Bull. Fac.Agric.,Cairo Univ., 61:309- 315 (2010) .

MICROPROPAGATION OF GINKGO (*Ginkgo biloba* L.) PLANT BY TISSUE CULTURE

(Received: 2.5.2010)

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ABSTRACT

Ginkgo biloba L., of the family Ginkgoaceae, is called a "living fossil". The present experiments were done in Plant Biotechnology Research Lab., Faculty of Agriculture, Cairo University during the period from 2004 to 2006.

The microcuttings were used as a source of explants at length (1.0 – 2.0 cm) which were removed from a tree then washed in soap water using septol soap for 30 min and rinsed with running tap water for 30 min. The explants were sterilized by Clorox [sodium hypochlorite (NaOCl)] at 20% for 20 min. Microcutting segments were cultured on Murashige and Skoog medium with various growth regulators, BA or Kin at different concentrations (0.0, 0.5, 1.0, 2.0 or 5.0 mg/l), the combination between 0.5 mg/l kin and different concentrations of NAA at (0.0, 1.0, 5.0 or 10.0 mg/l), the combination between 0.5 mg/l IAA with different concentrations of BA at (2.0, 4.0, 8.0 or 10.0 mg/l) and NAA at (1.0, 2.0, 3.0, 4.0 or 5.0 mg/l). The cultures were incubated in the light and morphological development was recorded as the shoot length, petiole length, number and size of leaves.

The maximum length of shoots was obtained when the microcutting segments were cultured on a medium supplemented with BA at 5.0 mg/l. However, shoots did not turn to plantlets, as they failed to root.

BA considerably enhanced shoot proliferation from microcutting segments. The best growth recorded as the highest length of shoots (3.0 cm), the highest length of petiole (5.3 cm) and highest number of leaves (12.0 leaf/explant) was noticed on microcutting cultured on a medium supplemented with BA at 5.0 mg/l after two recultures.

***Key words*:** *Ginkgo biloba ,growth regulators, microcutting segments, micropropagation.*

1. INTRODUCTION

Ginkgo biloba L. is resistant to insects, fungi and other pests. The leaves contain a lot of medicinal compounds, *i.e.* gingkolidies A, B, C, J and bilobalide (Jocelyne *et al*., 2002).

Carrier *et al.* (1991) cultured cells of *Ginkgo biloba* in MS medium supplemented with 1 mg NAA, 0.1 mg K and 30 g sucrose/litre. The young leaf and bud explants of *Ginkgo biloba* were cultured on MS medium supplemented with 2,4-D, kinetin, IBA, NAA and benzyladenine at 25 °C at a light intensity of 1600-2000 lux for 12 h daily. Cultures were examined after 15-20 days and the best combination of growth regulators for shoot and root induction was determined. Rooting was best (up to 80%) on MS media with either 1.0 mg NAA + 0.1 mg kinetin/litre or 2.0 mg NAA + 2.0 mg kinetin/litre.

The stem explants of *Ginkgo biloba* were cultured on MS, modified MS and DCR basal media, supplemented with various concentrations of phytohormones [NAA and ZT (zeatin) and two types of nutrient (CH (casein hydrolysate) and Ade (adenine)] (Hao *et al.,* 2000). Modified MS with CH at 500 mg/litre was the best medium for inducing the development of axillary buds. The shoots were induced to root on MS + NAA 0.1 mg/litre, and the rooting rate was 33.3%. Choi *et al.* (2003) reported that the immature zygotic embryos of *G. biloba* were cultured on Murashige and Skoog's medium (MS) supplemented with various combinations of naphthaleneacetic acid (NAA; 0.01 or 0.10 mg/l) and benzyladenine (BA; 0.1, 0.5 or 1 mg/l). The highest number (8 per embryo) of adventitious buds formed from cotyledons of heart stage embryos cultured on MS medium with 1 mg/l BA and 0.01 mg/l NAA. Adventitious buds transferred to hormone free MS medium grew into shoots, but did not produce plantlets because the shoots failed to root.

The present investigation aimed to find out the effect of different concentrations of various growth regulators under light condition on multiplication and development of ginkgo shoots, *in vitro.*

2. MATERIALS AND METHODS

Microcutting segments of ginkgo (*Ginkgo biloba* L.) were obtained from Orman botanical garden, Giza. They were washed with septol soap and water for 30 min and rinsed with running tap water for 30 min. Under aseptic conditions using laminar air-flow cabinet, they were firstly soaked in 70% ethanol solution for 30 sec., after which, explants were sterilized by Clorox [sodium hypochlorite (NaOCl)] at different concentrations for 20 min. All sterilization solutions were provided with a few drops of polyoxyethylene sorbitan monolaurate (tween-20). This was followed by three rinses with sterile distilled water. Microcuttings (2 – 3 mm long) were used as explants.

The explants were cultured on Murashige and Skoog (1962) (MS) medium with MS vitamins, 30 gm/l sucrose and 2 g activated charcoal. Plant growth regulators, [naphthalene acetic acid (NAA), indole acetic acid, (IAA), kinetin (Kin) and benzyladenine (BA)] were added alone or with various combinations at different concentrations (0.5 to 10.0 mg/l). The pH of the media was adjusted to 5.7. Agar was added at 7 g/l. The cultures were incubated in a growth chamber at 26± 2^{o}C under illuminated conditions. Cool white fluorescent lamps were used to give intensity of (3000 Lux for 16 hr, daily). Each treatment included 6 replicates (6 jars). The explants were transferred to fresh medium every 4 weeks; and each jar contained three explants. The explants were transferred to fresh media every 4 weeks. Shoot formation was recorded at the end of the experiment after 12 weeks in culture (through 2 re-cultures). Each experiment was repeated three times in a completely randomized design according to Snedecor and Cochran (1980).

2.1.Experiment1: Effect of different concentrations of 6-Benzyleaminopurine (BA) on micropropagation and growth of ginkgo microcutting

The aim of this experiment was to study the influences of different concentrations of BA on growth of shoots. The explants were cultured on ¾ MS basal medium supplemented with different concentrations of BA at (0.0, 0.5, 1.0, 2.0 or 5.0 mg/l).

2.2.Experiment2: Effect of different concentrations of 6-furfuryl amino-purine (kin) on growth of shoots

The aim of this experiment was to study the influences of different concentrations of kin on growth and development of shoots. The explants were cultured on ¾ MS basal medium supplemented with different concentrations of kin at (0.0, 0.5, 1.0, 2.0 or 5.0 mg/l).

2.3.Experiment 3: Effect of different combinations between kin and NAA on callus formation and growth of shoots

In this experiment the microcutting segments were individually cultured onto ¾ MS basal medium supplemented with 0.5 mg/l kin and different concentrations of NAA at (1.0, 5.0 or 10.0 mg/l).

2.4.Experiment 4: Effect of combinations between IAA and different concentrations of BA on growth of shoots

The aim of this experiment was to study the influences of combination between different concentrations of IAA and BA on growth of shoots. The explants were cultured on ¾ MS basal medium supplemented with 0.5 mg/l IAA and different concentrations of BA at (2.0, 4.0, 8.0 or 10.0 mg/l).

2.5.Experiment 5: Effect of ¾ MS basal salts supplemented with different concentrations of NAA (mg/l) on root initiation and growth of shoots.

In this experiment shoots were cultured on ¾ MS basal medium supplemented with different concentrations of NAA at (1.0, 2.0, 3.0, 4.0 or 5.0 mg/l) and activated charcoal at 2.0 gm/l.

The following data were recorded at the end of each experiment:

1-Shoot length (cm).
2-Petiole length (cm).
3-Leaf number (leaf/explant).
4-Leaf size (cm^2).

3. RESULTS AND DISCUSSION

3.1.Experiment1: Effect of different concentrations of 6-benzyleaminopurine (BA) on micropropagation and growth of ginkgo microcutting

The data in Table (1) show the effect of BA at (0.0, 0.5, 1.0, 2.0 or 5.0 mg/l) on shoot proliferation of explants cultured for 12 weeks. It could be noticed that increasing the concentration of BA increased the growth of the shoots.

There was a significant difference among different concentrations of BA, on shoot proliferation, expressed as shoot length. Adding BA at 5.0 mg/l produced the highest length of shoots (3.00 cm), the highest length of petiole (5.33 cm) and the highest number of leaves (12.0 leaf/explant). BA at 1.0 mg/l recorded the lowest length of shoots (0.40 cm) and the lowest length of petiole (2.67 cm).

it was concluded that applying BA considerably enhanced shoot proliferation from microcutting segments. The best growth recorded was noticed on microcuttings cultured on a medium supplemented with BA at 5.0 mg/l after

Table (1): Effect of different concentrations of BA on micropropagation of ginkg microcutting.

BA (mg/l)	Shoot length (cm)	Petiole length (cm)	Leaf number (leaf)	Leaf size (cm^2)
0.0	1.00 B	3.00 BC	10 AB	2.33 B
0.5	1.00 B	4.67 AB	8.00 B	3.33 A
1.0	0.40 B	2.67 C	9.67 AB	3.00 AB
2.0	0.90 B	3.00 BC	8.33 AB	2.67 AB
5.0	3.00 A	5.33 A	12.00 A	2.33 B
LSD	**1.15**	**1.88**	**3.89**	**0.74**

two recultures. By increasing BA concentration, shoot proliferation increased. Cytokinins play an important role in encouraging shoot and leaf proliferation. These results are in harmony with those obtained by Montes-Lopez and Rodriguez (2001) who evaluated the effect of different concentrations of BA (0, 5, 10, 15 and 20 mg/l) on axillary buds and shoot apices of *Ginkgo biloba*. On the other hand, Lazarte (1983) stated that good shoot multiplication of pecan cv. Desirable was achieved *in vitro* on modified Woody Plant Medium (WPM) containing 3.0 mg/l BA.

3.2.Experiment2: Effect of different concentrations of 6-furfuryl amino-purine (kin) on growth of ginkgo shoots

Data in Table (2) show the effect of different concentrations of kin at (0.0, 0.5, 1.0, 2.0 or 5.0 mg/l) on growth of microcuttings of *Ginkgo biloba* after 12 weeks in culture through 2 re-cultures. These data indicate that kin at all levels was not sufficient to improve growth of shoots derived from microcuttings. The shoot length did not show significant differences due to kin concentration. The shoot length was (0.53, 0.40, 0.47 and 0.23 cm) compared with the control which recorded 1.0 cm and the length of petioles (2.40, 2.00, 1.67, 2.33 and 2.50 cm), respectively, when different concentrations of kin (0.0, 0.5, 1.0, 2.0 and 5.0 mg/l, respectively) were used. It was concluded that using kin alone failed to produce adventitious shoots on the explants.

Gamborg *et al.* (1987) mentioned that cytokinins are derivatives of adenine. Several cytokinins occur in the cells of all organisms, but the hormone activity is detectable only in plants. Compounds most frequently used in culture media are benzyl adenine (BA), 6-frufuryl amino purine (kinetin), 6-4-hydroxy-3-methyl-trans-2-butenyl amino purine (zeatin) and isopentenyle adenosine (IPA). Many plant tissues have an absolute requirement of a specific cytokinin for morphogenesis to occur. The cytokinin are generally added to a culture medium to stimulate cell division, to induce shoot formation and axillary shoot proliferation.

3.3.Experiment 3: Effect of different combinations between kin and NAA on callus formation and growth of shoots.

Data in Table (3) show the influence of culturing explants on a medium supplemented with 0.5 mg/l kin and different concentrations of NAA at (0.0, 1.0, 5.0 or 10.0 mg/l) after 12 weeks in culture through 2 re-cultures. There was no significant difference between different combinations of 0.5 mg/l kin and NAA at (1.0, 5.0 and 10.0 mg/l) in their effect after 12 weeks in culture. However, the growth of shoots was better by using NAA at 1.0 mg/l together with kin at 0.5 mg/l. This combination resulted in the highest shoot length (1.50 cm), the highest length of petiole (2.83 cm), the highest number of leaves (12.33 leaf/explant) and the leaf size was (3.67 cm^2). On the contrary, the highest concentration of NAA (10.0 mg/l) resulted in the lowest growth of shoots, in the form of the lowest shoot length (0.50 cm), the lowest length of petiole (1.0 cm), the lowest leaf number (8.0 leaf/explant) and the leaf size (4.0 cm^2).

It was concluded that the best growth of shoots was recorded when ginkgo microcuttings were cultured on a medium supplemented with kin at 0.5 and NAA at 1.0 mg/l, after two recultures.

The obtained results agree with those of Carrier *et al.* (1990) who indicated that *G. biloba* cells could grow on a medium supplemented with NAA and kinetin in concentrations ranging from 0.1 to 2.0 mg/litre. The best growth and maintenance of callus cultures were achieved using a medium supplemented with 2 mg NAA and 1 mg kinetin/litre. Greater growth rates were obtained on MS medium containing 1 mg NAA, 0.1 mg kinetin and 30 g sucrose/litre.

Levi and Skin (1991) working on lateral bud-derived callus for embryo induction on MS medium containing 1.5 mg NAA and 0.1 mg kinetin/l, reported the formation of distinct groups of embryogenic cells of asparagus somatic embryos. In an additional study, the same authors found that NAA at 1.0-10.0 mg/l in combination with kinetin at 0.0-1.0 mg/l yielded means of 64, 175 and 225 small globular embryos per gram of callus on induction medium (IM) for spear cross section (SS), *in vitro* crown (IVC) and lateral buds (LB), respectively.

NAA promoted a higher rate of embryo development (0.5, 9.4 and 11.9 plantlets), from these respective callus sources of asparagus somatic embryos. There was no difference between kinetin levels of 0.0-1.0 mg/l on callus

Table (2): Effect of different concentrations of Kin on micropropagation of ginkgo microcuttings.

Kin (mg/l)	Shoot length (cm)	Petiole length (cm)	Leaf number (leaf)	Leaf size (cm^2)
0.0	1.00 A	2.40 A	6.00 A	2.30 C
0.5	0.53 A	2.00 A	8.33 A	2.67 BC
1.0	0.40 A	1.67 A	5.33 A	2.00 C
2.0	0.47 A	2.33 A	9.00 A	3.33 AB
5.0	0.23 A	2.50 A	5.00 A	4.00 A
LSD	**0.67**	**1.07**	**4.48**	**0.94**

Table (3): Effect of different concentrations of Kin + NAAon growth of ginkgo explants.

Kin+ NAA (mg/l)	Shoot length (cm)	Petiole length (cm)	Leaf number (leaf)	Leaf size (cm)
0.0 + 0.0	1.00 A	2.50 A	10.00 A	2.30 B
0.5 + 1.0	1.50 A	2.83 A	12.33 A	3.67 A
0.5 + 5.0	0.67 A	1.83 A	9.67 A	3.67 A
0.5 + 10.0	0.50 A	1.00 A	8.00 A	4.00 A
LSD	**1.15**	**3.75**	**7.11**	**1.19**

Table (4): Effect of the combination of IAA + BA on growth of ginkgo explants.

IAA + BA (mg/l)	Shoot length (cm)	Petiole length (cm)	Leaf number (leaf)	Leaf size (cm^2)
0.0 + 0.0	1.00 A	1.00 BC	5.00 B	2.67 B
0.5 + 2.0	0.50 A	0.58 C	4.00 B	1.00 C
0.5 + 4.0	0.50 A	1.00 BC	5.00 B	3.00 B
0.5 + 8.0	0.67 A	1.50 B	8.00 A	3.00 B
0.5 + 10.0	1.17 A	2.50 A	7.00 A	4.00 A
LSD	**0.958**	**0.852**	**1.730**	**0.002**

Table (5): Effect of different concentrations of NAA on roots induction and growth of ginkgo explants.

NAA (mg/l)	Shoot length (cm)	Petiole length (cm)	Leaf number (leaf)	Leaf size (cm^2)	Root formation
0.0	0.30 AB	2.80 A	6.00 AB	2.50 BC	0.00
1.0	0.50 A	3.00 A	7.00 AB	3.33 AB	0.00
2.0	0.30 AB	2.83 A	4.00 B	3.00 AB	0.00
3.0	0.20 B	2.87 A	7.00 AB	2.67 BC	0.00
4.0	0.20 B	2.50 A	9.00 A	2.00 C	0.00
5.0	0.20 B	4.00 A	8.00 A	3.67 A	0.00
LSD	**0.26**	**2.26**	**3.31**	**0.91**	**0.00**

growth and embryogenesis, whereas l0 mg/l in (IM) was inhibitory.

3.4.Experiment 4: Effect of the combinations between IAA and different concentrations of BA on growth of shoots

Data in Table (4) show the influence of the combination between different concentrations of IAA and BA on growth of shoots, when explants were cultured on medium supplemented with 0.5 mg/l IAA together with different concentrations of BA (0.0, 2.0, 4.0, 8.0 or 10.0 mg/l) after 12 weeks in culture through 2 re-cultures. There was a significant difference between the effect of different combinations of BA and IAA. Increasing the concentration of BA to 8 or 10.0 mg/l combined with 0.5 mg/l IAA, increased the development of shoots and induced the highest length of shoots (1.17 cm), the highest length of petiole (2.50 cm), the highest number of leaves (7.0 leaf/explant) and the leaf size (4.0 cm^2) after 12 weeks in culture. On the contrary, lower concentrations of BA (2.00 and 4.00 mg/l) resulted in the lowest growth of shoots, in the form of the lowest shoot length (0.50 cm), the lowest length of petiole (0.58 and 1.00 cm), the lowest of leaf number (4.0 and 5.0 leaf/explant), respectively and the lowest leaf size (1.0 and 3.0 cm^2) were obtained by BA in addition with IAA at 0.5 mg/l, respectively. The control recorded the lowest results.

It was concluded that the best growth was observed with microcuttings cultured on a medium supplemented with BA at 8.0 or 10.0 mg/l combined with IAA at 0.5 mg/l, after two recultures. By increasing BA concentration, shoot development increased.

These results are in agreement with those obtained by Agarwal and Ranu (2000). They investigated the *in vitro* plant regeneration potential of geraniums (*Pelargonium* x *hortorum*). Using various combinations of growth regulators, a regeneration protocol has been developed to raise *in vitro* plantlets from young petiole and leaf explants from three different cultivars of geraniums. Shoot buds transferred to a medium supplemented with 0.44 µM (0.1 mg/litre) N6-benzyladenine and 0.11 µM (0.02 mg/litre) IAA, grew vigorously and attained 1-2 cm in length in 3-4 weeks. On the other hand, Poulain *et al.* (1979) reported that when tissues taken from the soft parts of an adult date palm cv. Bou Feggous offshoots were cultured on a medium supplemented with auxin (IAA at 0.5-5.0 mg/litre) and cytokinin (BA at 0.1-1.0 mg/litre), buds were formed after 3 months. When these buds were transplanted to a medium where cytokinin (isopentenyl-adenine) was reduced to 0.1 mg/litre and the auxins were modified to 1 mg/litre naphthoxyacetic acid, 2 mg/litre IAA and 3 mg/litre IBA, the explants rooted. Ammar and Benbadis (1977) indicated that date seeds, from which the endosperms were removed, were cultured on media to which various amendments were added. In a medium augmented with IAA + BA, both male and female inflorescences were induced after a few weeks.

3.5. Experiment 5: Effect of ¾ MS basal salts supplemented with different concentrations of NAA (mg/l) on root initiation and growth of shoots.

The data in Table (5) show the influence of culturing shoots on a medium supplemented with different concentrations of NAA at (1.0, 2.0, 3.0, 4.0 or 5.0 mg/l) and activated charcoal at 2.0 g/l, after 12 weeks in culture through 2 re-cultures. There was a significant difference between the effects of different concentrations of NAA. The shoots underwent no development as they recorded low shoot length (0.30, 0.50, 0.30, 0.20, 0.20 and 0.20 cm) with different concentrations of NAA at (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l, respectively). The highest number of leaves (9.0 leaf) was recorded when NAA at 4.0 mg/l was used, while, the lowest number (4.0 leaf/explant) was observed when NAA at 2.0 mg/l was used.It could be mentioned that all concentrations of NAA failed to induce any roots and the development of shoots was low.

These results are in harmony with those of Choi *et al.* (2003). They cultured immature zygotic embryos of *G. biloba* on MS medium supplemented with various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D; 0.01 or 0.10 mg/l), naphthaleneacetic acid (NAA; 0.01 or 0.10 mg/dm-3), benzyladenine (BA; 0.1, 0.5 or 1 mg/l) and zeatin (0.1, 0.5 or 1 mg/l) or with various concentrations of 2,4-D alone. The highest number (8/embryo) of adventitious buds was formed from cotyledons in the heart stage embryos cultured on medium with 1 mg BA/l and 0.01 mg NAA/l. Adventitious buds transferred to hormone free MS medium grew into shoots, but did not produce plantlets because they failed to root. However, Jiang and Zhang (1998) cultured young leaf and bud explants of *Ginkgo biloba* on MS medium supplemented with 2,4-D, kinetin, IBA, NAA and benzyladenine. They indicated that rooting was best (up to 80%) on MS medium with either 1.0 mg NAA + 0.1 mg kinetin or 2.0 mg NAA + 2.0 mg kinetin/litre.

It can be concluded from the foregoing investigation that adding BA to ¾ MS medium considerably enhanced shoot proliferation from microcutting segments. The best growth recorded

with cultured cutting of ginkgo was on the medium supplemented with BA at 5.0 mg/l after two recultures. By increasing BA concentration the shoots proliferation increased but the shoots failed to induce any roots.

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إكثار نبات الجنكو (*Ginkgo biloba* L.) بإستخدام زراعة الأنسجة

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ملخص

يتبع نبات الجنكو عائلة (*Ginkgoaceae*) ،أجريت الدراسة الحالية في معمل زراعة الأنسجة بكلية الزراعة – جامعة القاهرة، خلال الفترة من 2004 - 2006 على نبات الجنكو (*Ginkgo biloba* L.).

تم إستخدام العقل الدقيقة كمصدر للأجزاء النباتية بطول (1-2سم) و التى فصلت من الشجرة ، ثم غسلت بالماء و الصابون لمدة 30دقيقة ثم تم شطفها تحت الماء الجارى لمدة 30 دقيقة. بعد ذلك تم تعقيم الأجزاء النباتية بإستخدام الكلوروكس بتركيز 20% لمدة 20 دقيقة. زرعت أجزاء العقل على بيئة MS تحتوى على منظمات النمو المختلفة ، البنزيل أدينين

أوالكينتين بتركيزات (0.0، 0.5، 1.0، 2.0 أو5.0 مجم/لتر) و تم إستخدام التركيبات المختلفة من 0.5 مجم/لتر كينتين مع نفثالين حامض الخليك بتركيزات (1.0، 5.0 أو 10.0 مجم/لتر)، و تم إستخدام التركيبات المختلفة من 0.5 مجم/لتر أندول حامض الخليك مع البنزيل أدينين بتركيزات (2.0، 4.0، 0.8 أو 10.0 مجم/لتر) و تم إستخدام النفثالين حامض الخليك بتركيزات (0.0، 1.0، 2.0، 3.0، 4.0 أو 5.0 مجم/لتر). تم تحضين الزراعات فى وجود إضاءة و تم تسجيل طول الأفرع، طول عنق الورقة، عدد الأوراق و حجم الورقة بعد 8 أسابيع من الزراعة. كانت أفضل النتائج المتحصل عليها من التجارب السابقة عند الزراعة على بيئة MS المحتوية على بنزيل أدينين بمفرده بتركيز 5 مجم/لتر. لم تنجح الأفرع فى إنتاج نبيتات لفشلها فى تكوين الجذور.

التوصيات: وجد أن إضافة البنزيل أدينين بمفرده بتركيز 5 مجم/لتر على ¾ قوة أملاح بيئة MS يعتبر مشجع لإكثار الأفرع من أجزاء العقل الدقيقة للجنكو . كانت النتائج المتحصل عليها كالأتى أعلى طول للأفرع (3.00 سم)، أعلى طول للعنق (5.33 سم) و أعلى عدد للأوراق (12.00 ورقة/للفرع).

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (61) العدد الثالث (يوليه 2010):309-315.