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BRIEF COMMUNICATION

In vitro propagation of *Ginkgo biloba* by using various bud cultures

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Abstract

The purpose of this research is micropropagation of *Ginkgo biloba* L. Apical and nodal meristems removed from plantlets or apical buds from a tree were used as explants. Meristems produced an extensive callus and single or rare multiple shoots on Murashige and Skoog medium with different growth regulators and endosperm extract (En) obtained from mature seeds of the same species. For successful root production it was necessary to transfer the shoots to a rooting medium with En.

Additional key words: endosperm extract, growth regulators, micropropagation.

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Ginkgo biloba L. attracts considerable attention as an ornamental tree and as a source of numerous chemical substances with pharmacological properties termed ginkgolides. Since its sexual reproduction shows some difficulties such as dioecy of species and low germinability of its seeds, which are recalcitrant and not able to maintain germinability for a long time (Tommasi *et al.* 1999), the vegetative reproduction of plantlets by means of *in vitro* techniques provokes interest. There are many reports of micropropagation protocols for ligneous species used for many purposes, such as the medicinal tree *Crataeva nurvala* (Walia *et al*. 2003), but there is little information in the literature on *G. biloba* cultivation. There are some reports regarding *in vitro* culture of embryos, male and female gametophytes and somatic embryogenesis (Bulard 1952, 1966, Webb *et al*. 1986, Camper *et al*. 1997). The use of different factors in complex media containing yeast extract, coconut milk, tomato juice, casein hydrolysate or glutamate has been reported for various *in vitro* cultures of *G. biloba* (Wang and Chen 1965, Wang and Lee 1966). The aim of this research was to improve micropropagation of *G. biloba*. We have considered the use of MS medium with growth regulators and endosperm extract obtained from mature seeds of the same species.

Seeds of *Ginkgo biloba* L. obtained from commercial sources (*Florsilva-Anzaloni*, Bologna, Italy), deprived of sarcotesta, were stored at 4 °C in trays at about 80 % humidity. Batches of seeds were deprived of their lignified tegument, sown in Petri dishes on sterile sand and cultivated at 22 °C in the dark for 8 d. Then the plantlets were transferred to a glasshouse for 30 d as a source of explants.

Our studies of *in vitro* culture of *G. biloba* were initiated by utilizing explants of apical and nodal buds with auxiliary meristems. Stems containing both types of buds were collected from plantlets of germinated mature seeds (Fig. 1*A*) or from a tree (only apical buds). All the explants were sterilized by rapid immersion in 70 % ethanol and then in an aqueous solution of sodium hypochlorite, NaOCl (active chlorine 1 %), for 15 min and rinsed three times for ten min each in sterile water. Meristem explants (4 - 5 mm) were cultured on Murashige and Skoog (1962) medium (MS) containing 35 g dm^{-3} sucrose and 10 g dm^{-3} *Difco-bacto* agar. The medium was adjusted to pH 5.8 before autoclaving at 115 °C for 20 min. Multiplication experiments were initiated with single explants in culture tubes for preliminary results and continued into 250 cm^3 Erlenmeyer flasks with 3 explants per container. The explants were cultured on a growth basal medium (MS) or on the same medium with growth regulators. Auxins and cytokinins were added to the medium prior to autoclaving in the various combinations selected from

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Abbreviations: MS - Murashige and Skoog medium; BAP - 6-benzyladenine; En - endosperm; IAA - indolacetic-3-acid; IBA - indole-3-butyric acid; Kin - kinetin, NAA - naphtaleneacetic acid.

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preliminary results. All the media were tested with or without endosperm extract (En) added to each medium prior to autoclaving at a concentration of 20 cm^3 dm^{-3}. The En was collected by grinding 20 g of endosperm tissues with 20 cm^3 phosphate buffer 0.2 M, pH 7.6, centrifuging at 6 000 *g* for 15 min and utilizing the supernatant. The following media were used: MS basal medium; MS+ indole acetic acid (IAA) + Kinetin (Kin); MS + IAA + 6-benzyladenine (BAP), MS + naphthalene-acetic acid (NAA) + Kin or BAP (multiplication media); MS + indole-butyric acid (IBA) (rooting medium). 30 explants for each treatment were tested and the experiments were repeated three times. The data were subjected to statistical analysis. All *in vitro* cultures were incubated under a 14-h photoperiod at an irradiance of 36 μmol $m^{-2}s^{-1}$ (white fluorescent lamps *Osram 36 W*) and a temperature 25 ± 1 °C.

Some cultured explants were collected for histological analysis to verify tissue modifications in consecutive cultures. They were fixed in Bouin's liquid (mixture 30:10:1 v/v of picric acid, formalin and glacial acetic acid), dehydrated in an ethanol series and embedded in paraffin. Sections (20 μm thick) were cut and stained with safranin-fast green.

On MS basal medium and MS medium with different combinations of growth regulators (Table 1) the cultures only produced a callus. The addition of En extract was important for shoot production: in fact, in media MS + IAA + Kin + En the explants from apical and nodal buds produced a dark green callus and one single shoot in 80 % and in 30 % of the cultures, respectively (Fig. 1*B*). On medium MS + IAA + BAP + En the explants produced a much larger callus and shoots in 60 % of the explants from apical buds and in 20 % from the nodal buds. The shoots were scarcely developed (sometimes multiple) and no roots were produced. Media MS + NAA + Kin or MS + BAP + En produced a large amount of dark green callus at the base of the cultures, but prevented shoot growth, evident only in 20 % of apical bud explants and in 10 % of nodal bud explants. The apical buds of the tree showed the same behaviour as the apical buds from the seeds but in a percentage of 10 % (Fig. 1*C*). In all cases the shoots obtained from various explants were removed from initial cultures and transferred onto a rooting medium MS + IBA + En where they produced many normal roots in 70 % of the cultures (Fig. 1*D*). Increasing concentrations of growth regulators prevented shoot formation in all types of explants. The callus was thick in longitudinal section and strongly coloured at the base of the cultures and very thin in the upper part. The shoot originated from the growth of the initial bud explant and never from the callus. The vascular bundles present in the basal callus were numerous and continued to the apex and the new leaflets with many branches (Fig. 1*E*).

The reported data for bud cultures of *Ginkgo biloba* L. show possible bud explant productivity and organo-genetic potentiality. Although the percentage of productivity was low, particularly with regard to nodal buds, complete plantlets from bud explants were obtained. In the literature it is reported that the production of whole plantlets of *G. biloba in vitro* is limited to cultures of intact embryos (Camper *et al.* 1997) even in media containing only various cytokinin/auxin levels. Since no data were reported for shoot tip cultures, our results are interesting although further studies will be required for a refinement of the micropropagation protocol.

Table 1. Development of callus and shoots on apical or nodal bud explants in MS media with various growth regulators [μM] and endosperm extract (2 months' culture). Means ± SE, $n = 20$. Value followed by the same letter(s) in each column are not significantly different according to Student's *t*-test ($P < 0.01$). + - large callus; ++ - very large callus; 0 - no development; – - not tested;

Medium composition	Callus	Shoots [%] apical buds plantlets	apical buds trees	nodal buds
4.6 Kin + 5.7 IAA –En	+	0a	0a	0a
4.6 Kin + 5.7 IAA +En	+	80 ± 4.1b	20 ± 2.4b	30 ± 2.1b
4.6 Kin + 11.4 IAA –En	+	0a	0a	0a
4.6 Kin + 11.4 IAA +En	+	78 ± 2.3b	19 ± 2.2b	33 ± 1.3b
4.4 BAP + 5.7 IAA –En	+	0a	0a	0a
4.4 BAP + 5.7 IAA +En	+	60 ± 3.2c	10 ± 2.4c	20 ± 2.1c
8.8 BAP + 11.4 IAA –En	+	0a	0a	0a
8.8 BAP + 11.4 IAA +En	+	56 ± 3.4c	10 ± 2.4c	20 ± 2.2c
4.6 Kin + 0.54 NAA –En	++	0a	-	0a
4.6 Kin + 0.54 NAA +En	++	20 ± 2.1d	-	10 ± 2.4d
4.4 BAP + 0.54 NAA –En	++	0a	-	0a
4.4 BAP + 0.54 NAA +En	++	20 ± 1.9d	-	10 ± 0.9d
8.8 BAP + 0.54 NAA –En	++	0a	-	0a
8.8 BAP + 0.54 NAA +En	++	10 ± 0.8e	-	10 ± 1.4d

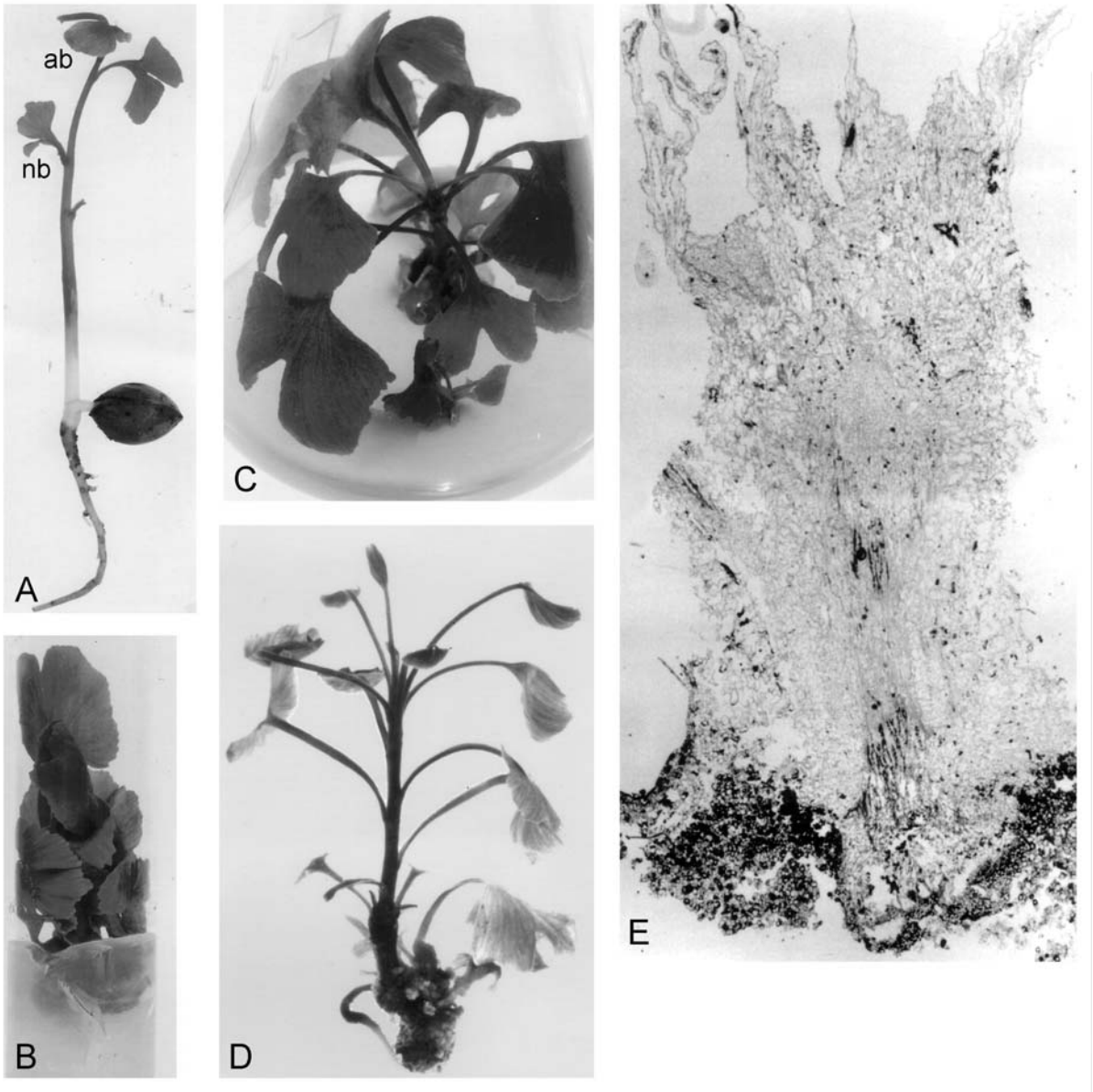

Fig. 1. *A* - Germinated seed, ab - apical bud, nb - nodal bud, used as explants; *B* - apical bud cultured on MS+IAA+Kin+ En showing growth of callus and one shoot; *C* - apical buds from a tree cultured on MS+IAA+Kin+En that produced one shoot and callus; *D* - the whole plantlet with roots obtained on MS+IBA+En from apical bud of germinated seed; *E* - longitudinal section of *Ginkgo biloba* cultured bud of after 4 weeks on MS+IAA+Kin+En: note the differentiation of the callus and its vascular bundles in connection with new leaflets (×10).

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