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PRELIMINARY STUDIES ON *IN VITRO* PROPAGATION OF *GINKGO BILOBA* L.

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ABSTRACT

In vitro shoot culture of *Ginkgo* so far is not adequate relative to it's medicinal and ornamental importance. The aim of the present study was to develop methods for in vitro micropropagation of this fossil plant. Different cultural media have been involved in serial experiments. As a result *in vitro* shoot culture of *Ginkgo* was initiated and maintained from 2-bud shoot apices on MS or WPM nutrient media. Our work would be considered pioneer under lack of information with this regard.

KEYWORDS: micropropagation, shoot culture, *in vitro*, nodal segments

INTRODUCTION

After registration of the *Ginkgo* leaf extract EGb 761 for human use in 1974 in France, the use of *Ginkgo* has been growing at a very rapid rate worldwide and accordingly the pharmaceutical industry needs huge quantities of leaves [5]. The conventional propagation methods are slow and wouldn't fulfill these demands. A quicker process could be guaranteed using plant tissue culture, but so far there is a lack of information regarding *in vitro* micropropagation of *Ginkgo*. It is obvious that *Ginkgo in vitro* reproduction is not adequate relative to the medicinal and ornamental importance of this fossil plant. *In vitro* micropropagation, in particular, faces some obstacles standing in the way of establishing an efficient mass micropropagation system for *Ginkgo*. Most of the studies conducted so far in this respect have used many types of explants including embryos. Meanwhile, using vegetative explants has not been widely studied. Tommasi and Scaramuzzi [8] studied the possibility of *Ginkgo* micropropagation using apical and nodal meristems removed from plantlets or apical buds from a tree. They found that meristems produced an extensive callus and single or rare multiple shoots on MS medium with different growth regulators and endosperm extract obtained from mature seeds of the same species. They considered their (poor) results interesting since no data had been reported for shoot tip cultures until then. In fact, as reported by Camper [1], the production of whole plantlets of *G. biloba in vitro* was limited to cultures of intact embryos even in media containing only various cytokinin/auxin levels.

The aim of the present study was to investigate the possibility of using shoot apices obtained from a single mature male *Ginkgo* tree as initial explants for establishing an efficient mass micropropagation protocol.

MATERIALS AND METHODS

Plant material

The explants were collected during March-April from a 17-year-old male *Ginkgo* tree. Two types of explants (shoot apices and shoot segments) were prepared from the apical shoots.

Disinfestation of plant material

Initial explants used in the experiment were obtained from the corresponding plant early in the morning and kept in distilled water-filled jars inside icebox to keep them vital. Explants were first given a 2-minutes fungicide treatment (0.1% kantus) followed by washing with liquid soap solution and rinsing with running water for one hour. After washing, five minutes treatment with a 5% (w/v) solution of calcium hypochlorite [$Ca(ClO)_2$] with constant agitation was given. Calcium hypochlorite was washed off three times with sterilized distilled water, 10 minutes each, under aseptic conditions. The explants were then dissected on sterilized paper cutting pads and inoculated on the corresponding medium (Table 1.).

Carbohydrates (sucrose and/or glucose) and 5 g/l agar (in case of solid media), were added after adjusting the pH to 5.6 using 0.1 N KOH or 0.1 N HCl. After it was steamed to melt the gelling agent, the medium was dispensed into the test tubes and autoclaved at 121°C at a pressure of 1.1 kg. cm^{-2} for 20 min.

Establishment and secondary sprouting

Initiated stem cultures of *Ginkgo* were subjected to various treatments in order to improve shoot growth, development and proliferation (Table 1.). Responses to zeatin and casein hydrolysate (CH) at different concentrations were studied as well as the effect of culture vessels' size on shoots growth. Influence of GA_3 on shoot growth and development of microshoots of *Ginkgo* was evaluated. A 0.58μM solution of GA_3 was prepared, cold-sterilized by a Millipore membrane and added to the shoot cultures under aseptic conditions.

In vitro cultures were kept at 22±2°C under 16-h photoperiod (fluorescent tubes OSRAM 40 W, 40 μmol m^{-2} s^{-1} PPED).

RESULTS AND DISCUSSION

Initiation of shoot culture from a mature male Ginkgo tree

As presented in Table 2, no bud induction was noticed with any of the used media. Most of the explants deteriorated and were swollen. Callus accumulations were noticed in almost the all media with a higher frequency with shoot segments cultured on 8.88μM BAP-supplemented medium. Liquid medium seems to be quite improper for either bud induction or explant survival in *Ginkgo*, and therefore it was excluded from further trials. Probably, relatively bad physical properties of liquid media comparing to solid ones are the main cause of culture deterioration. The proper media for bud induction were solid MS supplemented with 4.44μM BAP + 0.057μM IAA, hormone-free MS with 250mg $.l^{-1}$ CH or WPM with 6.84μM zeatin +

250 mg $.l^{-1}$ CH. When liquid media or high concentrations of BAP were employed most of the explants deteriorated and were swollen and callus accumulations were noticed (Fig. 1). Of the explant types used, only two-bud shoot apices proved to be adequate for axillary bud induction. Nodal segments, particularly those comprising one bud, deteriorated soon after culturing, turned brown and died at last. It is advisable, therefore, to use two-bud shoot apices for initiating *in vitro* shoot culture of *Ginkgo*. MS containing high BAP concentration was recommended by Montes-Lopez [6] for shoot culture of *Ginkgo* initiated from shoot tips of one-year-old branches. They postulated that media containing BAP at 66.6μM or 88.76μM resulted in 60% and 80% sprouting, respectively. Indeed, these results contradict those obtained by the present experiment where media containing 66.6μM resulted in nothing but callus accumulations, desiccated shoots, falling leaves and almost 100% dead cultures. Much lower concentrations of BAP, were recommended for shoot cultures of *Ginkgo* by many authors [2, 10, 11]. Possibly, an explanation of the differences among all these plant growth regulators (PGR) combinations would be the balance between the exogenous growth regulators and the endogenous hormones. That is, as the culture progressed, the available exogenous cytokinin was consumed or degraded, changing the cytokinin/auxin ratio to levels adequate for bud growth. Higher cytokinin concentrations appear to stimulate ethylene production which can be antagonistic or inhibitory to organized development such as shoot proliferation [3]. Van Staden [9] also indicated that levels of cytokinin, which are too high, cause many small shoots to be produced, which typically fail to elongate; they may also cause the leaves of some species to have an unusual shape, and/or induce shoots to become hyperhydric.

All explants were then transferred into MS [7]containing 44μM BAP + 0.057μM IAA. During subsequent subcultures, newly developed leaves were green, open and relatively big whilst no secondary proliferation could be obtained on the same medium. It could be concluded that although this medium was relatively proper for culture initiation, other media would be needed for the maintenance and establishment.

Secondary sprouting was feasible on WPM [4] containing 6.84μM zeatin with/without CH as one single shoot with low frequency (Fig. 2). Our results are promising comparing to those obtained by Tommasi and Scaramuzzi [8] who reported only callus in the shoot cultures of *Ginkgo* with a single shoot in 80 % in their best cases. We found that scrapping off the basal part rather than removing the whole shoot base was relatively much better for axillary buds induction. Behavior of shoots grown in big tubes was surprisingly better than that in jars. Shoots previously elongated by the addition of 4ml of 0.58µM GA3 to each jar were cut into apical and basal nodal segments and placed on different media (Table 1). It was noticed that apical shoots grown on WPM containing 6.84μM zeatin with the addition of CH survived though only single elongated shoot was obtained with no axillary bud induction. Basal segments deteriorated soon after culturing irrespective of the used medium (Fig 1.A) Horizontal orientation of explants culture proved unsuccessful for bud induction.

Table 1.

Basal media and plant growth regulators used for *Ginkgo in vitro* stem culture initiation and establishment.

Stage	Basal medium	Supplements	
		Growth regulator µM	Others [v≡ vitamins, CH≡ casein hydrolysate]
Initiation	¼ MS liquid	8.88 BAP	MS v + 20g/l sucrose + 20g/l glucose
	¼ MS liquid	13.32 BAP	MS v + 20g/l sucrose + 20g/l glucose
	MS	2.50 BAP + 0.05 IBA	MS v + 30g/l sucrose
	MS	4.44 BAP + 0.057 IAA	MS v + 40g/l sucrose
	MS	8.88 BAP + 0.057 IAA	MS v + 40g/l sucrose
	MS	66.60 BAP	MS v + 30g/l sucrose
	MS	3.11 BAP + 0.057 IAA	MS v + 40g/l sucrose
	MS	7.50 TDZ + 2.50 IBA	MS v + 40g/l sucrose
	WPM	1.33 BAP + 0.54 NAA	WPM v + 20g/l sucrose
	WPM	3.11 BAP + 0.057IAA	WPM v + 40g/l sucrose
Proliferation	MS	Hormone-free	MS v + 500 mg/l CH + 30g/l sucrose
	MS	Hormone-free	MS v + 250 mg/l CH + 30g/l sucrose
	MS	6.84 zeatin	MS v + 40g/l sucrose
	MS	6.84 zeatin	MS v + 250 mg/l CH
	WPM	6.84 zeatin	WPM v + 20g/l sucrose
	WPM	6.84 zeatin	WPM v + 250 mg/l CH + 20g/l sucrose
	WPM	Hormone-free	WPM v + 20g/l sucrose
	WPM	Hormone-free	WPM v + 250 mg/l CH + 20g/l sucrose
	WPM	2.50 TDZ	WPM v + 250 mg/l CH + 20g/l sucrose
	WPM	2.50 TDZ + 6.84 zeatin	WPM v + 250 mg/l CH + 20g/l sucrose
	WPM	13.69 zeatin	WPM v + 250 mg/l CH + 20g/l sucrose

Table 2.

In vitro performance of *Ginkgo* explants grown on MS- or WPM-based media containing different combinations of zeatin and/or CH.

Basal medium	Supplements	Development features	
		Shoot apices	***shoots from pervious cultures***
MS	Hormone-free + 250 mg l^{-1} CH	High frequency of bud induction (>90), big, green leaves, elongated shoots.	No further secondary sprouting, green leaves and elongated shoots
	6.84 μM zeatin	Bud induction (≈40%), average growth, not like MS+CH	No further secondary sprouting
	6.84 μM zeatin+ 250 mg l^{-1} CH	Bud induction (≈50%), average growth, not like MS+CH	No further secondary sprouting
WPM	Hormone-free	No development, slow growth	No further secondary sprouting
	Hormone-free + 250 mg l^{-1} CH	No development, slow growth	No further secondary sprouting
	6.84 μM zeatin	Bud induction (≈70%), frequent callus formation, generally adequate growth	Secondary sprouting with low frequency, single bud from the utmost basal part.
	6.84 μM zeatin+ 250 mg l^{-1} CH	Bud induction (≈70%), frequent callus formation	Good growth, green big leaves, secondary sprouting with low frequency

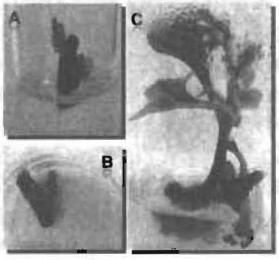

Fig 1. Different explant types of *Ginkgo* cultured on various medium types. A. basal segments on solid medium; B. basal segments on liquid medium;C. shoot apices on solid medium.

Fig 2. *Ginkgo* sprouting microshoots grown on WPM with 6.84 μM zeatin. The arrow points at a newly formed axillary bud induced from the utmost basal part.

CONCLUSIONS

In vitro shoot culture of *Ginkgo* could be initiated and maintained in an effective manner from 2-bud shoot apices on MS containing 250 g l^{-1} CH, or WPM containing 6.84μM zeatin with/without 250g l^{-1}CH. However, we prefer bud initiation on the former and culture maintenance on the latter. Our work would be considered pioneer under lack of information noticed with this regard.

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