IN VITRO CULTURE OF COTYLEDON TISSUE OF CASTANEA SATIVA MILL.

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ABSTRACT


Establishment of callus tissue culture from cotyledon fragments of Castanea sativa Mill. is described. The explants were grown on various combinations of auxins (IAA, IBA, NAA, 2,4-D) and cytokinins (K, BA) to induce callus and regeneration of organs. The best growth occurred on basal medium with 2,4-D (1 and 10 mg/l) plus both K or BA (0.5 mg/l). Root regeneration was achieved with both IBA and NAA (10 mg/l) supplemented with K or BA.

INTRODUCTION

The tissue culture in vitro of woody plants has, in general, been less well studied than that of herbaceous ones. Chestnut (Castanea sativa Mill.) has been proved to be nearly impossible to root from cuttings, the vegetative propagation of this species being very difficult (Vieitez, 1974). However, the successful propagation of woody plants by in vitro tissue culture methods has been reported by various authors for several species, e.g. aspen (Winton, 1970; Chalupa, 1974), almond (Mehra and Mehra, 1974), Eucalyptus (Cresswell and Nitsch, 1975), birch (Huhtinen, 1976) and others (see review by Murashige, 1974). Chestnut tissue culture in vitro has scarcely been reported. Previously, Jacquiot (1950, 1953, 1956), Trippi (1963) and Borrod (1971) have obtained chestnut callus, in vitro cambium being used as a source of callus tissue, but organ differentiation was never induced.

The present paper is a study of the in vitro organogenesis capacity of chestnut tissue, as a possible technique for the vegetative propagation of this species. Cotyledons as a source of callus tissue culture have been reported by Robbins and Whitwood (1974) for Arachis hypogea, Cucumis, Cucurbita, Phaseolus vulgaris and Glycine max. We have had success in culturing cotyledons of Castanea sativa in vitro for callusing. Root differentiation
Fig. 1. Tracheidal elements in callus subculture observed under a polarized light microscope (270 x).

Fig. 2. Meristematic pockets of cells surrounded by much larger ones induced by IBA (10 mg/l) plus CM (12%) treatment (82 x).

Fig. 3. Roots formed from primary cultures grown on the basal medium with the addition of IBA (10 mg/l) and K (0.5 mg/l).

Fig. 4. Root primordia into the mass of primary explants (34 x).

Fig. 5. Section of a root with 2 lateral root primordia, with visible provascular cylinders (76 x).
from callus culture in vitro, obtained for the first time in this species, is also reported.

MATERIALS AND METHODS

Nuts of Castanea sativa Mill. were collected in October from only one tree. The samples were quickly rinsed in 95% (v/v) alcohol, then further surface sterilised in calcium hypochlorite solution 7% (w/v) for 10 min, and then rinsed 3 times with sterile water, the nuts remaining in the third rinse until used. Sterilised nuts were dissected between sterile paper.

Explants from cotyledons about 150 mg were placed in 30 x 150 mm test tubes which contained a medium consisting of Heller's mineral salts, Nitsch's micronutrients, thiamine 1 mg/l, Ca-pantothenate 0.5 mg/l, biotin 0.1 mg/l, cysteine chlorhydrate 10 mg/l, m-inositol 500 mg/l, sucrose 3% (w/v) and agar 0.7% (w/v). Growth substances such as IAA, IBA, NAA and 2,4-D at concentrations of 0.1, 1 and 10 mg/l were added, in combination with 0.5 mg/l kinetin (K) or 6-benzyl-aminopurine (BA). Coconut milk (CM) 12% (v/v) also was added in combination with auxin. The pH of the medium was adjusted to 5.6 before autoclaving. The cultures were grown in a 16 h photoperiod. Temperature was maintained at 18°C during the dark period and 25°C during the light one. For each treatment 24 replicate cultures were kept.

For anatomical studies the explants were fixed in formalin-acetic-alcohol (5:5:90, v/v/v) embedded in paraffin, and microtome sections were processed in the conventional manner using safranin fast-green as the stain. For ensuring identification of lignified tissues, polarized light microscopy was performed.

RESULTS AND DISCUSSION

Early callus growth was slow, but increased after 3 weeks so that callus subcultures were possible after 6–7 weeks of incubation. Most of the callus tissue remained immersed in the agar. The callus was composed of very firm and compact tissue, with external nodular outcrops. Due to genetic variability of the cotyledons, there was a strong variation in callus growth and root differentiation as well as in the colour, which ranged from pale yellow to greenish.

Callus growth was slight in the absence of growth regulators, and IAA showed the least stimulation for it; 2,4-D promoted callus formation greatly, the optimum concentration being 1 mg/l. Concentrations of 1 and 10 mg/l of both IBA and NAA were also active in inducing callus formation, but 0.1 mg/l was not effective. However, root differentiation occurred only with IBA and NAA, the latter being less effective than the former. When cytokinin was replaced by coconut milk (12%), the growth was increased, and the best proliferation was obtained when 1 mg/l of 2,4-D was added.

Histological examination of the callus undergoing differentiation showed a lot of tracheidal elements in which 2 ordination types were observed:
(1) tracheidal elements with thick secondary walls with reticulate thickenings, disposed in long and gross bundles that probably originated from vascular bundles of cotyledon explants; (2) tracheidal elements organized in the form of distinct nests or nodules, that were characteristically amphicribal with a central tracheidal complex surrounded by a diffuse cambial zone cutting off tiered rows of cells on its outer surface (Fig. 1). A great number of tracheidal elements was also seen to originate from diffuse cambial tissue differentiated precisely in the zone between the primary explant and the newly formed tissue. Vascular nodules were not frequently observed with 2,4-D treatments, which showed random distribution of isolated tracheids.

Both meristematic nodules and zones were located at the peripheral layers of a callus tissue. IBA (10 mg/l) plus CM (12%) treatment differentiated isolated groups of unorganised meristematic pockets like spheres amongst the parenchyma (Fig. 2).

When IBA (10 mg/l) plus both K or BA (0.5 mg/l), and NAA (10 mg/l) plus K (0.5 mg/l) treatments were used, tender roots with a slow growth pattern were formed in the aerial zone of the primary explants cultures (Fig. 3). Some roots occasionally grew and introduced themselves into the agar, and some formed absorbent hairs. However, roots were never observed with 2,4-D and IAA treatments. Histological examination of those rooting-treatments showed a lot of primary meristems which were organized in root primordia, generally differentiated into the primary cotyledon explant (Fig. 4). Such root meristems occasionally developed lateral roots (Fig. 5). Most of these primordia did not arise at the periphery of the differentiating callus, but remain inside the callus mass.

It is emphasized that the auxins IBA and NAA produced root differentiation and are also the most effective auxins to root chestnut shoots by stooling (Vieitez, 1955).

Because of the quicker growth rate obtained, Murashige and Skoog's inorganic components (1962) were employed for subculturing, and biotin and cysteine chloride were suppressed. IBA (1 mg/l) and either K or BA (0.5 mg/l) were added to the medium. 2,4-D and NAA produced a soft callus and a poorer success percentage in subculturing. Callus growth was hastened in continuous darkness, but root primordia were never observed.

Root differentiation in chestnut callus cultured in vitro can be an important step in the root formation in this very difficult species for adventitious rooting.

REFERENCES


