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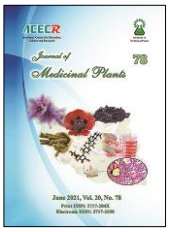

Research Article

The efficient method for *in vitro* micropropagation of *Ginkgo biloba* L.

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ABSTRACT

Background: *Ginkgo biloba* L. leaf extract is used in medicine due to its therapeutic actions such as regulating cerebral blood flow, lowering oxidative stress, delaying the progress of dementia and diabetes. **Objective:** At the current study, the efficient micropropagation of *G. biloba* has been optimized by applying tissue culture method. **Methods:** Different explants (leaves, stems and lateral buds) were disinfected superficially and were cultured in WPM supplemented with various types and concentrations of plant growth regulators for shooting and subsequently for root induction. **Results:** The best treatment for shoot induction frequency (100 %), regenerated shoot length (2.47 cm) and number of regenerated leaves per explant (6.5) was achieved by culturing the lateral buds on WPM medium having Kin at 1 mg/L and IAA at 0.5 mg/L. The best root induction medium on the basis of root induction (100 %) and regenerated root lengths (8.5 cm) was WPM medium with IBA at 1 mg/L and AC at 2 g/L. After acclimatization, 60 % of regenerated platelets were survived. Finally, based on HPLC analysis, no significant difference was observed between the amount of quercetin in the leaves of propagated seedlings under *in vitro* conditions and their mother base. **Conclusion:** The optimized protocol proposed to be used as an efficient method for commercial micropropagation of ginkgo tree.

1. Introduction

Ginkgo biloba L. is a Gymnosperm tree with no close living relatives. It is known as "a living fossil" because of its ancient presence on earth since 200 million years ago. It naturally grows in China, Japan and Korea and is commonly being cultivated in other countries for medicinal or ornamental purposes [1-3]. Ginkgo's extracts have been reported to have effect on the regulation of cerebral blood flow and protection against free radicals, reduction the progression of Alzheimer [4-7]. There are numerous chemical constituents contained in bark, roots and leaves of this precious plant such as ginkgolides, bilobalide and the flavonol glycosides including quercetin [8, 9]. *G. biloba* also contains abundant

Abbreviations: WPM, Woody Plant Medium; Kin, Kinetin; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; 2,4-D, 2,4-Dichrolophenoxyacetic acid; NAA, Naphthaleneacetic acid; BAP, 6-Benzylaminopurine; HPLC, High-performance liquid chromatography; ANOVA, Analysis of variance; SE, Standard error, AC; Activated charcoal

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endophytes and various secondary metabolites. According to the published literature about endophytics from *G. biloba* including *Chaetomium*, *Aspergillus*, *Alternaria*, *Penicillium* and *Charobacter* were isolated from the root, stem, leaf, seed and bark of *G. biloba*. The endophytics could produce lots of phytochemicals like flavonoids, terpenoids, and other compounds. These compounds have practically shown certain antibacteria, antioxidation, anticardiovascular, anticancer, and antimicrobial functions [10]. There are some difficulties in generative propagation of *G. biloba* including dioceous nature, long juvenile phase, long reproductive cycle, and low germination rate, short period of seeds viability and recalcitrant nature of seeds [11]. Otherwise, the vegetative propagation through cuttings has low income and is extremely dependent on the juvenile nature of explants [12]. In order to overcome referred obstacles and to conduct a massive and rapid production scheme of this medicinal plant, *in vitro* propagation were used as an alternative and effective way in previous studies. The important point in this research is that the introduced method is based on direct regeneration (without callus induction phase) which could be one of the most effective methods to conserve plant maternal germplasm. That is because the somaclonal variation in the callus tissue (Indirect regeneration) impairs the achievement of mass cloning while direct shoot induction doesn't encounter such problems [13, 14]. Optimizing the *in vitro* micropropagation condition depends on many factors such as explants type and hormonal composition of the culture medium and several previous studies as well as current investigation have shown the same. For example, apical and nodal meristems along with apical buds from plantlets of *G. biloba* were used as explants and were cultured on MS medium having different kinds of plant growth regulators including BAP, NAA, Kin and IAA as well as endosperm from mature seeds of the same species. Meristems produced calli with extensive range of texts and colors along with single or rare multiple shoots on MS medium with different growth regulators and endosperm extract. For instance, in media having IAA, Kin and endosperm extract, 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. On medium including IAA, BAP and endosperm extract, the explants produced a much larger callus and shoots in 60 % of the explants from apical buds and in 20 % from the nodal buds [11]. Another experiment being conducted in the context has also shown that how the explants type and plant growth regulators as well as endosperm have provided to possess important effects on shoot and root regeneration of *G. biloba*. The effect of two types of explants (woody and herbaceous nodal segments) from adult plants of *G. biloba* and the effect of hydrolyzed casein (500 mg/L), kinetin (0.46 and 4.65 μM) and AC (1.5 g/L) were evaluated upon new shoots induction and development. Results showed that woody nodal segments did not produce axillary shoots. However, nodal segments from herbaceous shoots were displayed high *in vitro* morphogenic capacity. Results also demonstrated the essential role of hydrolyzed casein for the axillary shoots induction and further multiplication [12]. In another study, different developmental stages of immature embryos of *G. biloba* were cultured on MS medium supplemented with various 2,4-D, NAA, BAP and zeatin or with various concentrations of 2,4-D alone. Maximum number (8 per embryo) of adventitious formed from cotyledons of heart stage embryos cultured on MS medium 1 mg/ dm^3 BAP and 0.01 mg/dm^3 NAA [15].

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Most of the previous researches being conducted in this field have been focused on applying zygotic embryos and *in vitro* germinated seedlings as explants which are difficult to collect in places like Iran where this species is not endemic and does not grow naturally. What makes this study prominent maybe is the use of vegetative segments as explants as well as the application of common cultural medium and plant growth regulators which are easy to be prepared.

2. Materials and Methods

2.1 Plant material and surface sterilization

Leaves, stems and lateral buds of young and herbaceous branches of a five-year old ginkgo plant in active growing season (spring, 2020, Karaj. Iran) were collected as explants. The explants were surface sterilized by treating a modified method including the following procedures: shaking with sterile distilled water and tween 20 (10 min); washing under running tab water (60 min); shaking with 70 % ethanol (2-3 min), shaking with 0.2 % (v/v) sodium hypochlorite solution (15 min), rinsing with sterile distilled water (2-3 min), shaking with 0.1 % Mercury (II) chloride solution (2 min). Finally, explants were rinsed again three times with sterile distilled water to remove any traces of the surfactants [9].

2.2 Shoot induction

Aseptically excised explants of leaves, stems and lateral buds (5-10 mm) were cultured in WPM [16] culture as a base medium for 10 days. Then, the healthy explants (without fungal or bacterial contaminations) were subcultrured in different shoot induction media consisting of WPM medium along with BAP, Kin and IAA (Sigma Aldrich Company, UK) at 0, 0.5 and 1 mg/L as plant growth regulators. The cultures were solidified with 7.0 g/L agar. The pH was adjusted to 5.7 with NaOH or HCl (0.1 N). The culture medium was distributed in aliquots of 50 mL in caped glass (230 mL) and autoclaved for 20 minutes (121 ºC). All cultures were kept in the growth chamber under at 25 ºC ± 2 and photo period of 16 hours light and 8 hours darkness intervals for 60 days. The subculture was performed once every month. The shoot induction (%), the number of leaves (shoots) per explants and the shoot lengths were taken under evaluation for the issue of shoot regeneration.

2.3 Root induction and acclimatization

For root induction, the regenerated shoots were transferred on WPM medium supplemented with IBA at 0, 0.5 and 1 mg/L and AC at 0 and 2 g/L. The culture was kept for 60 days with the same environmental conditions and then, the root induction (%) and the number of root per explants were measured. For acclimatization, rooted plantlets were washed in running tap water to remove the nutrient media to avoid the fungal attack of the root system. Cleaned plantlets were then planted in pots under plastic caps. Every day one hole was made on plastic caps to reduce humidity gradually. The soil mix prepared by mixing coco peat, peat moss and perlite at a 1:1:1 ratio. The soil was autoclaved at 121 $^{\circ C}$ for 45 min prior to planting of the rooted plantlets.

2.4 Analysis of total flavonoid by HPLC analysis

G. biloba extracts of leaves from both micropropagated plantlets and donner were prepared by mixing freeze-dried leaves (0.2 g) with 50 mL acetonitrile solution treated with 60 min sonication. Next, the extracts were centrifuged at 20,000 rpm at 4 ºC for 15 min. The supernatant was then collected and filtered for HPLC analysis. Querrcetin were quantified by

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their optical density peaks at 370 nm and using Querrcetin (Sigma Aldrich Company, UK) as standard compounds for calibration. HPLC analysis was performed on Knauer HPLC system (1200 series, UV detector K-2501). A volume of 50 µL of samples was injected in 18 reverse-phases Phenomenex column (Gemini NX-C18, 5 mm, 4.6 × 250 mm). The mobile phase for alkaloid elution was 0.3 g/L solution of phosphoric acid (pH: 2).

2.5 Experimental design and statistical analysis

The experiments (shoot induction and root induction) were setup on a completely randomized design with three replicates per treatment and three explants per replicate. Nine treatments (BAP, Kin and IAA at 0, 0.5 and 1 mg/L) for shoot induction experiment and four treatments (IBA at 0, 0.5 and 1 mg/L and AC at 0 and 2 g/L) for root induction experiment were studied. Statistical differences were assessed based on ANOVA and an independent sample T-Test using SPSS (version 18, USA). Means were compared using Duncan multiple rang test. Differences were considered significant at a probability level of $P < 0.01$.The values are expressed as the mean ± SE.

3. Results

3.1 Shoot regeneration

One month after transferring of Ginkgo explants into shoot induction media, new shoots appeared on the lateral bud explants and after another month, each shoot had a leaf (Fig. 1A). While, stem and leaf explants did not regenerate even after 60 days. Based on the results, the frequency of *in vitro* shoot induction in all treatments for the lateral bud explants was 100 % except hormone free WPM medium which showed no shoot induction even after 60 days.

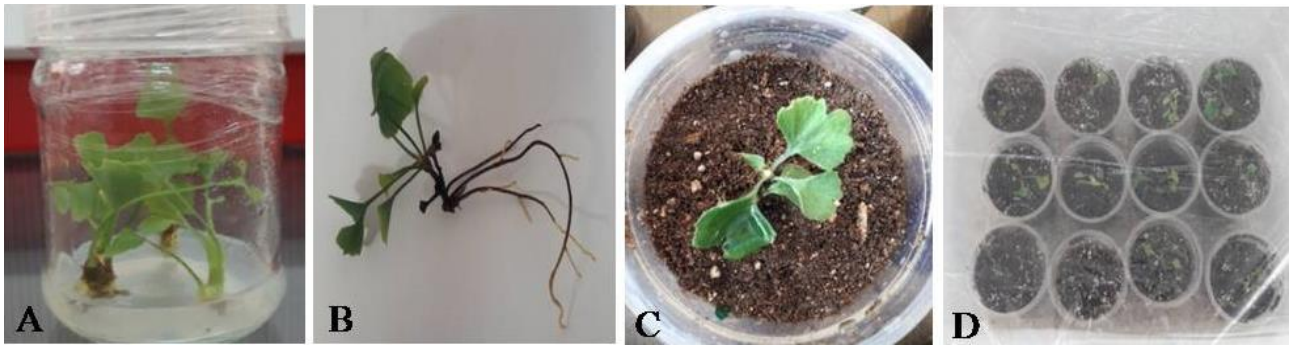

Fig. 1. *In vitro* micropropagtion of *G. biloba.* A) Shoot regeneration after 60 days of culturing; B) Root regeneration after 30 days of culturing; C) Transferring regenerated plantlets from *in vitro* culture to the soil; D) Keeping plantlets under the plastic cap for acclimatization

The results of variance analysis of *in vitro* regenerated shoot lengths and leaf numbers per explant (or shoot numbers) showed that the effect of Kin and IBA on shoot lengths and leaf numbers was significant at 5 % probability level (Table 1). The mean shoot lengths and leaf numbers in different shoot induction treatments were observed from 1.07 to 2.47 cm and from 4 to 6.5, respectively. The highest shoot length and leave number were both obtained in WPM medium supplemented with Kin at 0.5 mg/L + IAA at 1 mg/L (Fig. 2 and Fig. 3).

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Table 1. The variance analysis of the effect of different concentrations of BAP, IAA and Kin combinations on regenerated shoot lengths (cm) and leaf numbers per explant of *G. biloba*

Source of variation	df	Mean of Squares	
		Shoot Length	Leaf Number
BAP	2	0.554^{ns}	2.577^{ns}
IAA	2	1.598^{ns}	2.383^{ns}
Kin	2	1.618^{ns}	5.616^{ns}
BAP × IAA	4	0.150^{ns}	1.109^{ns}
BAP × Kin	4	0.290^{ns}	0.502^{ns}
IAA × Kin	4	2.822^{*}	5.803^{*}
BAP × IAA × Kin	8	0.955^{ns}	1.761^{ns}
Error	54	0.997	1.969

* Significant at $P \leq 0.05$; ns: not significant

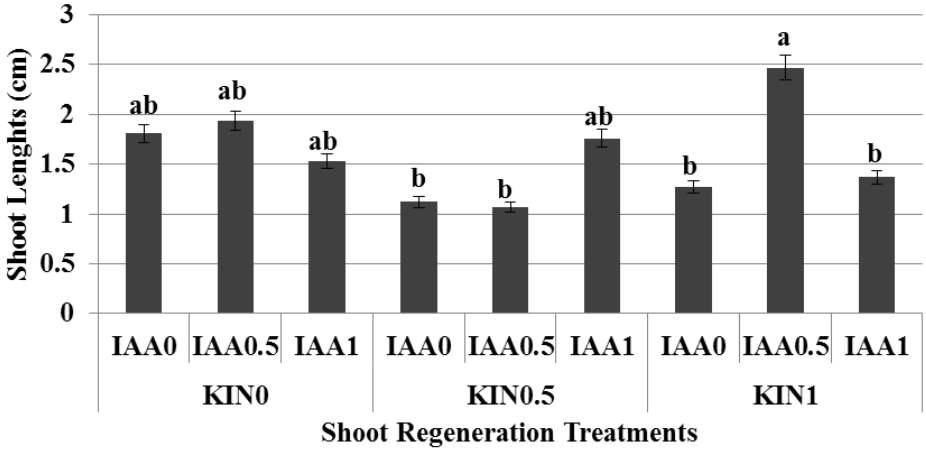

Fig. 2. Effects of different concentrations of IAA and Kin combinations on regenerated shoot lengths (cm) of *G. biloba*. Values followed by different letters in each treatment are significantly different at $P \leq 0.05$. The values are expressed as the mean ± SE

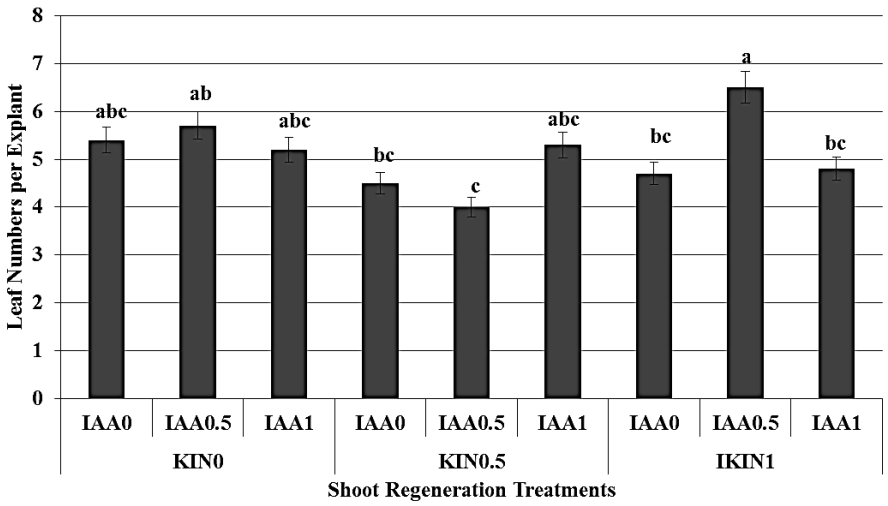

Fig. 3. Effects of different concentrations of IAA and Kin combinations on regenerated leaf number per explant of *G. biloba*. Values followed by different letters in each treatment are significantly different at $P \leq 0.05$. The values are expressed as the mean ± SE.

3.2 Root induction and acclimatization

In the next step, regenerated shoots were subcultured on WPM medium containing different concentrations and combinations of IBA and AC for root induction. They were rooted successfully with 100% efficiency after 60 days cultured on WPM medium with IBA at 1 mg/L and AC at 2 g/L (Fig. 1B). The variance analysis of the effect of IBA and AC on root induction (%) and root lengths were considered as significant at 1 % and 5 % probability level, respectively (Table 2). The maximum root length (8.5 cm) was both observed on WPM medium having IBA at 1 mg/L and AC at 2 g/L (Fig. 4 and Fig. 5).

Rooted plantlets were washed in running tap water to remove the nutrient media to avoid the fungal attack on the root system. Cleaned plantlets were then planted in small plastic pots with the soil (Fig. 1C).

All the plantlets were acclimatized for 40 prior to transferring them to greenhouse. The survival rate during the acclimatization procedure reached 60 %.

Table 2. The variance analysis of the effect of different concentrations of IBA and AC combinations on regenerated root induction (%) and root lengths (cm) per explant of *G. biloba*.

Source of variation	df	Mean of Squares	
		Root Induction	Root Length
IBA	2	6160.5**	37.340**
AC	1	3960.5**	30.681*
IBA × AC	2	3499**	18.029*
Error	12	181.5	3.519

** Significant at $P \leq 0.01$; * Significant at $P \leq 0.05$

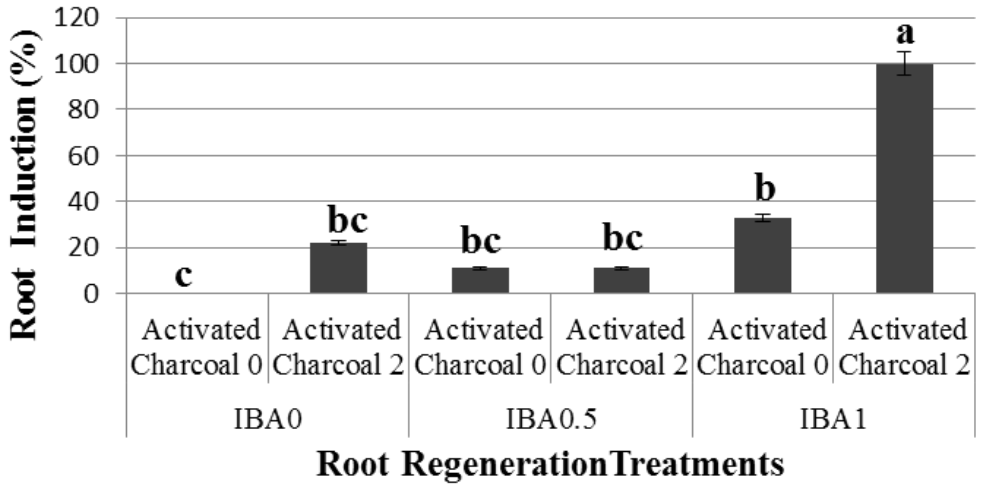

Fig. 4. Effects of different concentrations of AC and IBA combinations on Root induction (%) of *G. biloba*. Values followed by different letters in each trait are significantly different at $P \leq 0.05$. The values are expressed as the mean ± SE

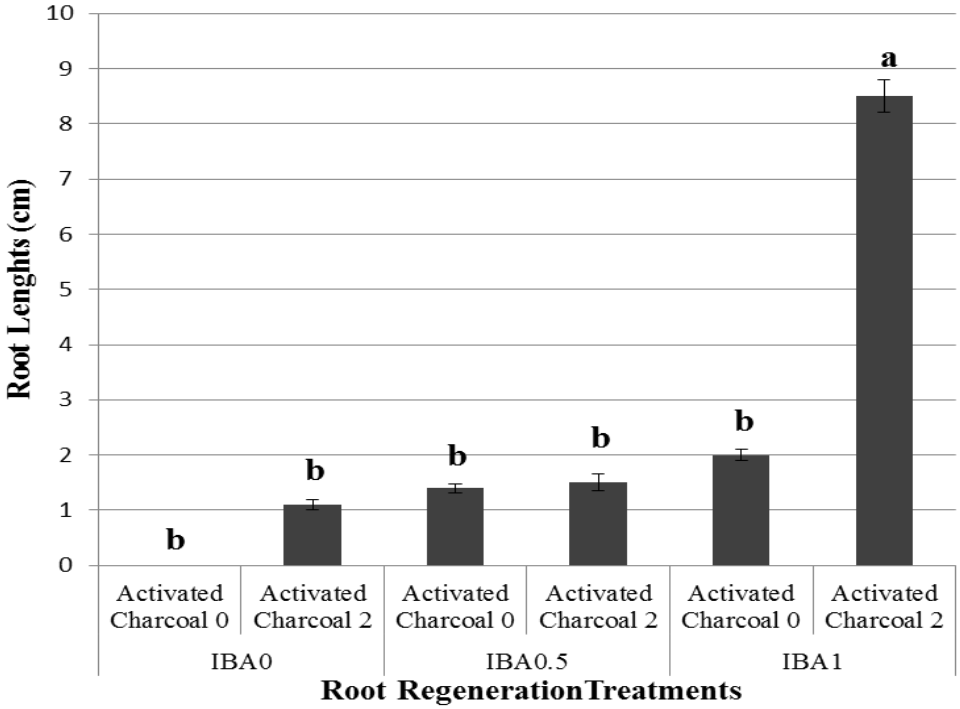

Fig. 5. Effects of different concentrations of AC and IBA combinations on regenerated root lengths (cm) of *G. biloba*. Values followed by different letters in each trait are significantly different at $P \leq 0.05$. The values are expressed as the mean ± standard error (SE).

3.3 HPLC analysis

At the final stage of the present study, quercetin contents in dried leaf samples of *in vitro* regenerated plantlets and the donor plant were measured by high performance liquid chromatography (HPLC) method. According to T-test analysis, there was no significant difference between them (Table 3). HPLC chromatograms of the extract of donor plant and *in vitro* regenerated leaves (one replication of each) were shown (Fig. 6).

Table 3. T-Test analysis of quercetin contents in dried leaf samples of *in vitro* regenerated plantlets and the donor plant

	Mean and SEM		**Mean Difference**	**t**	**df**	**Sig. (2-tailed)**
	***In vitro* regenerated plantlets**	**Donor plant**				
Quercetin concentration	0.233 ± 0.085	0.153 ± 0.045	0.080	1.439	4	0.223

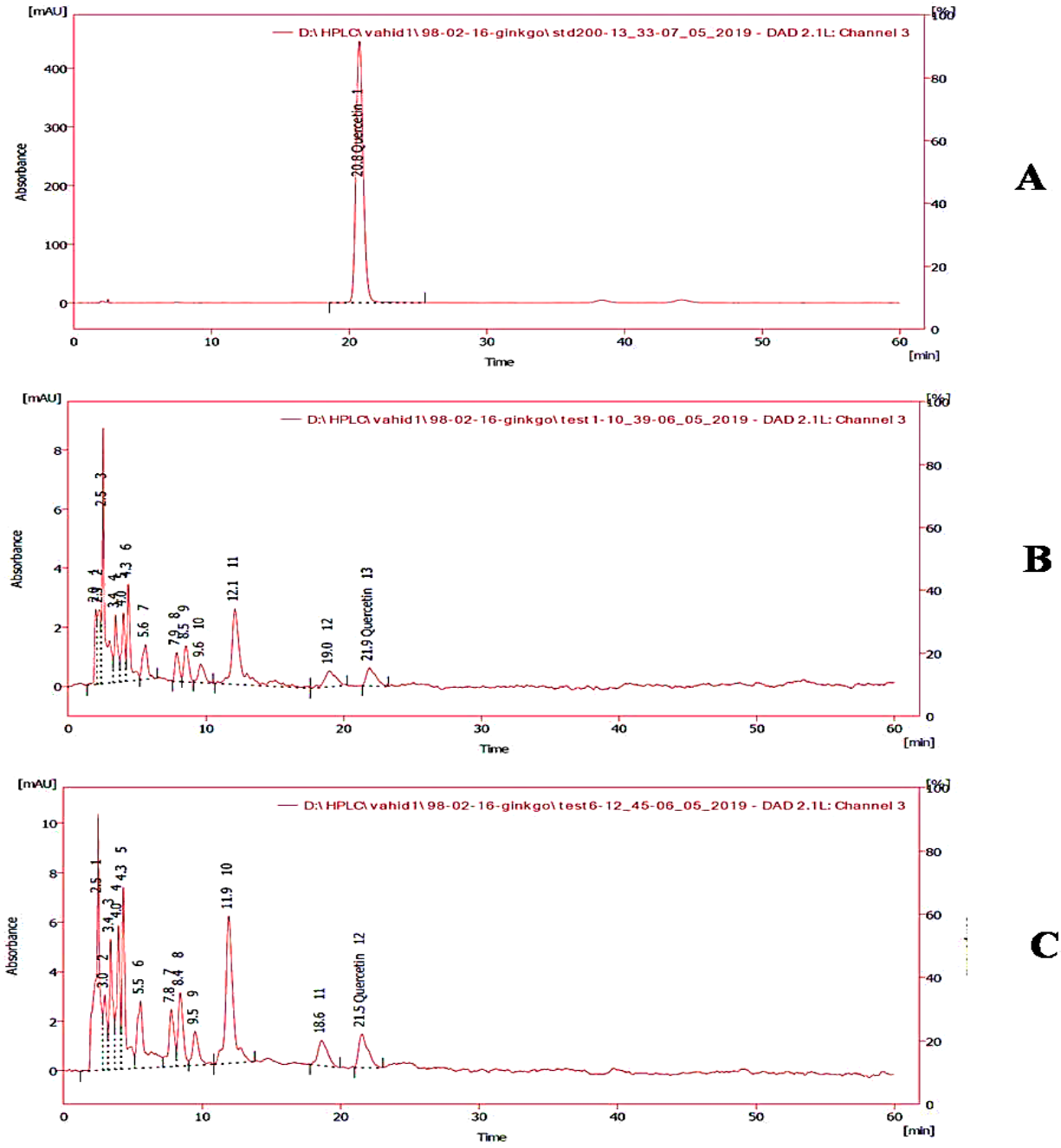

Fig. 6. HPLC chromatograms of *G. biloba* (A) Standard Quercetin (B) The extract of donor plant leaves (one replication) (C) The extract of *in vitro* regenerated leaves (one replication)

4. Discussion

In vitro culture has opened extensive areas of research for micropropogation, secondary metabolite production and biodiversity conservation. Plant *in vitro* regeneration is a biotechnological tool that offers a tremendous potential solution for the propagation of endangered and superior genotypes of medicinal

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plants as well as *G. biloba* which could be released to their natural habitat or cultivated on a large scale for the pharmaceutical product of interest [9, 17].

Plants have a remarkable regenerative capacity, but it varies widely among species and tissue types. Explant choice is one of the essential factor in establishing successful micropropagation protocol of *G. biloba* [9]. In the literature, *in vitro* regeneration of *G. biloba* is mostly limited to cultures of intact embryos [18- 19]. There are also some reports regarding applying male gametophytes [20], female gametophytes [21], nodal segments and buds [9, 11-12] as explants for *in vitro* culture and micropropagation of this medicinal plant. At the current study, among stem, leave and lateral bud as explants, only the last one showed positive response to *in vitro* regeneration. Stem and leave explants did not induce any shoot even 60 days after being cultured in different shoot induction treatments. While lateral buds induced shoots with the frequency of 100 % in all treatments expect hormone free WPM medium. This result shows the importance of explant type in *in vitro* regeneration of *G. biloba* and confirmed the previous studies. The role of explant type in micropropagation of various plant species has also been identified [22-24]. Another key factor for *in vitro* regeneration of *G. biloba* as well as different other plant species is plant growth regulators. Plant growth regulators are important biomolecules in plants which exist at low concentrations and act as important signaling compounds mediating almost all plant processes. They are important in not only day to day physiological functions, adaptations, growth and development processes, but may respond in specialized manners to developmental or stress related cues [9, 25-26].

In this study, the treatment containing Kin (1 mg/L) + IAA (0.5 mg/L) has shown the best response regarding shoot length (2.47 cm) and leaf number per explant (6.5). While Tommasi and Scaramuzzi [11] reported callus induction and low shoot regeneration by applying IAA + Kin (in MS basal medium with endosperm extract) and Mantovani *et al.*, [12] showed that hydrolyzed casein is essential for the axillary shoots and future mollification. The assessment of the effects of different cytokinins on shoot multiplication of ginkgo showed that Kin promoted the formation of multiple shoots [12]. Results of other researchers have also shown that the Kin increases shoot number proliferation. [27-28].

Induction of roots is among bottlenecks encountered in complete plant regeneration in woody plants that includes *G. biloba*, and in most of the cases the response regarding rooting percentage and quality are low [9, 19]. The efficiency/inefficiency for root initiation using natural and synthetic auxins have been reported to be associated with genotypic influence in the in vitro cultures of many woody species [9, 29-30]. According to results obtained in this investigation, the treatment having IBA (1 mg/L) + AC (2 g/L) were the best for root induction (100 %) and root length (8.5 cm). Tommasi and Scaramuzzi [11] suggested applying IBA + endosperm extract in MS medium to produce normal roots with the frequency of 70 %. Formation of a well-developed root system is as important as multiplication stage for plants cultivated in *in vitro* environment. A well-established *in vitro* rooting system is essential both for shoot survival and plant growth especially to next stages such as acclimatization and transplanting to field. For root formation, auxins are the most widely used regulators, and IBA is commonly used since it allows a better

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rooting capacity and is less susceptible to biological degradation as compared to other synthetic auxins [31]. The effect of AC is connected to the formation of a darkened environment, adsorption of undesirable and inhibiting substances and binding of growth regulators and other organic compounds which promotes changes that lead to the formation of roots [32].

The advantage of the introduced protocol at the current study could be its potential to enhance both number and length of the regenerated shoots and roots.

Finally, comparing the amount of quercetin present in the leaves of *in vitro* propagated platelets and the donor plant (according to HPLC analysis) showed that there was no significant difference between them. This is another reason for the role of direct regeneration without callus stage in increasing the similarity between the donor plant and the plants propagated from it.

5. Conclusion

In conclusion, culturing lateral buds of *G. biloba* as explants in WPM medium supplemented with the combination of Kin and IAA for shoot induction and the combination of IBA and AC for root induction is suggested for low cost and effective *in vitro* regeneration.

Author contributions

Experiment performing and data gathering: N. Z., writing: N. Z. & A.Q., editing: A. M. & Sh. R.

Conflict of Interest

Authors declare that there is no conflict of interest.

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This work was accomplished at Biotechnology department of Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran.

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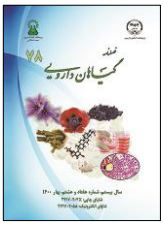

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مقاله تحقیقاتی

روشی مؤثر در ریزازدیادی درون شیشه‌ای گیاه ژینکو

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اطلاعات مقاله

گل‌واژگان:
درخت ژینکو
تنظیم‌کننده رشد گیاهی
القای ریشه
القای ساقه
کشت بافت

چکیده

مقدمه: عصاره برگ درخت ژینکو (*Ginkgo biloba* L.) به دلیل دارا بودن خواص درمانی چون تنظیم جریان خون مغزی، محافظت در برابر رادیکال‌های آزاد، تأخیر در پیشرفت زوال عقل و دیابت در پزشکی استفاده می‌شود. **هدف:** هدف از تحقیق حاضر، بهینه‌سازی تکثیر گیاه ژینکو به روش ریزازدیادی از طریق کشت بافت می‌باشد. **روش بررسی:** ریزنمونه‌های مختلفی چون برگ، ساقه و جوانه‌های جانبی، گندزدایی سطحی شدند. سپس به منظور القا ساقه و ریشه، بر روی محیط کشت WPM به همراه انواع و غلظت‌های متنوعی از تنظیم کننده‌های رشد گیاهی کشت داده شدند. **نتایج:** بهترین تیمار از نظر درصد القای نوساقه (۱۰۰ درصد)، طول نوساقه باززا شده (۲/۴۷ سانتی‌متر) و تعداد برگ‌های باززا شده (۶/۵)، حاصل کشت جوانه جانبی در محیط کشت WPM به همراه کینتین به میزان ۱ میلی‌گرم در لیتر و ایندول-۳- استیک اسید به میزان ۰/۵ میلی‌گرم در لیتر بوده است. بهترین محیط القا ریشه بر اساس ریشه‌زایی (۱۰۰ درصد) و طول ریشه‌های باززا شده (۸/۵ سانتی‌متر) نیز محیط کشت WPM به همراه ایندول-۳- بوتیریک اسید به میزان ۱ میلی‌گرم در لیتر و زغال فعال به میزان ۲ گرم در لیتر به دست آمد. پس از طی مرحله سازگاری، ۶۰ درصد از گیاهچه‌های باززا شده، زنده ماندند. در نهایت، بر اساس آنالیز HPLC، تفاوت معنی‌داری بین میزان کوئرستین موجود در برگ‌های گیاهچه های تکثیر شده در شرایط درون شیشه یا پایه مادری آنها مشاهده نشد. **نتیجه‌گیری: این** شیوه‌نامه بهینه‌سازی شده می‌تواند به عنوان روشی کارآمد در ریزادیادی تجاری درخت ژینکو مورد استفاده قرار گیرد.

مخففها: WPM، محیط کشت درخت چوبی؛ Kin، کینتین؛ IAA، ایندول-۳- استیک اسید؛ IBA، ایندول-۳- بوتیریک اسید؛ 2,4-D، ۲،۴- دی کلرو فنوکسی استیک اسید؛ NAA، نفتالن استیک اسید؛ BAP، ۶- بنزیل آمینوپورین؛ HPLC، کروماتوگرافی مایع با کارایی بالا؛ ANOVA، تجزیه واریانس؛ SE، خطای استاندارد؛ AC، زغال فعال

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