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Effect of some chemicals inducers on terpenes production (ginkgolide A and bilobalide) in callus of *Ginkgo biloba*L. plant

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Abstract:*The present experiment was carried out to study the effectof some inducers i.e. casein (0.5, 1, 1.5 and 2 g), gibberellic acid(0.1, 0.5,1 and 2 ppm) and sucrose(45,60, 75 and 90 g) on callus growth, terpenesproduction (ginkgolide A and bilobalide) and some biochemical constituents in Ginkgo biloba L. callus. Data showed that the highest callus induction was shown on Murashige and Skoog (MS) medium supplemented with 1.0 mg/l of NAA and 1.0 mg/l kinetin. The highest amount of ginkgolideA(0.269 mg/g fresh weight) was recorded at rate 0.5 g of casein. While, highest concentration of bilobalide (4.00 mg/g) was obtained with75 g/l sucrose. In the same direction, the maximum value of callus weight was associated with the highest concentration of gibberellic acid (2 ppm) compared with the control. On the contrary, the lowest fresh weight is obtained by the addition of the high concentration of sucrose 90 g/l. While, the maximum value of malondialdehyde and hydrogen peroxide are associated with the highest concentration of sucrose. In conclusion, Ginkgo bilobaL.callus cultured showed capacity for producing ginkgolide A and bilobalide and could be an alternative renewable natural source for its large scale production. Moreover, some of inducers may contribute to increase active constituents with reduced the cost production in callus culture of Ginkgo biloba L.*

Keywords:*Ginkgo biloba L., growth of callus, ginkgolideA,bilobalide, malondialdehyde, hydrogen peroxide andamino acids*

I. Introduction

*Ginkgo biloba*L. is a tree belonging to family (Ginkgoaceae), it is considered one of the endangered plants in Egypt which requires the conservation and maintenanceby conventional methods or using modern technologies in the field of biotechnology (Brenner *et al.,* 2005 and Bekhit*et al*., 2008).It is one of the most used medicinal plants for more than 2,000 years, which used for the improvement of the peripheral and central blood circulation, arterial occlusive disease, vertigo and against demential disorders (memory impairment and concentration difficulties) (Warrier and Corzine, 2000).In Europe and the USA have been interesting of production pharmaceutical products from *Ginkgo biloba*L.(Cassileth, 2011). Standardized ginkgo leaf extract obtained from dried leaves contain 3.1 % ginkgolides, 2.9% bilobalide and 2.4% flavonoids (Drieu*et al.*,2000;Van Beek and Montor, 2009). This extract used for treatment many diseases due to its therapeutic actions in regulating cereberal blood flow, protecting against free radicals and dealing the progress of dementia and diabetes (Cheng *et al.,* 2009; Weinmann*et al* .,2010 and Zhou *et al.,* 2011).

Bilobalide is considered sesquieterpene and have related to Ginkgolides but differ from ginkgolides by the absence of tetrahydrofuran ring(Tanaka*et al.,* 2011), where ginkgolides have been used in treating disorders such as asthma which inhibit allergic responses(Braquet*et al*., 1987).*Ginkgo biloba*extract contains twoimportant active pharmaceutical components; flavonoids and terpene lactones which are used in treatment of cerebrovascular diseases such as coronary heart disease and high blood pressure(Nakanishi, 2005; Mahadevan and Park, 2008; Lu *et al.,* 2011and Cheng *et al*., 2014). Also,Kanowski*et al.,* (1997) showed that *Ginkgobiloba* has therapeutic potential in slowing some of the symptoms associated to primary stages of Alzheimer's disease.

There are many researches which showed the importance of use chemical inducers such as (nitrogenic compound, hormones and sugars).In this regard, casein used by Abyari*et al*., (2016) and gibberellic acid byHendawey and Abo El Fadl (2014),and sucrose byThiruvengadam*et al.,* (2016) for increasing the content of active constituents in produced callus. Malondialdehyde is used as biomarker to measure the level of oxidative stress in organism (Del Rio *et al.,* 2005). Hydrogen peroxide is considered a weak oxidizing agent which cross cell membrane rapidly and reacts with Fe^{+2} and possibly Cu^{+2} ions to form the damaging toxic hydroxyl radical (Mandal *et al.,* 2012) and it may act as secondary messenger to regulate the antioxidant defense under stress conditions (Quan*et al.,* 2008).Amino acids can be used to synthesize the necessary proteins and other molecules to support growth(Javed*et al.,* 2013).

This research aims to study the effect of some chemical inducers (casein, gibberellic acid and sucrose) with different concentrations on callus induction, callus growth rate and the production of ginkgolide A and bilobalide. Also, biochemical constituents such as malondialdehyde, hydrogen peroxide and combined amino acids were determined in *Ginkgo biloba*L. callus.

II. Materials And Methods

Plant material collection

One year old branches were taken from up to 30 years old male tree of *Ginkgbiloba*L. grown in the Egyptian Botanical Garden(Orman). The leaves (green and healthy) were excised from the branches in the second week of April in season 2014.

Plant material sterilization

The leaves of *G. biloba* were washed under running tap water followed by detergent for 5 minutes. The surface of leaves were sterilized under aseptic condition by immersion in 70% ethanol for 30 seconds, and then rinsed three times with sterilized distilled water and transferred to 0.1% mercuric chloride (w/v) for 2 minutes, followed by rinsing sterile distilled water. All leaves explants sterilized in 40% (v/v) commercial bleach (Clorox) 5.25 %(w/v) available chlorine, sodium hypochlorite solution with one drop of Tween 20, vigorously shaken for 20 minutes, and rinsed three-four times with sterile distilled water. Subsequently, the leaves explant exposed to the sterilization agent were cut into 1 cm long under a laminar flow hood.

Culture conditions and callus induction

To induce callus formation, the sterilized leaves were cut length wise and cultured onMurashigeand Skoog (1962)basal medium (MS) supplemented with (100 mg/l) my-inositol, (30g/l) sucrose, (1.0 mg/l) naphthalene acetic acid , (1.0 mg/l) kinetin and solidified with (2.5g/l)phytagel. The pH of the medium was adjusted to 5.7 with 0.1N HCl and 0.1N NaOH, before autoclaving at 121°C for 20 minutes. The culture was incubated in dark at 25± 2°C and harvested after 30 days.

Active constituentspromotion

To promote active constituents in *G.biloba* callus, equal amount of callus (2g) was cultured on MS basal medium (as previously mentioned, which gave the best growth parameters) with some chemical inducers. The experiment included three treatments with different levels compared with control as follows:

- Control (without casein, gibberellic acid and sucrose)
- Casein hydrolysate at 0.5, 1.0, 1.5 and 2.0 g/l
- Gibberellic acid at 0.1, 0.5, 1 and 2 ppm
- Sucrose at 45, 60, 75 and 90 g/l

After 30 days from culture, the samples of fresh *G.biloba* callus were collected to determine some growth parameters, terpenesproduction (ginkgolide A and bilobalide) and some biochemical constituents.

Qualitative and quantitative identification of active constituents

Extraction of active constituents

Ginkgolide A and bilobalide were extracted from 6 g fresh weight of callus by mortaring in methanol 10ml after dilution with an equal volume of water. Ethyl acetate (20ml) was added to the resultant mixture, and the mixture was shaken on a vortex mixer for 40 minutes and then centrifuged at 3000 rpm; the ethyl acetate phase was transferred to a glass tube. The extraction procedure was repeated five times. Upon extraction, the combined ethyl acetate layer was evaporated by nitrogen at 50°C. The residue was dissolved in 2 ml of methanol, and the samples stored frozen in a sealed vial until separation of active constituents by High Performance Liquid Chromatography (HPLC) in Central lab of Desert research Center (DRC).

HPLC conditions

Active constituent of *G. biloba*L. callus were determined by High Performance Liquid Chromatography (HPLC) according to the method of Cheng *et al.*,(1998).The HPLC system was a Dionex Ultimate 3000 equipped with an auto- sampler, quaternary pump and a diode array detector. Samples were chromatographed BDShypersil C18 (4.6 x 250 mm, particle size 5µm). Separation was performed with methanol and water (23:77v/v) as the elusion solvent rate of 1ml/minute and the detection wave length was 205/220 nm. The peak area and the percentage of ginkgolide A and bilobalide were calculated using an external standard by computer software of HPLC.

Malondialdehyde content

The level of lipid peroxidation in *G.biloba*L. callus was quantified by determination of malondialdehyde content (MDA), breakdown product of lipid peroxidation according to Health and Packer (1968) and modified by Zaho*etal* (1994).Two gram of callus was homogenized in a 1 ml of 0.1 % (w/v) trichloroacetic acid with a prechilled mortar and pestle. The homogenate was spun at 14,000 rpm for 5 minutes. 2 ml of TBA reagent was added to 0.5 ml of the supernatant. The mixture was heated at 95^{o}C for 15 minutes and cooled immediately. The absorbance was read at 532 nm and the value was corrected for nonspecific absorption at 600 nm in spectrophotometer (Spectronic Genesys.5).The concentration of MDA-TBA complex in *G.biloba* callus was converted from ppm (calculated from MDA standard curve) to µmol / g fresh weight.

Hydrogen peroxide content

Hydrogen peroxide levels were determined according toVelikovea*et al*.,(2000). One gram of callus *Ginkgo biloba* was homogenized in an ice bath with 1 ml 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12.000rpm for 15 minutes and 0.5 ml of the supernatant was added to 0.5ml of 10 mM potassium phosphate buffer (pH 7.0) and 1ml of 1M KI. The absorbance of the supernatant was measured at 390 nm. The content of hydrogen peroxide was determined using standard curve.

Amino acids content

The hydrolyzed protein amino acids were determined according to the method described byPellet and Young,(1980).Amino acids composition was determined by amino acid analyzer apparatus model SYKAM (S4300) inCentral labof Desert Research Center (DRC).The peaks area of each amino acid were calculated using an external standard by computer softwareSYKAM.

Statistical analysis

The experiments were subjected to completely randomized design. Analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955), as modified by Snedecor and Cochran (1982), were performed to analyze the obtained data.

III. Results And Discussion

Fresh weight of *Ginkgo biloba* callus

Data recoded in Figure (1) showed the effect of different concentrations casein, gibberellic acid and sucrose on growth of*G.biloba* callus. The results showed that the treatment ofcasein had positive effect on fresh weight of callus compared with the control (without casein) and the maximum fresh weights of callus were recorded on the medium with 2 mg/l casein hydrolysate(Fig. 1B). These results agree withHegazi and EL-Lamey(2011) whofound that highest fresh weight in callus of *Ephedra alata* at 2 mg/l of casein. Onthe other hand, it was found the highest callus induction of *Elymusdahuricus*recorded using 1mg/l casein WonLee *etal*.,(2012).Also, the addition of casein to culture medium increased growth callus of *Sorghum bicolor*(Pola*etal*., 2009). Casein is milk protein and a rich source of amino nitrogen. It was added to culture medium to improve the growth of some tissues such as *Taxus*(Gibson *et al*., 1993). In the same direction, fresh weight of *Oryza sativa* callus was increased after application of casein (Khaleda and Al Forkan, 2006).

Moreover, all application of gibberellic acid leads to increasing callus weight comparing with the control and the highest concentration (2 ppm) gave the highest weight which recorded 5.0 g fresh weight (Fig.2B). On the otherhand,Hendaweyand Abo El Fadl (2014) showed that gibberellic acid (2 ppm) had negative effect on fresh weight *stevia rebaudiana* callus. There are some reports showed the parallel results with the present study. In this regard, FettNeto*et al*.,(1993) used gibberellic acid to develop callus of *Taxuscuspidata.* Also, Bekheet*et al*,(2014) found that application of gibberellic acid leads to increasing *Silybummarianum* callus.The addition of gibberellic acid to nutrient medium leads to increasing production of cell culture of *Coptis japonica*(Yasuhiro *etal.*,1998).Gibberellic acid has strong effect with exogenous auxin and cytokinin in promoting diameter growth in *Pinusstobus*(Savidge, 1990).The results are shown in Fig.2Cindicated that application of sucrose had negative effect on callus weight compared with control except at concentration 60 g /l which leads to increasing fresh weight of callus.In the same direction, Ml and WC (1986)showed that the highest growth of *Bambusabeecheyana* callus was observed on medium containing 60g/l of sucrose**.** Also,Verma*et al.*,(2012)who found that the best concentration of sucrose for growth *Catharanthusroseus* callus at rate 60g/l**. S**ucrose act as external energy source and contributes to the osmotic potential of the medium(Nowak *et al.*, 2004). In this study, the lowest weight observed at the highest concentration at 90 g/l. This result agrees with (Javed and Ikram 2008) found that the highest concentration of sucrose leads to decreasing the growth wheat callus. Also, the same result observed on growth of *Morindacitrifolia* root suspension culture (AbdullahiBaque*et al.*,2012).The high level of sucrose play important role in decreasing growth of cultured cells by causing stood up of the cell cycle when nutrients are insufficient(Wu*et al.*, 2006 and Gould *et al.*, 1981).The addition of high

concentrations of sucrose in the cultured media might have inhibitory effect on nutrient uptake by lowering water potential of the medium (Shim*et al.,* 2003).In general the highest weight of callus (5.0 g) was achieved withgibberellic acid at rate of 2 ppm while the lowest weight of callus (1.233g) was obtained with concentration of sucrose (90 g). It could be noticed that the fresh weight of callus was gradually decreased with high concentration of sucrose.

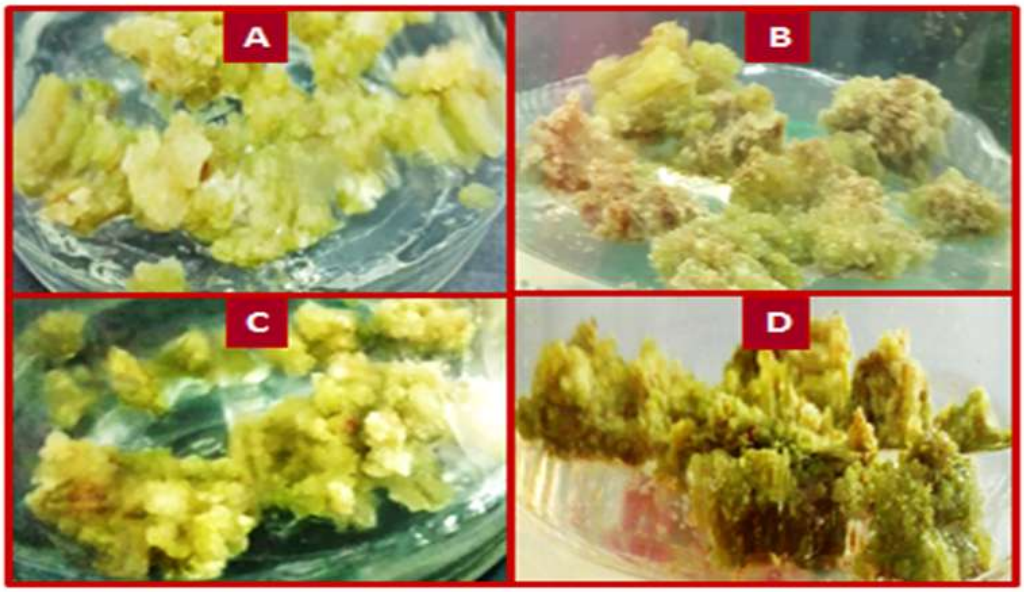
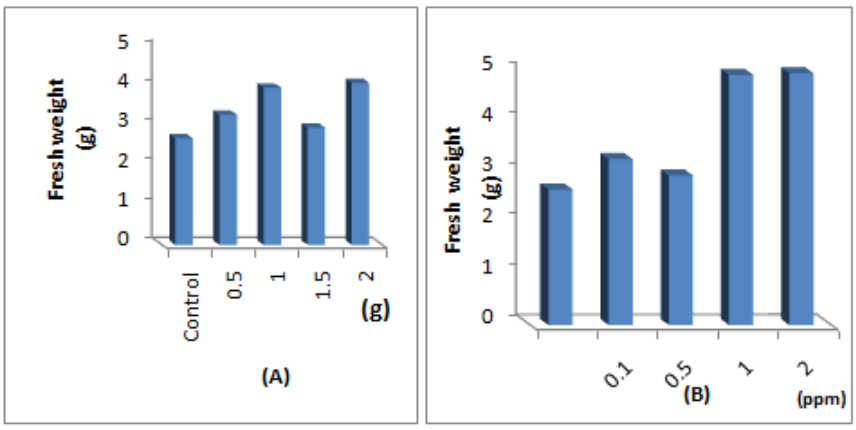


Figure (1): Effect of some inducers at the best concentrations on improvement of callus weight *Ginkgo biloba*. (A) Control, (B) Casein [2 g], (C) Gibberellic acid [2 ppm] and (D) Sucrose [60 g]



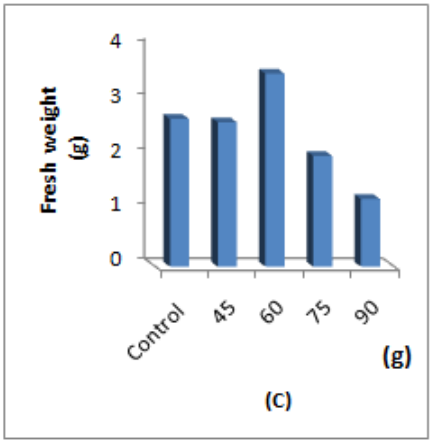


Figure (2): Effect of some inducers on fresh weight callus of *Ginkgo biloba*. (A) Casein [g], (B) Gibberellic acid [ppm] and (C) Sucrose [g]

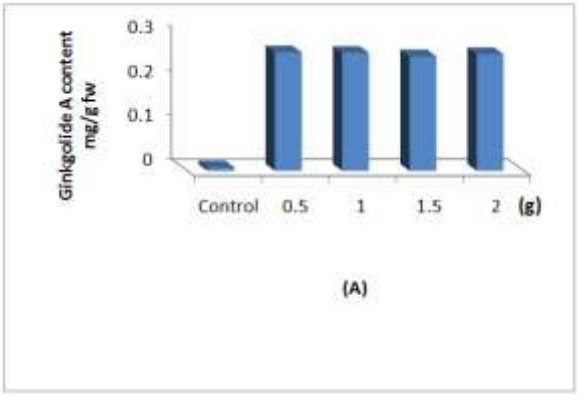
Ginkgolide A and bilobalidecontentin*G.biloba* callus

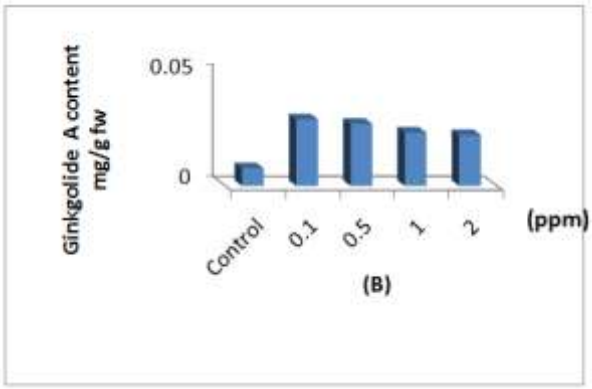
Effects of casein, gibberellic acid and sucrose on ginkgolide A and bilobalide contents in *G.biloba*callus are shown in (Figures3 and 4).The data obtained from HPLC analysis revealed that, *Ginkgo biloba*callus have higher bilobalide content (major compound) than ginkgolide A after applied all doses of inducers. Data shown in in Fig. (3A) demonstrated the effect different concentration of casein on Ginkgolide A production in *G.biloba* callus, it was found that all concentration leads to increasing Ginkgolide A contents. The maximum value (0.30 mg/g) was achieved when casein applied at 0.5 g/l compared with the control (without casein). This result agree with Wu *et al.,* (2005) they found that addition concentration of casein (0.5g/l) leads to increasing saponin yield in *Panax ginseng* cell culture. Also, in the same direction, Hegazi and El-lemely (2011)found that themaximum content of ephedrine in callus of *Ephedra alata*. Also, the maximum amount of alkaloid accumulation in *Catharanthusroseus* was noticed in callus cultures grown on MS supplemented with casein (Ahmed *et al.*,2000).Casein act as agood nutrient substrate for production secondary metabolite which consist of amino acids and peptides (Kayser and Quax, 2007).It was noted that the addition of casein to nutrient medium leads to increasing artemisinin production in cell culture of *Artmeisiaannua*(Woerdenbag*etal.,* 1993). The enhancement of secondary metabolites on MS medium supplemented with casein in cell culture might be due to its sterols or amino acids content (Heble, 1985).

In contrast, all treatment of casein leads to decreasing bilobalide content compared with the control .This results is agree with Hegazi and El-Lamey (2011)who found that the addition of casein (2.0g/l) gave the lowest content of ephedrine of *Ephedra alata*callus. There are some reports disagree with the results in the present study where Abyari*et al*., (2016) who found that casein leads to increasing of scopoletin in cell suspension culture of *Spilantheseacmella.*

Results in Fig.3Bshowed that, all treatments of gibberellic acid had a positive effect on the production of ginkgolide A. The highest value (0.269 mg/g) compared with the control. On the other hand, all concentrations of gibberellic acid leads to decreasing the active constituent (bilobalide) (Fig. 4B) in *G.biloba*callus. Gibberellic acid used as growth regulator for increasing the production of flavonoids and phenols in *Stevia rebaudiana* callus (Radic*et al.,* 2016).The same trends(Hendawey and Abo El Fadl, 2014) added gibberellic acid to basal media for improvement of stevioside production in *stevia* callus.Also, Banyai*et al* ., (2011)showed that artemisinin content was increased in *Aretmisiaannua* leaves after gibberellic acid was applied on plants. Also, the application of gibberellic acid on plant *Andrographispaniculata* promoted andrographolide accumulation (Vidyalakshmi and Anathi, 2013).Moreover,Modei*et al.,* (2011) found that gibberellic acid increased stevoisd content in stevia leaves. Gibberellic acidplay important role in activating the expression of gene where it was lowering the pH of the cell wall. This drop in the pH of cell wall leads to activation of cell wall hydrolysis (Lyndon,1997).It is considered elicitor for production secondary metabolite(Yuan *et al.,* 2008).

Data presented in Fig. 3C indicated that application of sucrose enhanced the content of ginkgolide A compared with the control (without sucrose). On the other hand, all application of sucrose had a negative effect on bilobalide content (expect 75 g/l) which recorded (4.0 mg/g) (Fig. 4C). These results confirmed by(Thiruvengadam*et al*, 2016) who used sucrose leads to increasing the anthraquinones and phenolic compound in cell suspension culture of *Polygoniummultiflorium*. Also, Soo- Jung *et al.,*(2004) found that the addition of 50g/l sucrose to nutrient medium leads to production high level of salidroside in *Rhodiolasachalinensis*callus. The highest value of Ginkgolide A was obtained when sucrose applied at rate 60g/l which reached (0.038 mg/g) in *G.biloba*callus. This result agrees with Verma*et al*., (2012)who found that the best concentration of sucrose at rate 60g/l for enhancement of alkaloid accumulation in *Catharanthusroseus* callus. Sucrose play important role on growth development and biosynthesis of secondary metabolites in cultured cells which is considered primary energy source for signaling molecules (Wang and Weathers, 2007).





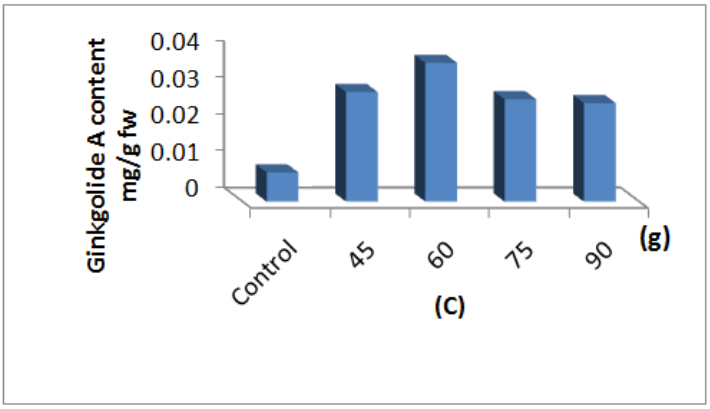


Figure. (3): Effect of some inducers on the production of Ginkgolide A in *Ginkgo biloba*callus (A) Casein [g], (B) Gibberellic acid [ppm] and (C) Sucrose [g]

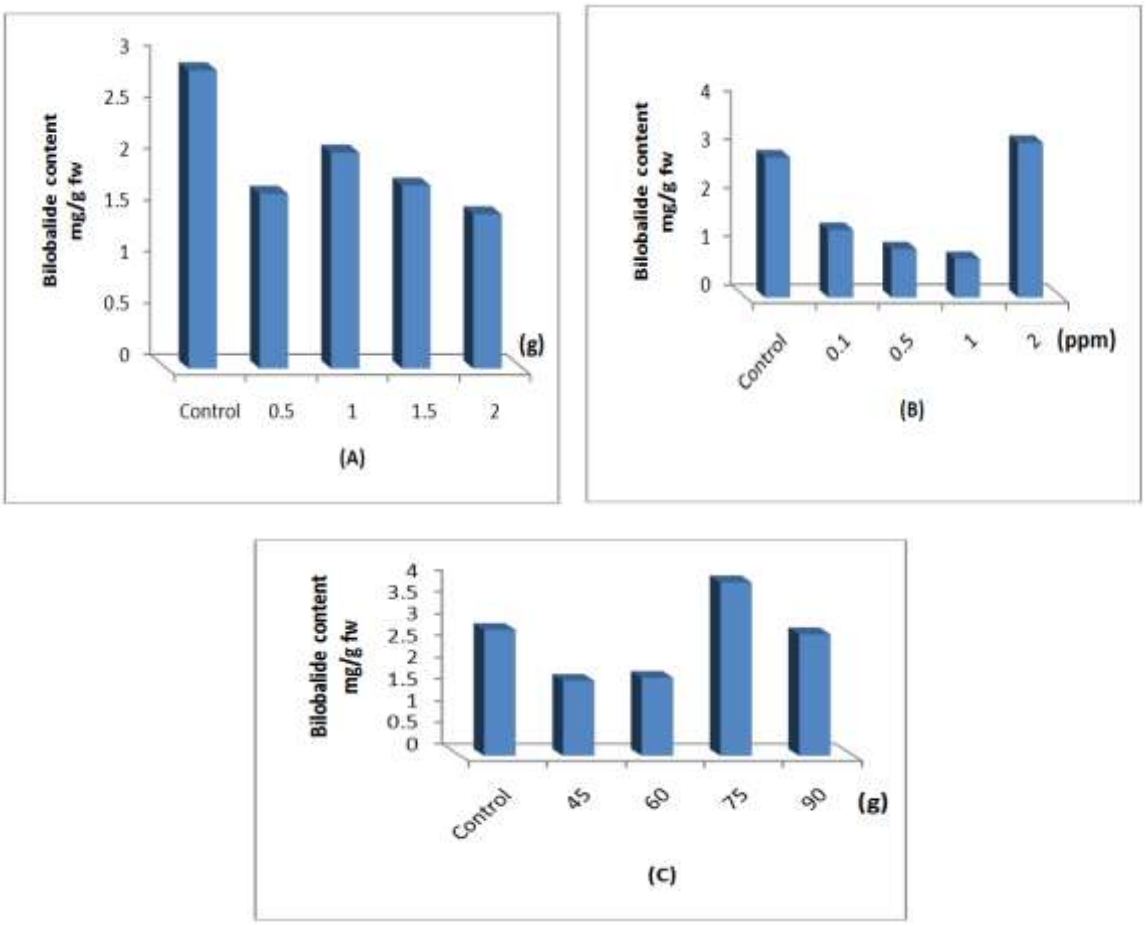


Figure. (4): Effect of some inducers on the production of bilobalide in *Ginkgo biloba* callus (A) Casein [g], (B) Gibberellic acid [ppm] and (C) Sucrose [g]

Malondialdehyde content in *G.biloba* callus

The level of lipid peroxidation in *G.biloba* callus was quantified by determination of malondialdehyde (MDA). The results in Fig.(5A) indicated that the lowest concentration of casein (0.5 g/l) leads to increasing of MDA which recorded (0.41µ mole /g fresh weight) compared with control. On the other hand, the highest concentration of casein (2g/l) leads to decreasing MDA content (0.20 µ mole /g fresh weight) compared with control (without casein). Also, the lowest concentration of gibberellic acid (0.1 ppm) gave the highest MDA content (0.33 µ mole /g fresh weight) (Fig.5B). The same observation was reported byHendawey and Abo ElFadal(2014) who revealed the lowest concentration of gibberellic acid gave the highest MDA content. In this regard, Khavri-Nejad*et al.,* (2013)observed MDA content in *Lycoperscianesculentum* was decreased by application of gibberellic acid.Also, Sayed and Gadallah (2013) found that gibberellic acid reduced MDA

content in *Helianthus annus* which protect the plant membrane from dehydration and heat stress injury where decreasing the negative effect of the acid mist and improved antioxidant defence. The treatment of sucrose at all concentrations showed increased of MDA content compared with the control. It was observed that MDA content increased with elevated sucrose concentration(Fig.5C). The same observation was reported by Cui *et al*.,(2010) who found that the addition high level of sucrose leads to increasing MDA content in root suspension cultures of *Hypericumperforatum*. Also, on *Morindacitrifolia*root suspension cultures (Abdullahi*et al*, 2012).Therefore, when the sucrose concentration higher than 30g/l,*G.biloba*calluswas affected by water deficit stress MDA content increased with addition increasing concentration of sucrose. Water deficit stress might be prevented by increasing non enzymatic scavenging system**.**

In general the highest MDA content was obtained at the highest concentration of sucrose at (90g/l) which recorded (0.83 µ mole /g fresh weight). While the lowest MDA content (0.11 µ mole /g fresh weight) was obtained with gibberellicacid (0.5 ppm)(Fig.5C and B,respectively).

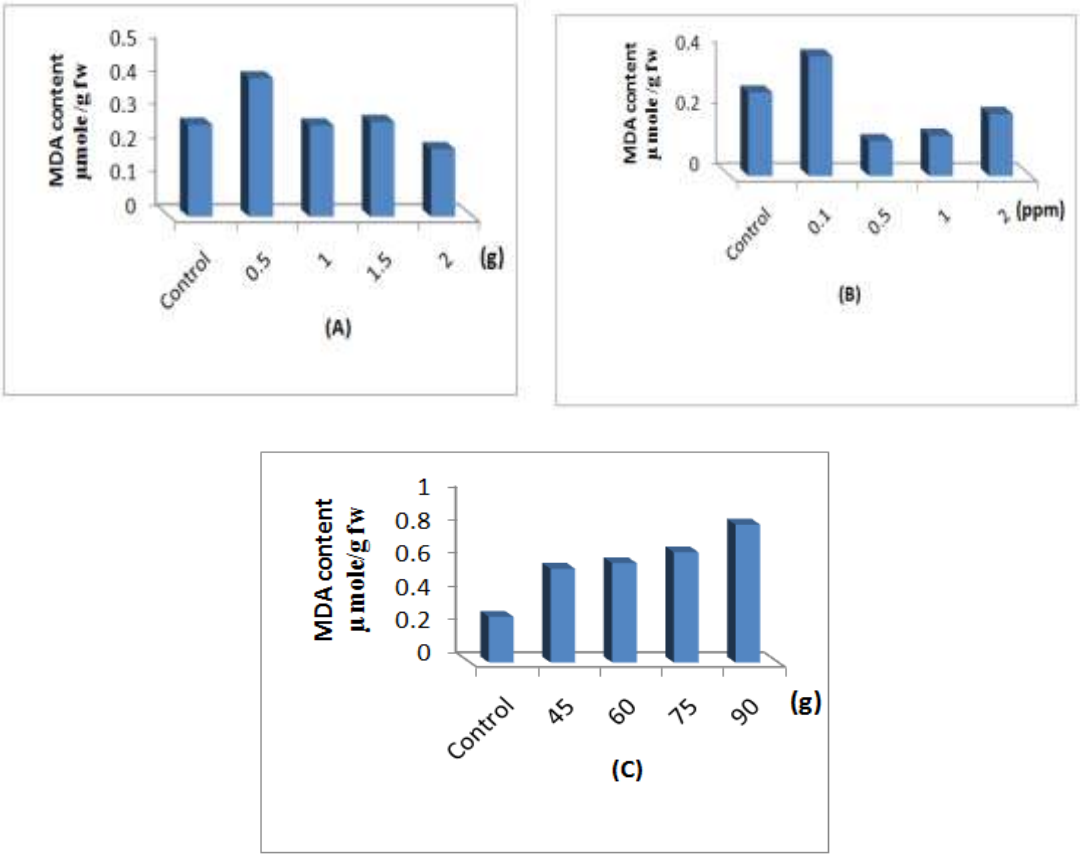


Figure. (5): Effect of chemical inducers on malondialdehyde in *Ginkgo biloba* callus (A) Casein [g], (B) Gibberellic acid [ppm] and (C) Sucrose [g]

Hydrogen peroxide content in *G.biloba* callus

The effects of casein, gibberellic acid and sucrose on hydrogen peroxide content in callus of *G.biloba* are shown in Fig. (6)**.** In this regard, the treatment with casein had a negative role on the accumulation of hydrogen peroxide. The lowest value of H_2O_2 (0.25 µ mole /g fresh weight) was obtained when callus treated with casein at concentration 1.5 g/l (Fig. 6A).Concerning the treatment with gibberellic acid, it was tended to accumulate hydrogen peroxide content in *G.biloba* callus especially at rate 0.5 ppm which recorded (0.41 µ mole /g fresh weight) (Fig. 6B). In the same direction,Sayed and Gadallah (2013)found that the treatment of *Helianthus annus* with gibberellic acid prevented the enhancement of H_2O_2 content caused by acid mist where gibberellic acid play important role in decreasing oxidative stress due to the production of reactive oxygen species. Application of sucrose leads to decreasing hydrogen peroxide content compared with control (without sucrose) except the highest concentration of sucrose (90g/l) which gave the highest accumulation of H_2O_2 (0.48 µ mole/g fresh weight) (Fig.6C). It is observed that H_2O_2 content is highly affected by the addition of sucrose at rate 90g/l. The same observation was reported by Cui *et al*., (2010)on root suspension cultures of *Hypericumperforatum* and Abdullahi*et al.*,(2012)on*Morindacitrifolia*root suspension cultures.

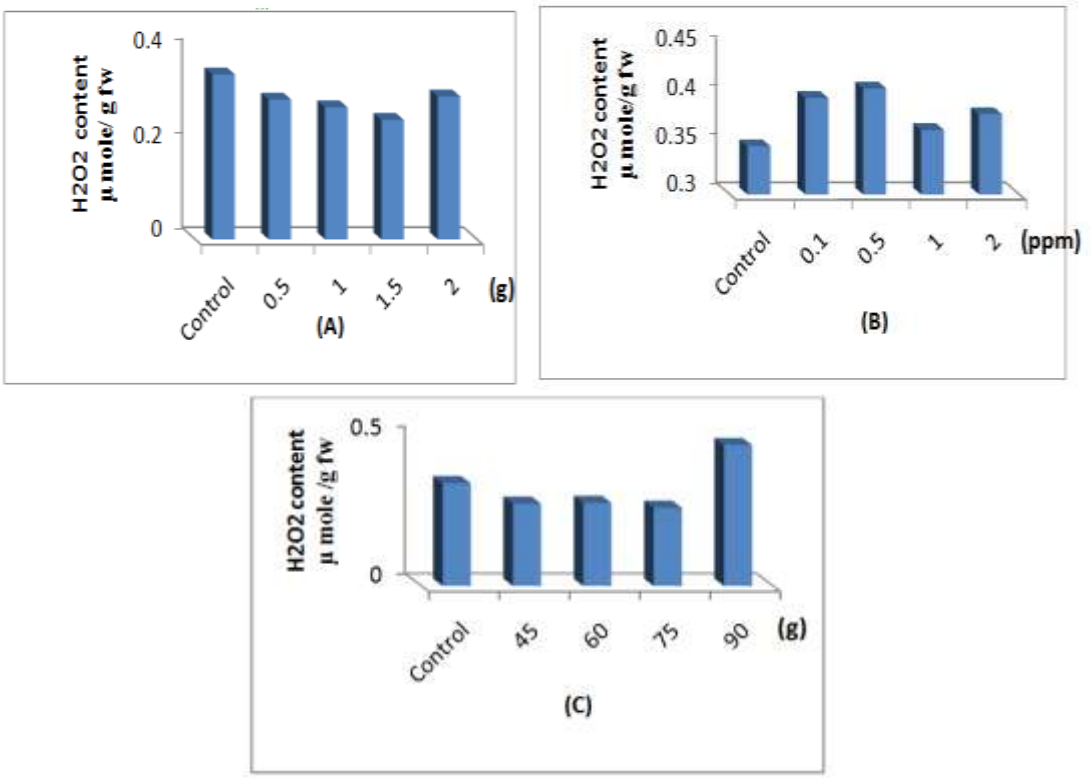


Figure. (6): Effect of chemical inducers on hydrogen peroxide in *Ginkgo biloba* callus
(A) Casein [g], (B) Gibberellic acid [ppm] and (C) Sucrose [g]

Amino acids in *G.biloba* callus

Data in Table (1) shows the effect of some inducers on the pattern of combined amino acid in *G.biloba* callus. Sixteen combined amino acids were detected in *Ginkgo* callus and the major amino acids in callus of *Ginkgo* were glutamic, aspartic, alanine andproline. Results indicated that glutamic acid was the most abundant amino acid and it was increased due to treatment with gibberellic acid compared with the control.

On the other hand, all application of casein leads to decreasing glutamic except the concentration 1.5 g/l which leads to increasing glutamic which recoded (1.2mg/g). In the same trend, all application of sucrose leads to decreasing glutamic except 45g/l which recorded (3.4 mg/g). This result disagree with Tsuchiya *et al.,* (2013) who found that the low glutamic acid content in *Bruguierasexangula*protoplast.

In this study, all gibberellic acid treatments leads to increasing aspartic acid compared with the control. This was true when *G.biloba* callus treated with casein except the concentration of 2.0 g/l. It was found aspartic acid increased by adding sucrose in medium at rates (45,90g/l). The highest value of alanine was observed at rate 2 ppm of gibberellic acid which recorded (2.2mg/g). On the other hand, the lowest value of alanine was recorded(0.77mg/g) at rate 75g/l of sucrose.

In this concern, all treatments of casein, gibberellic acid and sucrose leads to increasing of proline content compared with the control (without inducers).While the highest value of proline was obtained by adding gibberellic acid in the medium at rate 2ppm which recorded (1.7 mg/g) (Table, 1).This agree withHendawey and Abo ElFadl (2014) who found that the highest value of proline in *Stevia rebaudiana*callus at rate 2 ppm of gibberellic acid. The addition of sucrose in to medium had positive effect on proline accumulation in *Ginkgo* callus. This result are similar withLahrer*et al.*,(1993) who showed that sucrose had a positive effect on proline accumulation in higher plants. In this present work, it was observed the high proline level in *Ginkgo* callus. The high level of proline were associated with osmotic regulation and antioxidant protective roles (Du *et al.,* 2012). It was found the endogenous amino acids was regulated the protein synthesis during morphogenetic process as embryogenesis (Wetherell andDougall, 1976).It was noted that the amino acids of pyruvate family such as alanine, leucine, valine and isoleucine all increased with all concentration of gibberellic acid compared with control. This increasing in these amino acids may have been a result of protein hydrolysis (Fischer 1971). In the same direction, it was found that arginine increased with all concentrations of gibberellic acid compared with the control. These results are similar toKomamine*et al.*, (1992); Joy *et al.*, (1996) and Thrope and Stasolla(2001)who found that higher concentration of Arginine in embryogenic culture of carrots.

Table 1: Effect of chemical inducers on amino acids in *Ginkgo biloba* callus

Treatments		Amino acids content (mg/g FW)															
		Acidic		Basic			Neutral								Aromatic and imine		
Chemical inducers	Concentrations	Aspartic	Glutamic	Histidine	Lysine	Arginine	Glycine	Alanine	Valine	Isoleucine	Leucine	Threonine	Serine	Methionine	Tyrosine	Phenylalanine	Proline
Control		1.4	3.1	0.46	0.49	0.47	0.40	1.1	0.44	0.30	0.53	0.47	0.77	0.053	0.32	0.30	0.98
Casein (g)	0.5	1.5	3.0	0.51	0.59	0.52	0.51	1.2	0.53	0.39	0.65	0.58	0.85	0.070	0.40	0.36	1.0
	1.0	1.5	3.1	0.54	0.64	0.53	0.52	1.1	0.55	0.40	0.69	0.58	0.90	0.066	0.39	0.37	1.22
	1.5	2.2	4.7	0.80	0.95	0.77	0.84	1.9	0.86	0.61	1.0	0.87	1.3	0.092	0.54	0.56	1.81
	2.0	1.2	2.7	0.45	0.49	0.39	0.45	0.92	0.41	0.32	0.55	0.44	0.71	0.054	0.29	0.31	1.0
Gibberellic acid (ppm)	0.1	1.7	3.2	0.63	560.	0.59	0.59	1.4	0.55	0.41	0.67	0.60	0.95	0.078	0.40	0.40	1.19
	0.5	1.8	3.7	0.56	30.6	0.58	0.64	1.5	0.58	0.43	0.69	0.62	0.98	0.075	0.44	0.40	1.2
	1.0	1.6	3.5	0.44	60.6	0.52	0.57	1.1	0.53	0.39	0.59	0.58	0.91	0.065	0.36	0.37	1.17
	2.0	2.3	5.2	0.67	0.88	0.64	0.88	2.2	0.76	0.58	0.73	0.81	0.93	0.093	0.58	0.58	1.7
Sucrose (g)	45	1.6	3.4	0.42	0.54	0.47	0.54	1.2	0.48	0.36	0.64	0.55	0.83	0.060	0.28	0.36	1.50
	60	1	2.1	0.44	0.27	0.32	0.38	1.1	0.38	0.41	0.44	0.41	0.64	0.035	0.18	0.27	1.0
	75	1.1	1.7	0.45	0.41	0.32	0.46	0.77	0.37	0.27	0.48	0.44	0.67	0.035	0.23	0.30	0.99
	90	1.5	2.8	0.53	0.53	0.42	0.50	1.2	0.35	0.36	0.59	0.60	0.35	0.046	0.24	0.35	1.41

References

[1] AbdullahilBaque, M.; Elgirban, A.; Jung Lee, E. and YoeupPaek, K. (2012). Sucrose regulated enhanced induction of anthraquinone, phenolics, flavonoids biosynthesis and activities of antioxidant enzymes in adventitious root suspension cultures of *Morindacitrifolia*(L.). Acta Physiol. Plant., 34:405-415.

[2] Abyari, M.; Nasr, N.; Soorni, J. and Sadhu, D. (2016). Enhanced accumulation of scopoletin in cell suspension culture of *Spilanthesacmella*Murr. using precursor feeding. J. Braz.Arch. Biol. Technol., 59:e1650533.

[3] Ahmed, F.A.; Abdel- Fatah, O.M.; Kobeasy, M.I. and Ahmed, O.K. (2000). Factors affecting growth and indole alkaloids content of Catharanthuscalli (*Catharanthusroseus* L.) : amino acids, casein hydrolysate and irradiation . Biotechnol., 5 :379-384.

[4] Banyai, W.; Mii, M. and Supaibulwatana.(2011). Enhancement of artemisinin content and biomass in *Artemisia annua* by exogenous GA3 treatment .Plant Growth Regul.63: 45–54.

[5] Bekheet, S.A.; EL-Bahr, M.K.; Ali, Sanna, A. and Hamda, Manal. A. (2014). Callus production of globe Artichoke and milkthistle: *In Vitro*hypolipidemic and Antioxidant Activities.World. J.Pharm.Res. 3(4):1-17.

[6] Bekhit, M.A.; Gomaa, R.E.; Ibrahim,A.I. and Nasr, M.I. (2008). *In vitro* studies on *Ginkgo biloba*L. 2-Factors affecting callus production and Ginkgolide contents. J. Prod.Dev., 13(2):457-474.

[7] Braquet, P.; Touqui, T.; Shen, Y. and Vargaftig , B. (1987). Perspective in plantlet activating factorsrsearch. Pharmacol. Rev., 39: 97 - 145.

[8] Brenner, E.D.; Katari, M.S.; Stevenson, D.W.; Rudd, S.S.; Doulglas,.V.; Moss, W.N.; Twigg, R.W.; Runko, S.; Stellari, G.M.; Mccombie, W.R. and Coruzzi, G.M. (2005). EST analysis in *Ginkgo biloba*: an assessment of conserved developmental regulators and gymnosperm specific genes. Biomed. Cent. Genomics, 6:143.

[9] Cassileth, B. (2011). Ginkgo (*Ginkgo biloba*). Oncology (Williston Park) ., 25:971.

[10] Cheng, P.; Li su, X.; Hua Nie, L. and Zhuo, S.(1998). Analysis of ginkgolides and bilobalides in *Ginkgo biloba* L. Extract for its production process control by high performance liquid chromatography. J. Chromatogr. Sci., 36: 197-200.

[11] Cheng, S.; Xu, F. and Wang, Y.(2009). Advances in the study of flavonoids in *Ginkgo biloba* leaves.J. Med. Plant Res., 3:1248-52.

[12] Cheng, S.; Zhang, W.; Sun, N.; Xu., F.; Li,L.; Li Ao, Y. and Cheng, H. (2014). Production of flavonoids and terpene lactones from optimized *Ginkgo biloba* tissue culture. NotulaeBotanicaeHorti.Agrobotanici, 42(1):88-93.

[13] Cui,x.; Murthy, H.; Wu, C. and Paek, K. (2010). Sucrose induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of *Hypericumperforatum*L. Plant Cell, Tissue Organ Culture. 103:7–14.

[14] Del Rio, D.; Stewart, A.J. and Pellegrini. (2005). A review of recent studies on malondialdhyde as toxic molecule and biological markers of oxidative stress. Nutr. Metab. Cardiovasc. Dis., 15(4):316-328.

[15] Dewick, PM. (1997). The biosynthesis of C5-C25 terpenoid compounds. Nat. Prod. Rep., 14: 111-114.

[16] Drieu, K.; Jaggy, H. and Van Beek T.A. (2000). *Ginkgo biloba*. In: medicinal and aromatic plants-industrial profiles.Amsterdam: CRC Press; 2000.

[17] Duncan, B.D.(1995). Multiple Range and Multiple F Test. Biometric, 11:1-42.

[18] Du, H.; Wang, Z.; Yu, W. and Huang, B. (2012). Metabolic responses of hybrideBermudagrass to short-term and long –term drought stress. J.Am.Soc. Hort. Sci.137(6):411-420.

[19] FettNeto, A.G.; Melanson, S.J.; Sakata, K. and Dicosomo, F. (1993). Improvedgrowth and taxol yield in developing calli of *Taxuscuspidata* by medium composition modification. Biotechnol.,11 (6):731-4.

[20] Fischer, K. (1971). MethodenzurErkennung und Beurteilungforstschadlicherftverunreinigungen. Chemische und physikalischeReaktionen S02-begaster Pflanzen und Blatter.- Mitt. Forstl.Bundes-Versuchsanst. Wien (92): 209-231

[21] Gibson, D.M.; Ketchum, R.E.B.,; Vance, N.C.and Christen, A. (1993). Initiation and growth of cell lines of *Taxusbrevifolia* (Pacif yew). Plant cell Rep., (12):479-482.

[22] Gould, A.R.; Everett, N.P.; Wang, T.L. and Street, H.E. (1981). Studies on the control of the cell cycle in cultured plant cells:1. Effects nutrient limitation and nutrient starvation. Protoplasma, (106):1-13.

[23] Health, R.L. and Packer,L.(1968).Photoperoxidation in isolatedchloroplast. I. Kinetics and stoichiometry of fatty acidperoxidation. Archives of Biochem and Biophy., 125: 189–198.

[24] Hegazi, Gh.A. and EL-Lamey, T..M. (2011). *In vitro*production of some phenolic compounds from *Ephedra alata*Decne. J. Appl. Environ. Biol. Sci., 1(8)158-163.

[25] Heble, M.R.(1985). Multiple shoot cultures: aviable alternative *in vitro* system for the production of known new biologically active plant constituents In: Neumann K-H, Braz W, Reinhard E, editors. Primary and Secondary Metabolism of Plant Cell Cultures Germany : Springer Science &Busines Media. P377.

[26] Hendawey, M.H. and Abo EL Fadal, R.E. (2014). Biochemical studies on the production of active constituents in *Stevia rebaudiana* L. callus. Global J. Biotechnol. Biochem., 9:(3)76-93.

[27] Javed, F. and Ikram,S.(2008). Effect of sucrose induced osmotic stress on callus growth and biochemical aspects of two wheat genotypes.Pak. J. Bot., 40:1487-1495.

[28] Javed,S.; Asharf, M.Y.; Mahmood, S.; Bukhari, S.a.; Meraj, M. and Preveen, A. (2013). Comparative evaluation of biochemical changes in different sun flower varieties (*Cathamustinctorius* L.) under water deficit. J. Food Process Technol., 4-10.

[29] Joy Iv, R. w.;Mclntyre, D.D;Vogel, H. J. and Thorpe. T.A. (1996). Stage - specific nitrogen metabolism in developing carrot somatic embryos. Physiol.Plant., 97:149–159.

[30] Kanowski, W.N.;Hermann, K.; Stephan, W. and Wierich, R.H. (1997). Proof of the efficacy of the *Ginkgo biloba* special extracts EGb 761 in outpatients suffering from mild to moderate primary degenerative dementia of the Alzhimer type or multi- infract dementia. J. Phytomedicine, 4(1): 3-13.

[31] Kayser, O. and Quax, W.J.(2007). Medicinal plant biotechnology from basic research to industrial applications Wiley- Blackwell., P 187-199.

[32] Khaleda, L. and AL-Forkan,M.(2006) Stimulatory effects of casein hydrolysate and proline on *in vitro* callus induction and plant regeneration from five deep water rice (*Oryzasativa* L.). Biotechnol., 5:379-384.

[33] Komamine, A.; Kawahara, R.; Matsumoto, M.; Sunabori, S.; Toya, T.; Fujiwara, A.; Tsukahara, M.; Smith, J.; Ito, M.; Fukuda, H.; , Nomura, K. and Fujimura, T. (1992). Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry, and molecular biology. *In Vitro* Cell Dev. Biol. Plant, 28:11–14.

[34] Khavari-Nejad, R.A.; Najafi, F. and Ranjbari , M. (2013). The effects of GA_3 application on growth, lipid peroxidation, antioxidant enzymes activities, and sugars levels of Cadmium stressed tomato (*Lycopersiconesculentum* Mill.cv.CH) Plants. Rom . J. Biol. – Plant Biol., 58 (1): 51-60.

[35] Lahrer, F.; Leport, L.; Petrivalsky, M.andChappart, M.(1993). Effectors for the osmoinduced proline response in higher plants.Plant physiol. Biochem.31:911-922.

[36] Lu, S.; Guo, X. and Zhao, P. (2011). Effect *Ginkgo biloba* extract 500 immunity and antioxidant enzyme activities in ischemia reperfusion rats. J. Mol., 16 (11): 9194-9260.

[37] Mahadevan, S and Park, Y.(2008). Multifaceted therapeutic benefits of *Ginkgo biloba* L.: Chemistry, efficacy, safety, and uses. J. Food Sci., 73(1): 14-19.

[38] Ml, Y. and WC, C.(1986). Somatic embryogenesis and subsequent plant regeneration from inflorescence callus of *Bambusa*beecheyanaMunorvar- beecheyana. Plant Cell Rep., 5(6):409-11.

[39] Modi, A.R.; Shukla, Y.M.; Litoriya, N.S.; Patel, N.J.andSubhash, N.(2011).Effect of gibberellic acid foliar spray on growth parameters and stevioside content of ex vitro grown plants of *Stevia rebaudiana*Bertoni. Med.Plants Int. J. PhytomedicinesRelat. Ind., 3(2):157-160.

[40] Lyndon, R.F. (1997). Plant Development :The Cellular Basis. Translated by Majd A. and Ebadi M. Morvarid Press.

[41] Murashige, T. and Skoog F. (1962). Arevised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15:473-497.

[42] Mandal, N.; Chaudhuril, D.; Ghatel, N. and Sarkar, R. (2012). Phytochemical analysis and evalution of antioxidant and free radical scavenging activity of withanniasomniferaroot. Asian J. Pharm.Clin.,5(4):193-199.

[43] Nakanishi, K. (2005). Terpene trilactones from *Ginkgo biloba*: from ancient times to the 21 st century. Bioorg. Med. Chem., 13:4987-5000.

[44] Nowak, B..; Miczynski, K. and Hudy.L.(2004).Sugar uptake and utilization during adventitious bud differentiation on *in vitro*leafexplants of WegierkaZwykla plum (*Prunusdomestica*). Plant Cell Tissue Organ Culture,76:255-260.

[45] Pola, S.; Mani, N.S. and Ramana, T.(2009). Long Term Maintenance of callus cultures from immature Embyro of sorghum bicolor. World J. Agric. Sci., 5 (4): 415-421.

[46] Pellet, P. L. and Young, V. R. 1980. Nutritional evaluation of protein foods. Food and Nutrition Bulletin Supplement 4. United Nations University: Japan, 41-57.

[47] Quan, L.J.; Zhang, B.; Shi, W.W. and Li, H. Y.(2008). Hydrogen peroxide in pants:Aversatile molecule of the reactive oxygen species network. J.Integr. Plant Biol., 50:2-18.

[48] Radic, S.; Vujcicl,V.;Glogoskil, M. and Radic-Stojkovic, M.(2016).Influence of PH and plant growth regulators on secondary metabolite production and antioxidant activity of *Stevia rebaudiana* (Bert). Peroid biol., 1:9-19.

[49] Savidge, R.A.(1990). Phytohormonal regulation of cambial growth in trees,. In : Fast Growing Trees AND Nitrogen Fixing Trees. Wermer, D. and Muller, P.(Eds). Gustav Fisher Verlag, Sttutgart, New York. p.142-151.

[50] Sayed, S.A. and Gadalla, M.A.A.(2013). Gibberellic acid ameliorates the adverse effects of acid mist and improved antioxidant defense, water statues and growth of acid misted Sunflower plants. J.Biol. Earth Sci., 3(2):275-285.

[51] Shim, S.W.; Hahn, E.J. and Paek, K.Y.(2003). *In vitro* and *ex vitro* growth of grapevine rootstock '5BB' as influenced by number of air exchanges and the presence or absence of sucrose in culture media. Plant Cell Tissue Organ Culture, 75: 57-62.

[52] Snedecor, G.W. and Cochran, W.G.(1982). Statistical Methods. 7th Ed. Iowa State Univ. Press. Ames, Iowa, USA.

[53] Soo-Jung , K.; Baik, H.; Sun-Jin , H. and Jun-Cheul, A (2004). Production of salidroside from callus culture of *Rhodiolasachalinensis* A.Bor. Korean. J. Plant. Biotechnol., 31:(1):89-94.

[54] [54].Tanaka, A.; Arai, Y.; Kim, S.; Ham, J. and Usuki, T.(2011). Synthesis and biological evaluation of bilobol and adipostatinA.J. Asian Nat. Prod. Res., 13(4):290-296.

[55] Thiruvengadam, M.; Rekha, K.; Rajakumar, G,; June Lee, T.; Hyun kim, S. and Min Chung, L. (2016). Enhanced production of anthraquinoues and phenolic compounds and biological activities in cell suspension culture of *Polygoniummultiflorium*.J. Int.Mol.Sci., 17 (11):1912.

[56] Thorpe , T.A. and Stasolla, C (2001) .Somatic embryogenesis. In: Bhojwa SS, Soh WY (eds) Current trends in the embryology of angiosperms. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 279–236.

[57] Tsuchiya,S.; Ogita, S.; Kawana, Y.; Oyanagi,T, Hasegawa, A. and Sasamoto, H. (2013). Relation between amino acids profiles and recalcitrancy of cell growth or salt tolerance in tissue and protoplast Cultures of Three Mangrove Species, *Avicennia alba*, Bruguierasexangula, and Sonneratia. Am. J. Plant Sci., 4: 1366-1374.

[58] Van Beek, T.A., (2002). Chemical analysis of *Ginkgo biloba* leaves and extracts. J. Chromatogr A., 967:21-55.

[59] Van Beek, T.A. and Montoro, P. (2009). Chemical analysis and quality control of *Ginkgo biloba* leaves extracts and phytopharmceuticals. J.Chromatogr A., 1216: (11).2002-32.

[60] Velikova, V., Yordanov, I. and Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines.Plant Sci., 151, 59-66.

[61] Verma, A.K .; Singh , R.R. and Singh , S. (2012). Improved alkaloid content in callus culture of *Catharanthusroseus*.Botanica SERBICA, 36(2):123-130.

[62] Vidyalakshmi, A. and Ananthi,S. (2013). Induction of andrographolideAbiologically active ingredient in callus of *Andrographispaniculata* (Burm.F) Wallich Ex. Nees.Bioeng.Biosci., 1(1): 1-4.

[63] Wang, Y.and Weathers, P.J.(2007). Sugars proportionately affect artemisinin production. Plant Cell Rep., 26: 1073-1081.

[64] Warrier, G. and Corzine, A. (2000). The marketing and economic aspect of *Ginkgo biloba* product. in: Van Beek TA, editors. Ginkgo biloba in Medicinal and Aromatic Plants-Industrial Proles.Amsterdam: Harwood., 517-519.

[65] Woerdenbag, H.J.; Luers, J.F.; Van Uden, W.; Pras, N.; Malingre, T.M and Alfermann, A.W.(1993). Production of the new antimalaria drug artemisinin in shoot cultures *Artemisia annua* L. Plant Cell, Tissue and Organ Culture., 32(2): 247-257.

[66] Wetherell, D.F.andDougall, D.K.(1976). Sources of nitrogen supporting growth and embryogenesis in cultured wild Carrot tissue. Physiol. Plant., 37:97-103.

[67] Weinmann, S.; Roll, S.; Schwarzbach, C.; Vauth, C. and Willich, S.N. (2010). Effects of *Ginkgo biloba* in dementia: Systematic review and meta-analysis.BMC Geriatrics, 10-14.

[68] Won Lee, K.; Chinzorg, O.; Choi, G.J.; Yong Kim, K.; Ji, H.C.; Park, H.S.; Kim, W.H and Hoon Lee, S. (2012). Factors influencing callus induction and plant regeneration of Dahurianwildrye grass (*Elymusdahuricus* L.) Afr. J. Biotechnol., 11(4): 815-820.

[69] Wu, J.Y.; Wong, K.; Ho, K.P. and Zhou, L.G.(2005). Enhancement of saponin production in *Panaxginsnong* cell culture by osmotic stress and nutrients feeding. Enzyme Microb. Technol., 36:133-138.

[70] Wu, C. H; Dewir, Y.H.; Hahn, E.J. and Paek, K.Y.(2006).Optimization culturing conditions for the production of biomass and phenolics from adventitious roots of *Echinacea aungustifolia*. J. Plant Biol., 49:193-199.

[71] Yasuhiro, H.; Toshihiro, Y.; Teijiro, M.; Yasuhiro, F. and Yasuyuki, Y.(1998).Enhancement of berberine production suspension culture of *CoptisJaponic*by gibberellic acid treatment. J. plant Physiol., 13:12-15.

[72] Yuan, Y.; Huang, L.; Cui, G.H.; Mao, Y. and He, X.(2008). Effect of gibberellins and its synthetic inhibitor on metabolism of Tanshinons. Chin J. Exp. Trad. Med., 15: 1-3.

[73] Zaho, S.J.; Xu, C.C and Zou, Q. (1994).Improvement of method for measurement ofmalondialdehyde in plant tissue. Plant Physiol. Commun., 30:207-210.

[74] Zhou, l.; Meng, Q.; Qian, T.and Yang, Z.(2011). *Ginkgo biloba* extract enhances glucose tolerance in hyper insulinism-induced hepatic cells. J. Nat. Med., 65:50-5

Abd El menim M. Sharaf . "Effect of some chemicals inducers on terpenes production (ginkgolide A and bilobalide) in callus of Ginkgo biloba plant." IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 12.4 (2017): 08-18.