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Multiple Shoot-Bud Formation and Plantlet Regeneration on *Castanea sativa* Mill. Seeds in Culture

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ABSTRACT

Primordial initiation and development of shoot-buds has been accomplished by using shoots derived from chestnut (Castanea sativa Mill) seedlings cultured with added 6-benzylaminopurine (BAP). Germination of chestnut seeds in the presence of BAP (4 - 40 μM) stimulated varying numbers of shoot-buds in those areas of the main axis that were favorably altered. When excised single shoots from these treated seeds were subcultured on a fresh medium containing BAP (4 - 40 μM) continual shoot production was observed. Bud growth and shoot elongation were stimulated by transferring cultures to a reduced concentration of BAP (2 μM) plus indole-3-butyric acid (IBA 0.4 μM). Plant regeneration occurred in the presence of IBA (0.8 μM) after a preconditioning treatment in which naphthaleneacetic acid (NAA 50 μM) and kinetin (k 2 μM) were applied to the tissue culture shoots for 7 days in light.

INTRODUCTION

Chestnut trees are important economically for their fruits, woods and their ecological influence on soil and landscape. Unfortunately, chestnut copses have been severely damaged by several fungal diseases and have been replaced by foreign species. There is the possibility of introducing varieties which show a certain degree of resistance against some of the most common diseases of chestnut. In this way rapid asexual multiplication of such varieties might be advantageous.

Although the potential use of tissue culture techniques for propagation of woody plants has been described (Bonga, 1974; Mehra and Mehra, 1974; Brown, 1976; Cheng, 1976; Druart, 1980), and chestnut tissues have been frequently cultured (Jacquiot, 1956; Trippi, 1963; Borrod, 1971; Vieitez, 1978, 1980), only limited success in plant regeneration has been reported for Castanea sativa Mill. Thus, in the present report, the successful establishment of in vitro requirements for multiple shoot formation and plantlet regeneration from excised single shoots of chestnut are described.

MATERIAL AND METHODS

After removing the testa, seeds of European chestnut (Castanea sativa Mill) were submerged in 95% ethanol for 5 min., disinfected by immersion in 20% clorox (5.25% sodium hypochlorite, NaOCl for 30 min.), rinsed three times with autoclaved distilled water, and planted directly on half-strength K(h) medium. Growth substances, such as BAP (6-benzylaminopurine), IBA (indole-3-butyric acid), NAA (naphthaleneacetic acid), and K (kinetin) were added both individually and in combination to the defined basal medium.

The conditions for growth of seedlings and the composition of the cited K(h) basal medium have been established and described in detail (Cheng, 1975, 1977). Media were adjusted to pH 5.5 and then autoclaved for 10 min. at 121 C at 1 kg/cm^2 pressure. The cultures were incubated in a growth chamber maintained at 25 C with an 18 light h. photoperiod.

RESULTS AND DISCUSSION

The chestnut shoot segments used in these studies were obtained from seedlings cultured with various concentrations of cytokinin (BAP). The purpose of using these tissues from BAP treated seedlings was to determine whether such treatments could make tissues become more responsive to in vitro treatments for initiation and multiplication of shoot-buds. To obtain treated seedlings, European chestnut seeds were sown on the defined half-strength K(h) medium, supplemented with various concentrations of cytokinin (0 to 40 μM).

The effect of added BAP on seed germination and multiple shoot formation was examined (Table I). The results show that the percentage of seed germination remained high regardless of the cytokinin concentrations tested. Axillary bud development and initiation of adventitious bud primordia were favorably influenced. However, a differential effect on shoot bud formation was detected. When the seeds were grown in a basal medium, most of the seedlings gave rise to a main shoot with several inhibited axillary nodes along the axis. The root

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Table I. Effect of BAP on Stimulation of Shoot-Bud Formation.

BAP Concentration (μM)	Germination rate %	Seeds Resulting in Multiple Shoots %	No. Shoots/Seed >2 cm	<2 cm	Total
0	80	0	1	-	1
4	80	2	1	-	1-3
20	75	95	19	8	25-30
40	70	95	10	16	10-32

Chestnut seeds were cultured on half-strength K(h) medium supplemented with various concentrations of BAP as listed. After 6 weeks in culture the number of shoots was determined for at least 10 cultures.

system usually consists of a long root which produces extensive laterals. In the presence of the lowest concentration of BAP (4 μM) seedlings developed normally. In contrast, at a high concentration of BAP (20 and 40 μM) the morphological development of shoot and root apices was altered and multiple shoot formation was achieved (Figs. 1a and 1b).

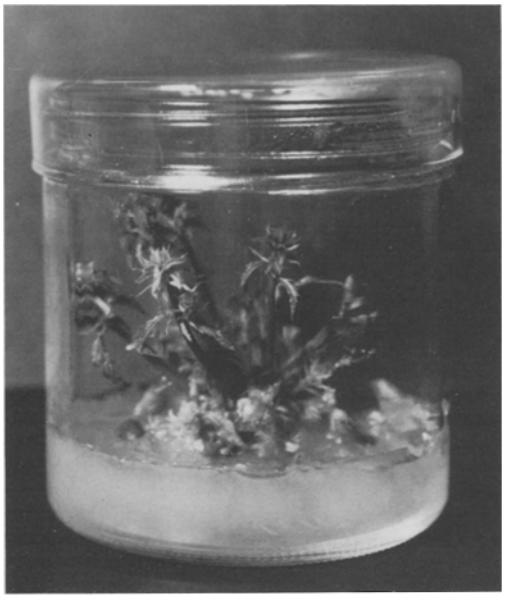

Fig. 1a.

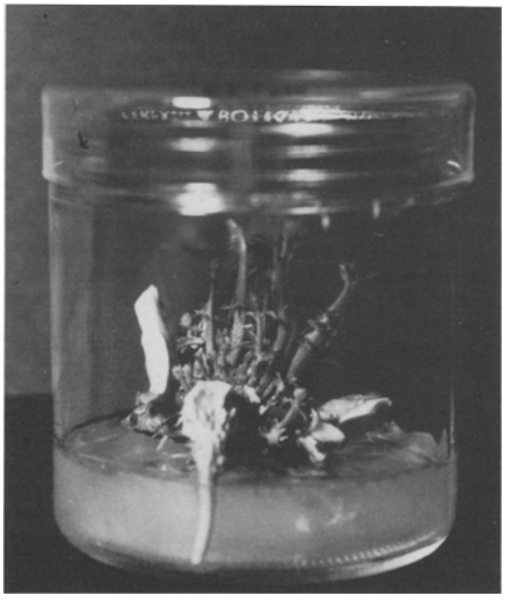

Fig. 1b.

The observed responses of chestnut seeds in culture are similar to those described for both in vitro and in vivo systems of various other plants (Cheng, et al, 1980) where a high concentration of cytokinin resulted in the growth of axillary buds through the elimination of plant apical dominance.

The developmental behaviour of seedlings germinated in the presence of a high concentration of BAP (20 μM) indicates definite advantages in the use of excised shoot segments to establish in vitro methods for the regenerative expression of multiple shoot-bud formation.

Subculturing excised regenerated shoots in the presence of a high concentration of BAP (Fig. 2) resulted in continual production of shoot-buds but inhibited subsequent shoot development. Unhealthy and curled leaves resulted. Conversely, a low concentration of BAP (2 μM) was less effective in stimulating shoot-bud formation, but growth of buds proceeded normally.

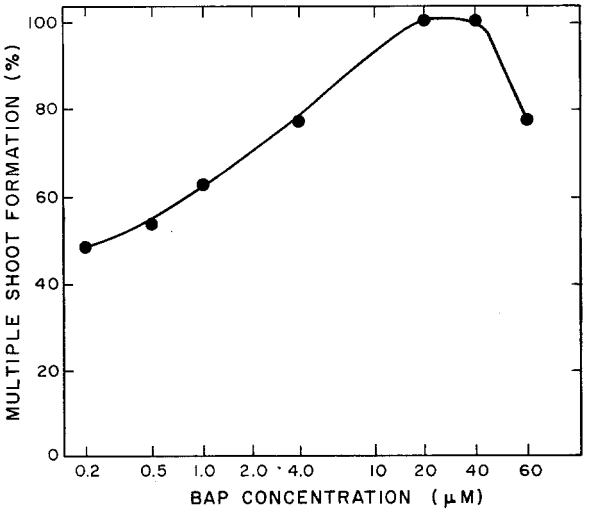

Fig. 2. Establishment of a continual shoot-bud producing culture. Excised single shoots from seeds cultured in the presence of BAP (20 μM) for 5-7 weeks were subcultured onto fresh medium supplemented with different concentrations of BAP as specified by the points in the Figure. The number of shoots were measured against the control group of seeds not treated with BAP 4 weeks after transfer.

Fig. 1. Shoot-bud formation of chestnut seeds stimulated by BAP at a) 20 μM and b) 40 μM.

Since stimulation of bud formation and bud growth in excised single shoots was achieved with BAP at relatively low concentrations (2 μM), the effect of different concentrations of auxin (IBA) but low concentrations of both IBA (0.4 μM) and BAP (2 μM) produced an optimum growth rate (Fig. 3).

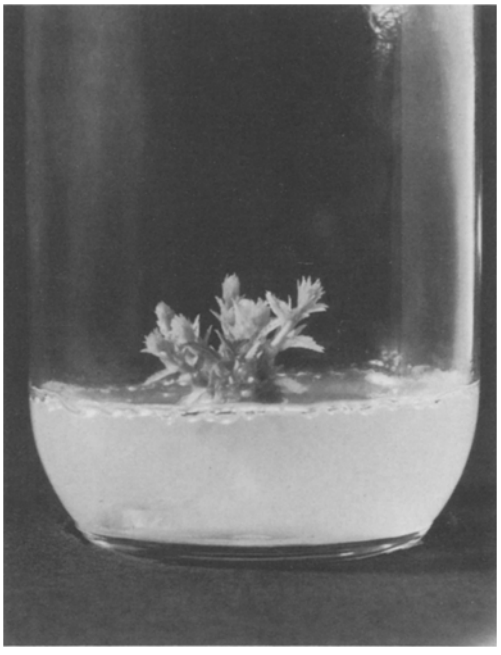

Fig. 3. Stimulation of shoot-bud elongation. Excised shoot segments were transferred from a high concentration of BAP (20 μM) onto a fresh medium supplemented with a reduced concentration of BAP (2 μM) plus IBA (0.4 μM).

The optimal conditions for obtaining multiple shoot formation in chestnut seedlings can be divided into three distinct steps: 1) initiation on half-strength K(h) medium in the presence of BAP (20 μM) for 4 to 7 weeks; 2) further stimulation of shoot-bud production through the subculture of single shoot segments for four weeks in a fresh K(h) medium containing the same concentration of BAP; 3) stimulation of shoot buds by the transfer of the cultures to modified K(h) medium containing IBA (0.4 μM) plus BAP (2 μM).

Under these conditions one can expect production of more than 50 shoots per seed within 9 to 11 weeks.

Rooting in this species has been found to be very difficult in the course of the experiments conducted. NAA (50 μM) plus K (2 μM) promoted root initiation after massive callus developed from the explants (Fig. 4a). In the presence of IBA (4.10 μM), small roots originated from the excised end of the shoot (Fig. 4b). However, growth of these newly-formed roots did not continue and the frequency of rooting was low. The following two-step procedure was used for optimum root formation: Excised single shoots were preconditioned for root initiation in a medium containing NAA (40 μM) and K (2 μM) for 7 days. After this period, the shoots were transferred to a fresh medium where application of IBA as low as 0.8 μM permitted subsequent root development (Fig. 4c) in half of the explants transferred.

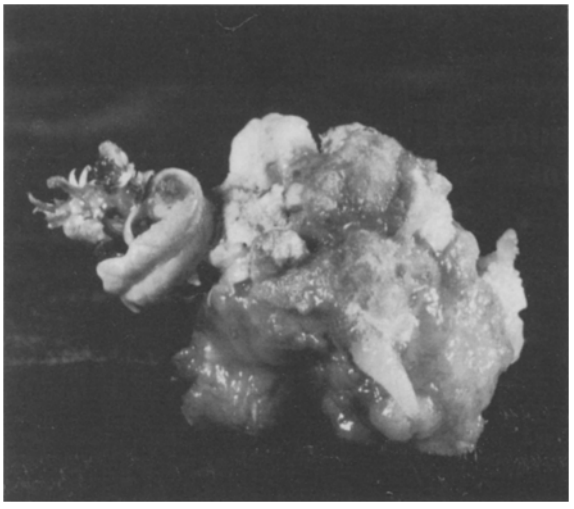

Fig. 4a

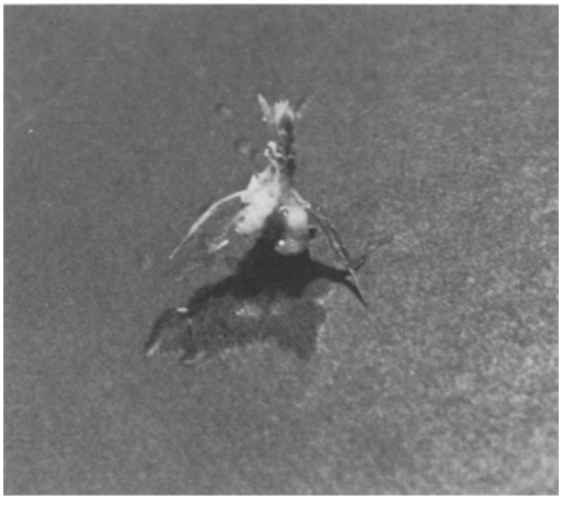

Fig. 4b

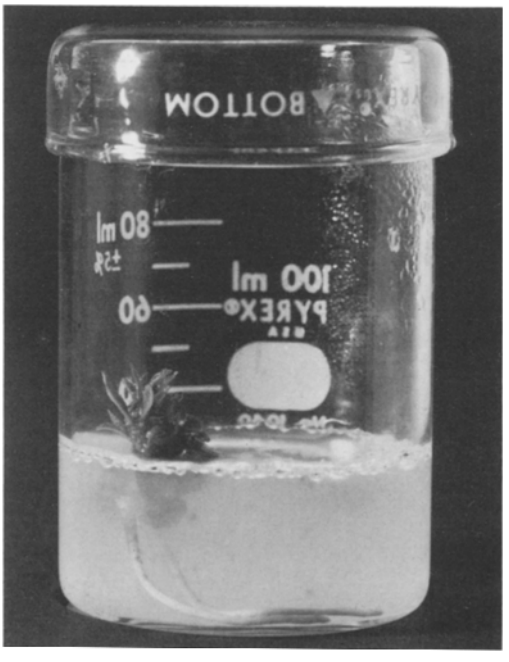

Fig. 4c

Fig. 4. Plantlet regeneration:

a) Root initiation obtained with NAA (40 μM) plus K (2 μM) after massive callus development.

b) Roots derived from the base of the shoot in the presence of IBA (4-10 μM).

c) Plant regeneration occurring in the presence of IBA (0.8 μM) after 7 days in the presence of NAA (50 μM) and K (2 μM).

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