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Meta-topolin improves lateral bud proliferation in micropropagation of *Ginkgo biloba* L.

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Abstract

In vitro shoot tip culture of *Ginkgo biloba* L. so far is not adequate relative to its 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 with different plant growth regulators have been involved in serial experiments. Meta-topolin improves lateral bud proliferation of shoot tip culture of *Ginkgo biloba*.

Keywords: micropropagation, shoot culture, in vitro, nodal segments

INTRODUCTION

The use of *Ginkgo* has been growing at a very rapid rate worldwide. The pharmaceutical industry needs huge quantities of leaves, especially after registration of the *Ginkgo* leaf extract EGb 761 for human use in 1974 in France (Masood, 1997). The conventional propagation methods are slow and are not able to meet these demands. The use of tissue culture to multiply woody species has become increasingly significant within the last 20 years but *Ginkgo* in vitro reproduction is not yet adequate relative to the medicinal and ornamental importance of this fossil plant. 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 investigated. Tommasi and Scaramuzzi (2004) 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. They considered their (poor) results interesting since no data had been reported for shoot tip cultures until then. In fact, as reported by Camper et al. (1997), 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. Our previous study showed that *in vitro* shoot culture of *Ginkgo* could be initiated and maintained in an effective manner from 2-bud shoot apices on MS or WPM containing 6.84µM zeatin with/without 250g L^{-1} casein hydrolysate, although no bud stimulation was observed. Basal segments deteriorated soon after culturing irrespective of the used medium (Ibrahim et al., 2011). Cultivation of Ginkgo shoot explants on MS or WPM enriched with TDZ (2.50 or 7.50 µM) and as well as with BAP (2,5 – 8,88 µM) had no effect since no secondary proliferation could be obtained on these media (Ibrahim et al., 2011).

The aim of the present study was to improve lateral bud proliferation of shoot apices in micropropagation protocol of *Ginkgo biloba* L.

MATERIALS AND METHODS

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The explants were collected during March-April from a 17-year-old male *Ginkgo* tree. Explants were prepared from the apical shoots, disinfected with solution of calcium hypochlorite and inoculated on the corresponding medium as described previously by Ibrahim et al. (2011). For each variant of nutrient media 30 tubes with a single explant contained in each tube in three repetitions were established(fig.1). The experiment was repeated twice (in the spring of 2013 and 2014).

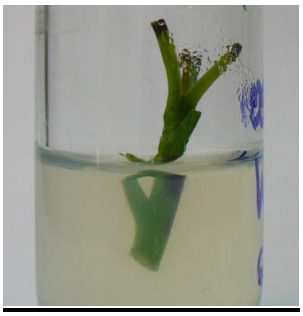

Fig.1. Two-bud shoot apex of *Ginkgo biloba* L. on the initiation medium.

In order to improve shoot growth, development and proliferation nutrient media based on both MS (Murashige and Skoog, 1962) or DKW (Driver and Kuniyuki, 1984) formulations were used (Table 1). They were enriched with different cytokinins: BAP (2.5 µM or 4.44 µM) or 2-isopentenyladenine (2iP, 50 µM or 75 µM) or meta-topolin (mT, 2.5; 4; 5.5; 7; 8 µM), 30.0 g L^{-1} sucrose or 30.0 g L^{-1} glucose and 6.5 g L^{-1} agar (Phytoagar, Duchefa). The pH of the media was adjusted to 5,6.

Table 1. Nutrient media for micropropagation of *Ginkgo biloba* L.

Variants	Basal medium and vitamins	Supplements	
		Growth regulators	Others
I	**MS**	**-**	**30.0 g L^{-1} sucrose**
II	**DKW**	**-**	**30.0 g L^{-1} glucose**
III	**MS**	**2.5 µM BAP**	**30.0 g L^{-1} sucrose**
IV	**DKW**	**2.5 µM BAP**	**30.0 g L^{-1} glucose**
V	**MS**	**4.44µM BAP**	**30.0 g L^{-1} sucrose**
VI	**DKW**	**4.44µM BAP**	**30.0 g L^{-1} glucose**
VII	**MS**	**50 µM 2iP**	**30.0 g L^{-1} sucrose**
VIII	**DKW**	**50 µM 2iP**	**30.0 g L^{-1} glucose**
IX	**MS**	**75 µM 2iP**	**30.0 g L^{-1} glucose**
X	**DKW**	**75 µM 2iP**	**30.0 g L^{-1} glucose**
XI	**DKW**	**2.5 µM mT**	**30.0 g L^{-1} glucose**
XII	**DKW**	**4 µM mT**	**30.0 g L^{-1} glucose**
XIII	**DKW**	**5.5 µM mT**	**30.0 g L^{-1} glucose**
XIV	**DKW**	**7 µM mT**	**30.0 g L^{-1} glucose**
XV	**DKW**	**8.5 µM mT**	**30.0 g L^{-1} glucose**

In three passages of three weeks on the respective nutrient media, the number of newly formed shoots was recorded. In vitro cultures were kept in a growth chamber at 22±2°C under 16-h photoperiod (fluorescent tubes OSRAM 40 W, 40 µmol m^{-2} s^{-1} PPED).

RESULTS AND DISCUSSION

After initiation, bud induction was noticed on some of used media supplemented with cytokinin. No bud induction, no development and slow growth was noticed with any of the used media without plant growth regulators (Fig.2, Variants I, II). Application of cytokinins BAP and 2iP did not lead to the expected result. Bud induction was about 40% in variants on media containing 44µM BAP (variants V, VI). During subsequent subcultures, newly developed leaves were green, open and relatively big whilst no secondary proliferation could be obtained on the same medium. This confirms the conclusion of Ibrahim et al. (2011) that although this medium was relatively suitable for culture initiation, other media would be needed for the maintenance and establishment.

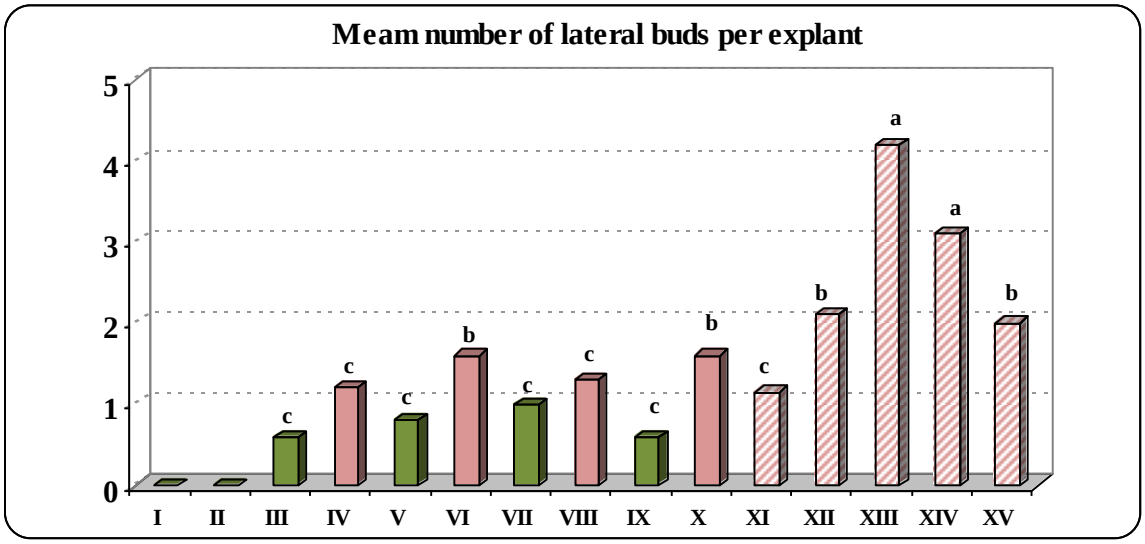

Fig.2. Mean number of lateral shoots per explant.
□ MS based media; ■ DKW based media; □ DKW based media with *meta*-topolin

When using the cytokinin 2iP relatively high percentages of sprouting were achieved, about 50%, but produced shoots were small and typically failed to elongate (Fig.2,3). Presumably the concentrations used (50µM, 75µM, variants VII-X) were too high. Van Staden (2008) indicated that high levels of cytokinin may induce the production of many small shoots, which typically fail to elongate. Excessive cytokinin levels may also cause leaves unusual shape, and/or induce shoots to become hyperhydric.

Fig.3. Ginkgo plantlets cultivated on DKW based media with 50 and 75 µM 2iP, respectively.

Taking into account the appearance and development of plants we can conclude that DKW based media seems to be quite better for either bud induction or explant survival in *Ginkgo*, and therefore it was preferred for further trials with mT. Basal medium DKW was developed and has been used successfully in many tree species, mainly nut.

All explants cultivated on DKW basal medium enriched with *meta*-topolin had good growth, big green leaves (Fig.2, variants XI-XV, Fig.4.).

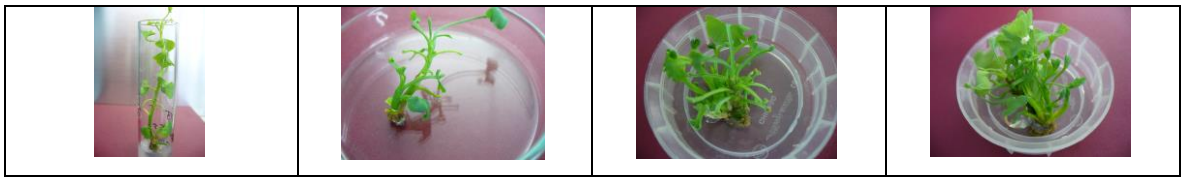

Fig.4. Plantlets cultivated on DKW based media with different concentrations meta-topolin (2.5; 4; 5.5 and 7 µM mT, respectively).

During subsequent subcultures, newly developed leaves on the lowest concentration of mT (2.5µM mT) were green, open and relatively expanded whilst no secondary proliferation could be obtained on the same medium. It could be concluded that although this medium was relatively suitable for stem elongation, further media would be necessary for the multiplication and maintenance steps (fig. 4.). The increase in the concentration of mT up to 5.5 µM lead to larger number of lateral buds on the explant (fig. 2,4). The best results from the examined media were received on media containing 5,5 µM mT (variant XIII), which demonstrated the best mean number of shoots per explant (4.2) and good physiological status (fig. 4.). Further increasing in the concentration of mT up to 8.5 µM had not produced greater number of lateral buds.

According to some authors hydroxylated analogs of BA have been proposed as alternative cytokinin substitutes to BA in plant tissue systems (Werbrouck et al. 1996; Strnad 1997). Shoot multiplication, rooting and acclimatization can be increased by substituting BA with N6-(3-hydroxybenzyl)adenine (*meta*-topolin, mT), a naturally-occurring BA analog in which the primary mT metabolite, although also deleterious, degrades more rapidly during acclimatization (Werbrouck et al., 1996; Strnad et al., 1997). More recently, mT has been shown to be the preferred cytokinin over BA for shoot multiplication, enhanced rooting, reduced hyperhydricity, and enhanced acclimatization ex vitro of *Aloe polyphylla* (Bairu et al., 2007).

Our results indicate that substituting BA with mT could significantly enhance in vitro lateral bud induction and shoot multiplication of Ginkgo plants. Inclusion of mT in media may provide a method to ensure efficient commercial in vitro propagation of a large number of valuable *Ginkgo biloba* L. genotypes. However, further screening of the efficacy of mT with a wide range of genotypes is required to confirm this broader application.

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