 2.5 cm in length were excised from seedlings with multiple shoot/buds as shown in Figure 1 and cultured in the medium containing IBA 0.25 μ M for 3 weeks.

To establish entire mass propagation of chestnut plants, rooting single shoots is essential. Rooting excised single shoots of *Castanea sativa* Mil (Vieitez and Vieitez, 1983) and *Castanea mollissima* Blume (Qi - Guan *et al,* 1986) *in vitro* has been reported. However our trials have not succeeded in rooted excised single shoots of Japanese chestnut as yet.

Generally speaking, *in vitro* mass propagation through reproductive organs may be useful in both breeding and propagation programs (Hisajima *et al,* 1986, Tanaka *et al,* 1988, Hisajima *et al,* 1987), e.g. (1) It may save F_1 hybrid seeds in the plantlet - propagation and seed/fruit production sites and it may save time for breeding new varieties through cross breeding programs. (2) It may provide rapid industrial application of F_1 hybrid lines established through embryorescue. (3) It may maintain parent strains for F_1 hybrid or cross breeding programs on a small scale. (4) It may minimize the period required for the release of newly bred lines to the market by eliminating seed production and, (5) It may provide rapid industrial application to increase the number of clonal plants from a single seed of non - bred lines which has individual superior traits. The authors have reported *in vitro* mass propagation of different species through reproductive organs, induction of female reproductive organs *in vitro,* and haploid plants via unfertilized ear/ovule culture (Hisajima *et al,* 1986, Tanaka *et al,* 1988, Hisajima *et al,* 1987).

The present tissue culture technique may lead to new opportunities in propagation and breeding of chestnut plant. eg., (1) As multiple shoots formed from embryonic parts of chestnut seeds in the present experiment, it is expected to establish micropropagation through immature embryos (including embryorescue). (2) As chestnut is a diclinous plant so that unfertilized ovaries are easy to get for culture and unfertilized ovule culture may lead to establishment of haploid plants and successive establishment of homogeneous lines of woody species which are difficult to be established by conventional methods, and (3) As seeds are free from disease (generally speaking). The technique may be useful to establish disease free lines.

Usually *in vitro* mass propagation systems are not employed for the establishment of variant lines. Trials are in progress to establish salt - tolerant lines through successive shoot multiplication with mutagen treatment, with rice as the model system (Tanaka *et al,* 1988). Our unpublished results revealed that variant plants could be regenerated from mutagen - treated cultures. Similar ideas may be applied to a chestnut breeding program.

Reproductive organs such as flower and pod (Harada, 1967, Wardel and Skoog, 1969, Narasimhula and Reddy, 1984), ears, globular embryos (Fujishita and Saito, 1988), and tassels (Kaseyama and Yabe, 1988), were induced *in vitro*. These findings and techniques of using reproductive organs may lead to other opportunities in chestnut breeding programs, eg. alteration of generation *in vitro*.

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