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Response of *Ginkgo biloba* L. plant to growth regulators during *in vitro* propagation

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Abstract
Ginkgo biloba L. is a deciduous, multipurpose plant and very rare in Egypt, belongs to the family Ginkgoaceae. It has many great medicinal properties, which have massive application in the pharmaceutical industries in addition to its decorative importance. There is a need for applying modern conventional methods of propagation for conservation and sustainable utilization of the plant, so, an efficient protocol for in vitro propagation of G.biloba has been established. Stem nodal segments were used as explants for micropropagation. Murashige and Skoog (MS) medium supplemented with 0.54 µ M α- naphthalene acetic acid (NAA) and 8.90 µM 6-benzyl adenine (BA), the best initiation medium for induced to bud break and growth (72.2% growth percentage). Autumn was the most suitable time for explants collection, giving the highest survival and growth percentage (72.2% and 84.61%, respectively) and average shoot length (1.2 cm). MS medium supplemented with several concentrations of BA, kinetin (Kin) and thidiazuron (TDZ) were used to determine their influence on shoot multiplication. The medium supplemented with 2.22 µM BA gave the highest average number of shoots (1.94 shoots per explant) and length of shoots (2.84 cm), BA was the most efficient cytokinin for the multiplication stage. The effect of different types of auxins on the rooting of shoots was investigated. Half-strength MS medium supplemented with 2.46 µM Indol-3-butyric acid (IBA) gave the highest rooting percentage (66.7%) with an average root number (2 roots per shoot) and length of roots (1.3cm). The effect of silver nitrate ($AgNO_3$) at different concentrations in MS medium containing IBA at 2.46 µM were evaluated for root improvement and acclimatization of rooted planets. The concentration of 23.6 µM $AgNO_3$ significantly increased the rooting percentage, it reached the maximum value of 88.9%, in addition to significantly of enhancement the number of roots (4.63 roots/ shoot) and root length (3.51cm). A percentage of 53% of rooted plantlets were successfully acclimatized in the greenhouse.

Keywords*: Ginkgo biloba, Micropropagation, Stem nodal segments, Thidiazuron, Silver nitrate.*

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I. Introduction

Ginkgo biloba L. is a gymnosperm tree that is only living member of Ginkgoaceae family. Moreover, the Gkingo plant has magnificent ornamental and medicinal value. The roots bark and leaves extract of this unique plant are used as an important source of medicinal material to treat many diseases (Zhou *et al.*,2011). *G. biloba* extract contains two important active pharmaceutical components, flavonoids and lactones which are used in treatment of cerebrovascular diseases such as coronary heart disease, high blood pressure and slowing some of Alzheimer`s - type dementia symptoms (Weimann *et al.*,2010, Lu *et al.*,2011 and Cheng *et al.*,2014) *Ginkgo biloba* is naturally propoation by seeds, however, seed set is poor, seed viability and germination percentage are low (Tommasi and Scaramuzzi, 2004). Also, the conventional propagation methods, are slow and a long juvenile phase, which led to endangered (Isah, 2016). Therefore, *in vitro* propagation technique was shown to be more suitable (Nacheva *et al.*,2017 and Isah, 2020). Also, Bekhit *et al*, (2008) who mentioned that in Egypt, Ginkgo tree is very rare because there is only one male tree. In addition, it is dioecious and prone to extinction. Hence, to overcome referred obstacles, *in vitro* propagation can play a vital role for mass propagation and production of this important medicinal plant. Although, earlier attempts have been made for propagation of Ginkgo through tissue culture (Tommasi and Scramuzzi, 2004; Tolyat *et al.*,2009; Ying *et al*.,2010; Mantovani *et al.*,2013 and Isah, 2020) but a considerable effort is still required to make it more practical and to use it as a commercial method for large- scale production of Ginkgo plant. However, the published methods were unsuccessful in mass production, due to the difficult rooting and low acclimatization of plants. Therefore, the present study was conducted to develop a refined micropropagation protocol via stem nodal segments of *G. biloba* and to plant growth regulators for production and conservation of this unique plant.

II. Materials And Methods

This study was carried out in the Plant Tissue Culture Unit, Plant Genetic Resources Department, Desert Research Center, Matareya, Cairo, Egypt, during the period from 2017 to 2020.

Plant material and sterilization

Nodal explants (as starting plant materials) were collected from young branches of a *Ginkgo biloba* male tree growing in the Orman Botanical Garden, Giza, Egypt. The stem nodal segment explants were chosen for *in vitro* propagation of *G. biloba* because shoot tip explants were very difficult to be sterilized (higher contamination) or survive, and all shoot tips cultured on nutrient medium deteriorated and failed to grow (Figure 1A). The explants were washed under running tap water followed by detergent in Savlon disinfectant solution 3 % for 20 min, then washed with running tap water for one hour. Surface sterilization was soaked for one min in 70% ethanol under aseptic conditions in a laminar airflow cabinet (Holten LaminAir HVR 2448, USA). The explants were then immersed for 5 min in a sterile solution of mercuric chloride ($HgCl_2$) at a concentration of 0.1% (w/v), followed by rinsing sterile distilled water. Then the explants were sterilized in 40% (v/v) commercial bleach solution (Clorox containing 5.25% (w/v), sodium hypochlorite solution; NaOCl) with a few drops of Tween- 20 for 20 min and finally rinsed thoroughly five times with distilled sterile water.

Culture media and Conditions

Explants were cultured on a basal MS medium (Murashige and Skoog, 1962; Duchefa, Haarlem, Netherlands) supplemented with 3 % (w/v) sucrose, 0.1 % myo-inositol, and in addition, 0.3% activated charcoal (AC). Plant growth regulators (PGRs, Sigma Cell Culture, min. 90%, St. Louis, USA) were added to culture medium according to the growth stage. The pH was adjusted to 5.7 ± 0.1, before autoclaving at a pressure of 1.06 kg/ cm^2, and 121°C for 15 min (Harvery Sterilemax autoclave, Thermo Scientific, USA), then the medium was galled with 0.3% phytagel (Duchefa, Haarlem, the Netherlands). Anti browning agents, viz. ascorbic acid (0.1%) and citric acid (0.15%) were added to reduce phenolic compounds. Culturing was done in culture tubes (25 x 150 mm) containing 15 ml of MS medium (for initiation and rooting stages) or large jars (330 ml) containing 50 ml of the medium (for multiplication stage). All cultures were incubated in a culture room at 24 ± 2°C with a 16- h photoperiod under cool white fluorescent lamps with a light intensity of 3000 Lux (F140 t/ 38, Toshiba).

Culture establishment

Various concentrations of 6-benzyl adenine (BA) (0.0, 2.22, 4.44, and 8.90 μM) in combination with α- naphthalene acetic acid (NAA) (0.0, 0.27, and 0.54 μM) were added to the MS medium for the establishment and bud break. The survival and growth percentage (%) and average shoot length (cm) were recorded after five weeks of culture.

Effect of seasonal variation on bud break

In order to obtained the best regeneration capacity, the stem nodal segments were used from apical shoots of *G. biloba.* Explants were collected in four seasons (spring, summer, autumn, and winter) and cultured on the medium with the best PGR concentration determined in the establishment experiment. Survival, growth percentage (%) and shoot length (cm) were recorded after five weeks of culture.

For multiplication of shoots, MS medium supplemented with 0.54 μM (NAA), 0.58 μM Gibberellic acid (GA_3; 0.45 μM flitter sterilized), 0.01% Casein hydrolysate (CH) and different concentration of cytokinins; BA (2.22, 4.44, and 8.90 μM), kinetin (Kin;2.32,4.60 and 9.30 μM or Thidiazuron (TDZ; 2.27, 4.55 and 9.1 μM), individually. MS medium without PGRs served as a control. The number of shoots per explant and shoots length (cm) were recorded after five weeks of culture.

Rooting induction

Individual shoots were transferred for rooting on half- strength MS medium containing 0.15% Activated Charcoal (AC) and supplemented with different concentrations of Indol-3- butyric acid (IBA; Sigma Cell Culture, min. 90%, St. Louis, USA) at 2.46, 4.92 and 9.84 μM and NAA at 2.69, 5.38, and 10.75 μM. MS medium without plant growth regulators (PGRs) is used as a control. Rooting percentage (%), average number of roots per shoot and average roots length (cm) were recorded after six weeks of culture. An experiment for rooting improvement was carried out using silver nitrate ($AgNO_3$), at different concentrations (5.9, 11.8, 23.6,47.1, and 94.2 μM) added to the best rooting induction medium (half- strength MS supplemented with 2.46 μM IBA and 0.15% AC). After six weeks of culture, the rooting percentage (%), average number of roots per shoot, and average root length (cm) were measured.

Acclimatization

Rooted microshoots were removed from the culture medium and washed thoroughly in sterile distilled water. The plantlets were then transferred to plastic pots containing a sterile mixture of peat (peat moss, PROMIX) and sand (3: 1 v/v) in the greenhouse (26 ± 2°C, 70-80% relative humidity). The potted plants were irrigated and covered with transparent plastic bags, which were gradually holes by making a hole in the plastic bag one hole/ three days for five weeks. After six weeks, the plastic bags were removed completely. Tow month-old plantlets were transferred outside the greenhouse.

Experimental design and statistical analysis

Experiments were subjected to the completely randomized design. All the experiments were repeated twice and each treatment had 18 replicate cultures. Analysis of variance (ANOVA) was used to evaluate significant differences between the mean values of different treatments, using Duncan's Multiple Range Test (Duncan ,1955) as modified by Snedecor and Cochrane (1990). The differences between means were compared at $p \leq 0.05$.

III. Results And Discussion

Culture establishment

MS basal medium supplemented with various cytokinin concentrations in addition to NAA showed that axillary shoots could form from stem nodal segments on all the tested media. However, the percentage of explants that grew ranged from 20 to 70% (Table 1). Data obtained after five weeks of culture revealed that shoots could be induced on all tested media with a 72.2% growth percentage on MS medium supplemented with 0.54 µM NAA and 8.90 µM BA (Figure 1B). However, the survival percentage on this medium was 94.4% and the average shoot length was 0.39 cm. Decreasing concentrations of BA for each NAA concentration brought about a decrease in the growth percentage. By taking into consideration the stem length it was found that among the concentrations tested, 0.54 µM NAA produced a higher average length of shoots than 0.27 µM NAA.

Table (1): Effect of MS medium and growth regulators (NAA and BA) on the *in vitro* establishment of *Ginkgo biloba* after five weeks from culture.

PGRs concentrations (µM)		Survival %	Growth % to survival	Average shoot length (cm)
NAA	BA			
0.0	0.0	61.1 f	20.0 g	0.11 e
0.27	2.22	72.2 e	30.8 f	0.90 c
0.27	4.44	77.8 d	42.9 e	0.34 d
0.27	8.90	83.3 c	53.3 d	0.13 e
0.54	2.22	77.8 d	64.3 c	1.52 a
0.54	4.44	88.9 b	68.8 b	1.27 b
0.54	8.90	94.4 a	72.2 a	0.39 d

Means in the same column with different letters are statistically significantly different at $P < 0.05$

The highest average length of shoots (1.52 cm) was obtained on MS medium supplemented with 0.54 µM NAA and 2.22 µM BA, and it decreased gradually with an increase in BA concentration (Table1). On the other hand, the lowest growth percentage and the least average shoot length were recorded on MS medium without PGRs (control treatment). A synergistic effect BA in combination with an auxin has been demonstrated in many trees of Pear; *Pyrus betulefolia* (Hassanen and Gabr, 2012), Sapota; *Casimiroa edulis (*Abd Elmeged *et al*.,2015), Plum; *Prunus domestica* (Wolella,2017), Almond × Peach hybrid rootstock (Ritterbusch *et al*.,2020) and Paulownia; *Paulownia tomentosa* (Taha and Seleem, 2021). Endogenous hormones of Ginkgo tissue are unable on their own to stimulate the sprouting of axillary buds. The addition on a cytokinin is thus necessary. Several studies have shown that combining a cytokinin (BA) and an auxin (NAA) has a positive effect. According to Hassanen and Gabr (2012); Attia *et al*., (2014) and Isah (2020), who mentioned that the combination of an auxin with a cytokinin is accompanied by an increase in the number of bud, also, it seemed necessary for the induction stage and gave very satisfactory results. Finely, concerning the stem nodal segment explants, cultured on MS medium supplemented with 0.54 µM NAA and 8.90 µM BA, they recorded the highest growth percentage. This result is in harmony with that obtained by Radomir and Tudor (2011) and also, Isah (2020) reported that green woody nodal segments from adult *G. biloba* plants cultured, for shoot induction produced better results than non-green woody nodal segment explants.

Effect of seasonal variation on survival and bud break

The effect of seasonal variation on survival and growth was investigated after determining the optimum cytokinin and auxin levels for bud sprouting in order to determine the best season for culture establishment.

One of the most significant factors to consider for the success of the initial culture of Ginkgo plant is the date of taking the explants. This might be considered as a disadvantage that inhibits the success of the micropropagation process.

Data in Table 2 shows that stem nodal segment explants of *G. biloba* taken in Autumn surpassed the other seasons and gave the highest significant survival percentage (72.2%), growth percentage (84.6%), and average shoot length (1.2 cm). Autumn was the optimum time for explants collection for active growth. This may be due to the low levels of endogenous phenolic compounds in the explants during this season. However, in Spring (active growth phase) phenolic compounds were high and explants turned brown (Figure 1 C). and callus induction was quicker from the cut surface of the explant (Figure 1 D). On the other hand, explants taken in Winter gave the least survival and sprouting percentage (33.3% and 16.7%), respectively, without any length and eventually died.

Table (2): Effect of seasonal variations on the survival and growth of *Ginkgo biloba* cultured *in vitro* after five weeks from culture.

Seasons	Survival (%)	Growth % to survival	Average shoot length (cm)
Spring	61.1 b	72.7 b	0.3 c
Summer	50.0 c	66.7 c	0.7 b
Autumn	72.2 a	84.6 a	1.2 a
Winter	33.3 d	16.7 d	0.0 d

Means in the same column with different letters are statistically significantly different at $P < 0.05$

These results may be due to phytohormones, endogenous contamination, or phenolic compounds (data not shown), the variation in the physiological status of the mother plant, and the collected explants during various seasons of the year. These results are in agreement with those obtained by Hassanen *et al.,* (2017) who found that the explants of guava (*Psidium guajava)* collected after the growing season in Autumn gave the best *in vitro* response. On the other hand, Saha (2013) reported that the best season for the initiation of axillary bud sprout from the node cultures of *Schleichera oleosa* under *in vitro* conditions was during April-May. Therefore, Mantovani *et al.*, (2013) revealed that the nodal segments of *G. biloba* excised in January (Summer in Brazil), the period of active growth of plant, were disinfected and successfully established *in vitro*. Moreover, Isah (2020) mentioned that the nodal explants were collected in the Winter (in India) season, and were more responsive to the *in vitro* shoot morphogenesis. With regard to the browning phenomenon, sometimes during *in vitro* culture of some plant species, the media will become brown and the explants will be unable to grow further and eventually die. Some explants leach some phenolic substances or secondary metabolites from cut surfaces, which oxidize later and turn the media brown and are toxic to the explants (Aliyu,2005). Browning of media is common, especially from tree species and mature tissues of woody species. The problem of browning during tissue culture was reported in many plant species including fruit trees like cashew (Aliyu, 2005), pear (Poudyal *et al.,* 2008; Hassanen and Gabr,2012), date palm (Diab, 2015), nabq (Hegazi *et al*.,2016) and guava (Hassanen *et al.*, 2017).

During tissue wounding, browning of tissue is caused by the oxidation of tannin and polyphenols and the formation of quinones, which are highly reactive and toxic to the plant tissues. Addition of antioxidants (ascorbic, citric acid)and activated charcoal (AC) as adsorbent agents to the establishment medium has been reported as effective in preventing oxidation of phenol(Ahmed *et al*., 2013 and Ahmed *et al*., 2016).Therefore, oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethality for explants (Singh, 2018).As browning of the media prevents further progress in biotechnology of woody trees. From the previous results, it was clear that high levels of growth promoting substances and low growth inhibitors during Autumn, antioxidant addition, and activated charcoal may be responsible for the high survival percentage of explants *in vitro* establishment.

Shoot multiplication

To achieve optimal multiplication of *in vitro* established axillary shoots cultured in MS basal medium containing 0.54 µM NAA, 2.89 µM gibberellic acid (GA3), 0.01% casein hydrolysate (CH), and supplemented with different concentrations of BA, Kin, or TDZ (Table 3), *in vitro* established axillary shoots cultured in MS basal medium containing 0.54 µM NAA, 2.89 µM gibberellic acid (GA3),The significantly highest average number of axillary shoots (1.94 shoots /explant) was recorded on MS medium containing 2.22 µM BA. (Figure 1E). followed by MS medium without PGRs and MS medium containing 4.44 µM BA which is insignificantly different from all tested cytokinins.

Table (3): Effect of MS medium supplemented with 0.54 µM NAA, 0.58 µM GA_3, 0.01% CH and growth regulators (BA, Kin and TDZ) on the *in vitro* shoot multiplication of *Ginkgo biloba*. after five weeks from culture.

Cytokinin concentrations (µM)			Average no. of shoots/explant	Average shoot length (cm)
BA	Kin	TDZ		
0.0	0.0	0.0	1.07 b	0.63 c
2.22	0.0	0.0	1.94 a	2.84 a
4.44	0.0	0.0	1.13 b	1.56 b
8.90	0.0	0.0	1.00 c	0.30 d
0.0	2.32	0.0	1.00 c	1.40 c
0.0	4.60	0.0	1.00 c	1.40 c
0.0	9.30	0.0	1.00 c	0.80 c
0.0	0.0	2.27	1.00 c	0.60 c
0.0	0.0	4.54	1.00 c	0.0 e
0.0	0.0	9.08	0.0 d	0.0 e

Means in the same column with different letters are statistically significantly different at $P < 0.05$

Increasing BA concentration up to 8.90 µM in medium decreased the average number of shoots. With respect to the average length of axillary shoots, it reached a significantly value (2.84 cm) on MS medium containing 2.22 µM BA compared to the other tested media. The average shoot length decreased gradually with an increase in BA or Kin and TDZ. Both high concentrations of TDZ (4.54 and 9.08 µM) gave negative growth values (0.0). The high concentrations of BA (4.44 and 8.90 µM) caused the vitrification of shoots and leaves (data not shown). Moreover, concentrations of Kin (4.60 and 9.30 µM) and TDZ (all concentrations) gave deteriorated cultures yellowish leaves without new growth, callus accumulations, weak stems, and eventually died. This finding is in agreement with authors who indicated that higher cytokinin concentrations appear to stimulate ethylene production, which can be antagonistic or shoot proliferation and cause vitrification or somaclonal variation (Van Staden *et al*., 2008 and lebedev *et al*.,2019). Cytokinins (BA, Kin and TDZ) had multiplication in the physiological processes and development of the plant such as cell division, enlargement, stimulation of protein synthesis, enzyme activity, apical dominance suppression and stimulation of lateral buds (Arab *et al*., 2014).

In this study, BA is more suitable and the most effective tested cytokinin for shoot multiplication of *ginkgo biloba* than Kin or TDZ. The significantly highest average number of axillary shoots per explant and shoot length were recorded on MS medium supplemented with 2.89 µM GA_3, 0.54 µM NAA, 0.01% CH and 2.22 µM BA. This treatment has shown the best response for multiplication. These results are agreed with those obtained by Hassanen *et al*., (2017) and Tsafouros and Roussos, (2019) who mentioned that, BA was the optimum cytokinin for shoot proliferation. Regarding TDZ, its minimum effect in comparison with BA, Kin and 2iP may be due to that in some systems, the synergistic effect of TDZ with other cytokinin or auxin was found to be more effective than using it individually (Deepa *et al*., 2018). On the other hand, Isah (2020) mentioned that, TDZ stimulated morphogenic capability with an enhanced shoot formation response, but these shoots were stunted. Regarding GA_3 and CH, it is the stimulation of cell division, elongation and the presence of auxin (NAA)in the medium that enhances the action of GA_3 (George *et al*., 2008). This was confirmed by Gonbad *et al*., (2014), successfully micropropagated *Camellia sinensis* by using GA_3 and auxin in the medium in combination with cytokinin. Also, Preece (2008) reported that the addition of 0.58 µM GA_3 into the MS medium was successful in elongation of shoots for *G. biloba.* As for casein hydrolysate (CH), the effect of CH with PGRs can be strongly modified by the growth medium. CH can be used as a relatively cheap source of a mix of amino acids, also, to stimulate cell growth and facilitate plant regeneration. In this regard, Mantovani *et al*., (2013) found that hydrolyzed casein is essential for axillary shoots and future multiplication. They, however, used higher concentration of CH (500mg/l) supplemented to MS medium which induced 85% of nodal segments developed shoots from axillary buds, and 35% formed multiple shoots. In contrast, the addition of casein hydrolysate (100mg/l) to growing culture increased the shoot number of *Elaeocarpus sphaericus* (Sakalani *et al*., 2015). Wherease,Saha, (2013) reported that the MS medium with or without CH were capable indusing of sprouting bud induction with high frequency (>90% of explant). On the other hand, the highest proliferation of *Chlorophytum borivilianum* shoots was recorded on MS medium supplemented with 10 mg/l casein hydrolysate (Khatr *et al*., 2019). They also confirmed that the inclusion of additives such as; adenine sulphate, casein hydrolysate, and putrescine had a promoting effect on multiple shoot induction and shoot proliferation.

Rooting stage

The concentrations of the two auxins tested for rooting of *Ginkgo biloba*, are presented in Table (4), 66.7% of the shoots rooted in the half-strength MS medium supplemented with 2.46 µM IBA and 0.15% AC. This medium was the best for rooting. It gave the highest average number (2.0 roots/shoots) and length (1.30

cm) of roots (Figure 1F), but these roots were short and thin. While, half- strength MS medium supplemented with NAA at 2.69 or 5.37 gave the results not encouraging.

Table (4): Effect of different concentrations of auxins (IBA and NAA) in half-strength MS medium on *in vitro* rooting of *Ginkgo biloba* after six weeks from culture.

Auxin Concentrations (μM)		Rooting (%)	Average number of roots/shoot	Average root length (cm)
IBA	NAA			
0.0	0.0	0.0 f	0.0 c	0.0 e
2.46	0.0	66.7 a	2.0 a	1.3 a
4.92	0.0	44.4 b	1.0 b	0.8 b
9.84	0.0	33.3 c	1.0 b	0.5 c
0.00	2.69	27.8 d	1.0 b	0.2 d
0.00	5.38	16.7 e	1.0 b	0.2 d
0.00	10.75	0.0 f	0.0 c	0.0 e

Means in the same column with different letters are statistically significantly different at $P < 0.05$.

These results agreed with the Ying and FuLiang, (2005), who reported that MS medium containing 0.5 mg/L IBA gave 66.7% rooting. On the other hand, in the present study, a high concentration of NAA (10.75 μM) or control medium inhibited rooting and root growth. Auxins are important PGRs for *in vitro* culture systems for root formation. IBA is a more potent auxin, effective rooting induction and more stable than NAA (Hassanen and Gabr, 2012). Housman (1993) has shown that IBA slowly oxidizes in tissue culture medium. Therefore, it is high stability, and slow degradation in the plant *in vitro* culture may be the reason for better performance of IBA as compared to other auxins. Low rooting efficiency has been a major problem for *in vitro* production of Ginkgo plants. In this experiment, all media were produced the lowest rooting percentage, number and length of roots. So, for increasing the root system and better acclimatization of plantlets in the greenhouse, well developed and improvement rooting are necessary. Data in Table (5) presented the effect of silver nitrate ($AgNO_3$) at different concentrations (0.0, 5.9, 11.8, 23.6, 47.1 and 94.2 μM) in half- strength MS medium and containing 2.46 μM IBA and 0.15 % AC on rooting improvement of *G. biloba* shoots before acclimatization. It was clear that addition, $AgNO_3$ in the medium was successful in promoting rooting percentage from 66.7% to 88.9% and enhanced the average number and length of roots, compared with the control medium or high concentrations of $AgNO_3$ (47.1 or 94.2μM). The rooting percentage reached 88.9% using 23.6 μM $AgNO_3$ in addition mean number and length of roots per explant were maximum values (4.63 roots / shoot and 3.51 cm, respectively) (Figure 1 G), followed by half-strength MS medium containing 11.8 μM $AgNO_3$, 2.46 μM IBA and 0.15% AC, which gave 83.3% rooting percentage with an average root number per shoot (4.50), and length of 2.93 cm.

Table (5): Effect of $AgNO_3$ at different concentration in half- strength MS medium on reached with IBA at 2.46 μM and 1.5% AC on rooting improvement *Ginkgo biloba* after six weeks of culture.

$AgNO_3$ concentrations (μM)	Rooting %	Average number of roots / shoot	Average length of root (cm)
0.0	66.7 d	1.92 d	1.33 d
5.9	72.2 c	2.31 c	2.70 c
11.8	83.3 b	4.50 b	2.93 b
23.6	88.9 a	4.63 a	3.51 a
47.1	61.1 e	1.64 e	1.17 e
94.2	44.4 f	0.88 f	0.71 f

Means in the same column with different letters are statistically significantly different at $P < 0.05$

On the other hand, high $AgNO_3$ concentrations (47.1 and 94.2μ M) did not stimulate adventitious rooting of the shoots and rooting was very low in comparison to the control treatment. It was 61.1% and 44.4% rooting percentage with an average root number per shoot (1.64 and 0.88 roots/ shoot) and length of roots (1.17 and 0.71 cm), respectively. Parimalan *et al.*, (2010) pointed out *in vitro* plant, the mode of action of $AgNO_3$ is assumed to be associated with the physiological effects of ethylene, silver ions are acting as a competitive inhibitors of ethylene action rather than inhibiting ethylene synthesis. Normally, ethylene inhibits S-adenosyl methionine decarboxylase, which in turn promotes polyamine (Parimalan *et al.*, 2010). Thus, the present study suggests that it is possible to improve the rooting of *G.biloba* by supplementing the growth medium with AgNO3. Many researchers have reported the positive effect of $AgNO_3$ on plant tissue culture and have enhanced the rooting frequency as well as length of the roots (Sirision and Techato, 2012; Hassanen and Gaber, 2013; Venkatachalam *et al.*, 2015 and Hegazi *et al.*,2016). Generally, it could be concluded that providing half-strength MS medium with$AgNO_3$ improved rooting measurements (number of roots /shoot and their average length) of *G. biloba*. This result is in agreement with those obtained by Hassanen and Gaber (2013) who reported that, silver nitrate had a significant effect on the rooting of pecan and increased the color of leaves and

root thickness. Also, Hegazi *et al.*, (2016) found that $AgNO_3$ had a positive effect on root formation of *Ziziphus spina Christi*. Fifty-three percentage of rooted plantlets were successfully acclimatized in the greenhouse (Figure1 H and I). The acclimatized plantlets have been transferred outside the greenhouse. (Figure 1 J).

Figure 1. *In vitro* propagation of *Ginkgo biloba* L.

(A) Different explant types (SA) shoot apices (NS) nodal segment on initiation medium showing bud break**.**
(B) Establishment of nodal segments on MS medium supplemented with 0.54 µM NAA and 8.90 µm BA.
(C) Shoot browning and treated with the activated charcoal (AC) in order to resolve the browning problem .
(D) Callusing at the base of leaf and node or shoot
(E) Multiplication of axillary shoots on MS medium supplemented with 2.22 µM BA.
(F) Rooted plantlets on half-strength MS medium supplemented with 2.46 µM IBA and 0.15% AC.
(G) Root improvement of plantlets on rooting medium supplemented with 23.6 µM $AgNO_3$.
(H) Acclimatization of trasplantlet after five weeks old in the greenhouse.
(I) Acclimatization of trasplantlet after six weeks old in the greenhouse.
(**J)** Acclimatization of trasplantlet after eight weeks old grown outside the greenhouse.

IV. Conclusion

To conclude, a reproducible and efficient micropropagation protocol has been developed using nodal segment explants of a selected adult plant of *Ginkgo biloba*, and it was taken in Autumn season. For the establishment stage, MS medium supplemented with 0.54 μM NAA and 8.90 μM BA is the best medium. A high multiplication rate with uniform growth and shoot length has been achieved. The BA has a positive effect on the multiplication and growth, but high concentrations decrease the growth. The rooting could be obtained using half-strength MS medium supplemented with 2.46 μM IBA and 0.15% activated charcoal. Silver nitrate treatments proved to be optimum for rooting improvement and the survival of plantlets in acclimatization. So, this protocol will help with mass propagation of woody plant culture, pharmaceutical industries, and *in vitro* germplasm conservation of *Ginkgo biloba* L. plants.

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