Effect of chitosan and light conditions on the production of callus biomass, total flavonoids and total phenolics in *Ginkgo biloba* L.

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ABSTRACT

Ginkgo biloba L. is a well-known tree for its high medicinal value owing to the presence of terpene trilactones, unique active metabolites, and other phytochemicals, including flavonoids and phenolics in its leaves. In Egypt, *G. biloba* has been reported as one of the endangered trees. Hence, plant tissue technique could be a potential alternative system to produce ginkgo's pharmaceutical compounds. In this study, an elicitation strategy using the biotic elicitor chitosan was employed to enhance the productivity of certain metabolites in *G. biloba* leaf-derived callus incubated in light (16 h photoperiod) or darkness. Combination of 1.0 mg/L α-naphthalene acetic acid (NAA) and 2.0 mg/L 6-benzyl adenine (BA) added to Murashige and Skoog (MS) medium resulted in the highest callogenesis percentage in both light and dark (95.00 and 94.43%, respectively) with a compacted callus texture but a different color. Significant differences were recorded between chitosan levels concerning callus biomass production and total flavonoids and phenolics accumulation. The highest yield of callus fresh and dry weights, growth index, and relative growth rate were recorded for light-grown cultures elicited with 100 mg/L chitosan followed by 50 mg/L. Same concentrations were also superior in enhancing the content and productivity of total flavonoids and total phenolics. Light incubation was more favorable than darkness for callus growth, flavonoid, and phenolic biosynthesis as well as antioxidant activity. The current study revealed the possibility of using calli culture as a biotechnological approach for the industrial production of *G. biloba* phytoconstituents.

Keywords: *Ginkgo biloba*; chitosan; biotic elicitation; flavonoids; phenolics; antioxidant activity; callus culture; light photoperiod.

INTRODUCTION

Ginkgo (*Ginkgo biloba* L.), family Ginkgoaceae, is a dioecious, deciduous, and gymnosperm tree known as maidenhair tree in English. Worldwide, it is well known for its high medicinal value and is thought to be the oldest living tree on earth. Although the ginkgo tree is native to China and commonly cultivated there, however, it is also grown as an important medicinal plant in Korea, India, France, Germany, and Untied States (Gopichand and Meena, 2015). Phytochemical screening of the ginkgo plant demonstrated the presence of various bioactive phytomolecules e.g., amnio acids, organic acids, polyphenols, and terpenoids. In *G. biloba* leaves, flavonoids (quercetin, kaempferol, and isorhamnetin), flavonoid glycosides (quercitrin, quercetin-3-β-D-glucoside, and rutin), phenolic acids (caffeic acid, *p*-coumaric acid, vanillic acid, and ferulic acid), and terpene trilactones (diterpene ginkgolides and sesquiterpene bilobalide) are reported to be the major and important phytoconstituents responsible for the pharmacological activity of ginkgo extract (Singh *et al.*, 2008 and El-Beltagi and Badawi, 2013). The highest content of terpene trilactones was recorded in the leaves, roots, and stem, respectively (Lu *et al*., 2017). Chemical synthesis of the diterpene ginkgolides has been performed and is academically successful, but it is still far from practical commercial use (Crimmins *et al*., 2000 and Sabater-Jara *et al*., 2013).

Leaf extracts of *G. biloba* exhibited antidementia, antiviral, antitumor, antioxidant, antibacterial and antiparasitic activities (DeFeudis *et al*., 2003; Weinmann *et al*., 2010; Sati and Joshi, 2011; El-Beltagi and Badawi, 2013 and Haruyama and Nagata, 2013). The anticancer properties of ginkgo leaf extract are related to its anti-angiogenic, antioxidant and gene-regulatory activity (DeFeudis *et al*., 2003). The extracts of ginkgo protect the lipid component of cell membranes from damage by scavenging various reactive oxygen species (ROS) (Maitra *et al*., 1995). Ginkgo extract has been shown to improve blood circulation by improving the opening of blood vessels, especially in the brain for the treatment of dementia and vasoregulating diseases (Weinmann *et al*., 2010). Moreover, its beneficial effect for Alzheimer's disease has

also been proven by inhibiting amyloid-β aggregation, the possible causative agent of Alzheimer's disease (Luo *et al*., 2002). Besides, bioactive ingredients of ginkgo protect mitochondria from oxidative stresses that may be part of the chronic oxidative stress spectrum in Alzheimer's disease (Eckert et al., 2003).

Globally, the demand for *G. biloba* products is increasing at a rate of 26-32% every year (Gopichand and Meena, 2015). The ginkgo tree was considered a living fossil under threat due to illegal exploitation and lack of knowledge about its sustainable harvest (Purohit *et al*., 2009). In Egypt, *G. biloba* has been reported as an endangered plant species that need the development of biotechnological approaches for its propagation, conservation and large-scale production of the high-value bioactive pharmaceuticals (Bekhit *et al*., 2008 and Sharaf *et al*., 2017). The biosynthesis and accumulation of plant phytochemicals are fluctuated and affected by several factors, such as species, ecotype, growth stage, pathogens, and environmental conditions (Isah *et al*., 2018). Various factors, including the tree gender of ginkgo, growth stage, tree age, soil characteristics, and other natural variation due to allogamous status of the species, all cause great changes in the content of plant phytoconstituents, especially terpene content; however, the tree age is the main determining factor (Balz *et al*., 1999). Furthermore, differences in the cultivation site of *G. biloba* and surrounding climate, slow plant growth, seasonal fluctuations of flavonoids and terpene lactones contents, as well as the complexity of the extraction process are limiting factors for the large-scale production of leaves from field-cultivated trees (Cheng *et al*., 2014 and Sukito *et al*., 2016).

Plant biotechnological approaches, including callus, cell suspension, and root cultures carried out *via* tissue culture technique offer an attractive and alternative system to conventional cultivation to produce biologically active by-products from medicinal plants (Sukito and Tachibana, 2016; Rady, 2019 and Elateeq *et al*., 2020). Such strategies allow obtaining plant material in large quantities in a seasonally independent way, with a rapid processing technique and easy isolation methods, under controlled and constant conditions, and in a short period, as well as without relying on field-grown mother plants (Karuppusamy, 2009; Elateeq, 2017 and Chandran *et al*., 2020). Therefore, production, isolation, and application of pharmaceutical components of *G. biloba* would be facilitated using a tissue culture system. Researchers have paid more attention towards scaling up the sustainable production of biomass and bioactive metabolites for several medicinal crops in bioreactor systems that would provide optimal conditions for maximizing production in cell (Park *et al*., 2004b and Werner *et al*., 2018), root (Cui *et al*., 2014 and Wu *et al*., 2018), and shoot cultures (López *et al*., 2018 and Krol *et al*., 2020). Applications of biotechnology approaches to ginkgo have been implemented since the 1970s to study the possibility of producing the unique active ingredients (ginkgolides and bilobalide) and other phytochemicals, like flavonoids through tissue culture technique (Nakanishi and Habaguchi, 1971 and Schrall and Becker, 1977). These studies examined the influence of nutrients, plant growth regulators (PGRs), chemical, physical, precursors, biotic and abiotic factors on enhancing the biosynthesis and accumulation of *G. biloba* promising bioactive metabolites in callus (Jeon *et al*., 1993; Camper *et al*., 1997; Yu *et al*., 1999; Park *et al*., 2004b; Bekhit *et al*., 2008; Hao *et al*., 2009; Cheng *et al*., 2014; Sukito *et al*., 2016 and Sharaf *et al*., 2017) and cell cultures (Carrier *et al*., 1991; Jeon *et al*., 1993; Kim *et al*., 1999; Park *et al*., 2004b; Kang *et al*., 2006; 2009; Chen *et al*., 2015; Sukito and Tachibana, 2016 and Sukito *et al*., 2016).

Light irradiation is one of the physical factors that influence the growth and development of plant tissues as well as the biosynthesis of various phytomolecules (El-Dawayati *et al*., 2020 and Youssef *et al*., 2021). Among the widely used tools of biotechnology, elicitation is an important strategy that stimulates the production of high-value compounds in medicinal crops. Elicitor is a biological (biotic) or non-biological (abiotic) agent that enhances the high expression of specific genes and acts as a signal, which is recognized by specific receptors on the membrane of a plant cell and induces defense responses leading to an increase in the biosynthesis and accumulation of plant by-products (Zhao *et al*., 2005 and Halder *et al*., 2019). Various biotic and abiotic elicitors could be applied to different *in vitro* cultures to enhance the biosynthesis and productivity of secondary metabolites in satisfactory quantities within a short period (Sivanandhan *et al*., 2012; Gabr *et al*., 2016; Toaima *et al*., 2017; El-Ashry *et al*., 2019 and Udomsin *et al*., 2019). Chitosan (β-1,4-linked glucosamine) is a chitin-deacetylated derivative mainly extracted from the exoskeletons of some crustaceans (Hadwiger,

2013). It has been verified that chitosan can be used as an effective, low-cost, and non-toxic biotic elicitor to enhance the production of pharmaceutical constituents for some medicinal plants (Udomsuk *et al.*, 2011; Sivanandhan *et al.*, 2012; Jiao *et al.*, 2018 and Udomsin *et al.*, 2019). However, to our knowledge, no studies have been done on the effect of chitosan on the production of secondary metabolites in *G. biloba* callus tissue. Hence, the present work aims to establish callus culture of *G. biloba* and enhance the accumulation of flavonoids and phenolics by chitosan elicitation under light and dark incubation to provide suitable biological material for efficient production of phenolics and flavonoids with a high capacity of antioxidant activity.

MATERIALS AND METHODS

The experiments and chemical analysis were carried out in the Laboratory of Biotechnology, Horticulture Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt.

Plant material

Explants of *G. biloba* (leaf blade) were excised from plants (4 years-old produced by seeds) grown in the greenhouse of Horticulture Farm, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt.

Explant preparation

Leaf blades were washed with a commercial detergent and tap water, and surface sterilized with ethanol (70%) for 1 min, followed by 20% commercial Clorox solution (containing 5.25% NaOCl) with 2 drops of Tween 20/100 mL for 20 min. The explants were then rinsed 3 times in sterile distilled water.

Effect of growth regulators on callus formation

Sterile uniform explants i.e., 1.0*0.5 cm^2 for leaf blade, were aseptically transferred to jars containing 30 mL of Murashige and Skoog (Murashige and Skoog, 1962) (MS) medium, 100 mg/L myo-inositol, 0.5 g/L casein hydrolysate, 30 g/L sucrose, solidified with 2.0 g/L gelrite, and supplemented with different combinations of auxins (2,4-dichlorophenoxy acetic acid; 2,4-D and α-naphthalene acetic acid; NAA) and cytokinins (kinetin; kin and 6-benzyl adenine; BA) as follow: 1.0 mg/L 2,4-D + 1.0 mg/L kin, 2.0 mg/L NAA + 1.0 mg/L kin, 0.5 mg/L NAA + 0.5 mg/L BA and 1.0 mg/L NAA + 2.0 mg/L BA, in addition to control medium (free hormones).

Effect of chitosan on bioactive metabolites accumulation and antioxidant activity

Leaf callus induced on medium fortified with 1.0 mg/L NAA and 2.0 mg/L BA was subcultured one time for 4 weeks on the same medium formula to provide an adequate amount of calli. Callus pieces (0.4 g fresh weight) taken from leaf calli were transferred to solid MS medium containing 100 mg/L myo-inositol, 0.5 g/L casein hydrolysate, 30 g/L sucrose, and supplemented with 1.0 mg/L NAA and 2.0 mg/L BA. Chitosan was added at concentrations of 0, 25, 50, 100, and 200 mg/L.

Culture condition

The pH value of culture media was adjusted to 5.8 with NaOH (1N) or HCl (1N) and autoclaved at 121^{o}C and 1.2 kg.F./cm^2 for 20 min. Three explants were planted per jar. Each treatment comprised 4 jars and repeated twice. Cultures were incubated for 4 weeks in a growth room under 25±2^{o}C and complete dark or 16/8 h light/dark cycle using cool white light 40 μmol^{-2} s^{-1}.

Measurements

Callus biomass estimation

Callus tissue was collected after 4 weeks of incubation period and the fresh weight (FW) was recorded. Dry weight (DW) of callus was determined after drying in an oven at 45^{o}C for 2 days. Dry matter percentage, growth index, and relative growth rate were calculated as follow:

Dry matter (%) = (final DW/final FW) x 100

Growth index = (final DW – initial DW)/initial DW

Relative growth rate = [(ln final DW) – (ln initial DW)]/incubation period, where ln: natural log, and incubation period is 4 weeks.

Determination of total flavonoids

Dried callus tissue (100 mg) was extracted with 5 mL of ethanol (95%) for 24 h at room temperature (~25^{o}C). After filtration, total flavonoid content in callus extract was determined by following the aluminum chloride colorimetric method described by Chang *et al*. (2002) and Madaan *et al*. (2011). In brief, 0.5 mL of ethanol extract was mixed with 1.5 mL of ethanol (95%), 0.1 mL of $AlCl_3$ (10%), 0.1 mL of potassium acetate (1 M) and 2.8 mL of distilled water. The reaction mixture was incubated for 30 min. at 25±2^{o}C. The

absorbance was measured using a spectrophotometer (JENWAY 6800 UV/Vis. spectrophotometer) at 415 nm against blank. Quercetin was used to establish the calibration curve, and total flavonoid content was calculated and expressed as mg quercetin equivalents per g DW of callus tissue.

Total flavonoids productivity (mg/L of culture medium) = total flavonoids content (mg/g DW) x callus biomass yield (DW g/L of culture medium).

Determination of total phenolics

Dried callus tissue (100 mg) was placed in 5 mL of ethanol (95%) for 48 h at 0°C. The tubes containing samples were then homogenized and centrifuged for 8-10 min. Total phenolics content in the supernatants was determined using the Folin-Ciocalteu method described by Chandler and Dodds (1983) and Singleton and Rossi (1965) with a slight modification. One mL of the extract supernatant was mixed with 1 mL of ethanol (95%), 5 mL of distilled water, 0.5 mL Folin-Ciocalteu reagent (50%). After 5 min, 1 mL of Na_2CO_3 (5%) was added and mixed well. The solution mixture was incubated for 60 min at 25±2°C, and the absorbance was read spectrophotometrically (JENWAY 6800 UV/Vis. spectrophotometer) at 725 nm against blank. Gallic acid dilutions were used to draw the standard concentration curve, and total phenolics content was expressed as mg gallic acid equivalents per g DW of callus tissue.

Total phenolics productivity (mg/L of culture medium) = total phenolics content (mg/g DW) x callus biomass yield (DW g/L of culture medium).

Determination of free radical scavenging activity

Samples of dried callus (100 mg) were extracted with 5 mL of ethanol (95%) for 24 h at room temperature. After filtration, the antioxidant activity of callus extract was assayed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test according to Wu *et al.*, (2018) with minor modifications. Ethanol extract (0.7 mL) of the callus samples was mixed with 3 mL of DPPH ethanol solution (200 µM). The mixture was shaken thoroughly and incubated for 30 min. in the dark at 25±2°C. The absorbance was recorded at 517 nm using a spectrophotometer (JENWAY 6800 UV/Vis. spectrophotometer). The percentage of DPPH radical scavenging activity was calculated using the following formula:

DPPH activity (%) = [(A control – A sample)/A control] x 100

where A control is the absorbance of the DPPH solution with 0.7 mL Ethanol (95%) free sample, and A sample is the absorbance of callus extract mixed with DPPH solution.

The statistical analysis

All experiments were conducted in a complete randomized design (CRD). The statistical analysis of data was subjected to Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) (Duncan, 1955) at $P<0.05$ using COSTAT package ver. 6.4 (CoHort software Monterey, USA). The results were represented by means with Standard Deviation (±SD) (n = 3).

RESULTS AND DISCUSSION

Effect of plant growth regulators and light conditions on callus formation in *G. biloba*

Callus is a mass of undifferentiated plant cells induced from wounded sites of various explants cultured on modified media under controlled conditions. Callus tissue can be used in different biotechnological approaches and biological studies, and also as a potential tool for the production of bioactive pharmaceutical compounds (Bosila *et al.*, 2012; 2016). *G. biloba* leaves excised from field-grown trees have been used in previous investigations as a suitable source for callus induction and medicinal compounds production (Chen *et al.*, 1997; Bekhit *et al.*, 2008; Chen *et al.*, 2015; Sukito *et al.*, 2016 and Sharaf *et al.*, 2017). To investigate the effect of PGRs on callus formation from leaf taken from intact plants, 4 combinations of auxins (NAA and 2,4-D) and cytokinins (kin and BA) were selected based on screening the previous studies on ginkgo. As displayed in Table (1) and Figure (1), different combinations of PGRs affected significantly ($P<0.05$) the percentage of leaf formed callus, callus grade, callus texture, and color after the 4 weeks incubation period. On the other side, the condition of light did not show significant variations concerning callogenesis except the cultures planted on MS supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L kin where 69.33 and 39.83% of leaf explants have produced callus when grown under 16/8 h light/dark cycle and complete dark, respectively. However, the color of the callus was distinctly different. Most of the calli produced from light-incubated cultures were green and yellowish-green while those produced in the dark were white (Table 1 and Figure 1 a, b, c, d). Again, an exception was

recorded for callus originated on MS medium with 1.0 mg/L 2,4-D and 1.0 mg/L kin where the color of the callus appeared yellowish-white. Callus texture differed due to the difference in PGRs and was not correlated with light conditions. Compacted callus was appeared on media fortified with NAA and BA, while friable and semi friable textures were observed for callus developed on media augmented with 2,4-D + kin and NAA + kin, respectively. Bekhit *et al*., (2008) also reported the same texture and color for leaf callus grown on MS with NAA and kin. Necrosis in leaf segments cultured on a PGR-free medium (control) was occurred (Figure 1 e), indicating that the physiological status and the endogenous hormones in the leaf blade are inadequate to support the process of callus induction. A combination of 1.0 mg/L NAA and 2.0 mg/L BA resulted in the highest callogenesis percentage in both light and dark (95.00 and 94.43%, respectively) with a compacted callus and a high callus degree (+++++) making it a suitable candidate for further experiments with callus cultures. Park *et al*., (2004a) found that NAA was better than 2,4-D with regard to callus formation from petiole explants of *G. biloba*. Similar behavior of NAA and BA were noticed with Cheng *et al*., (2014) on callus originated from ginkgo embryo. The friable callus obtained here can be subjected to experiments with cell suspensions in ginkgo.

Cheng *et al*., (2014) reported that embryo-derived calli of ginkgo were achieved on MS supplemented with 2.0 mg/L NAA + 2.0 mg/L BA, however, the subculture of callus was carried out on a medium containing 2.0 mg/L NAA + 1.0 mg/L BA. Plant cell division is regulated by the combined action of cytokinin and auxin. While auxin influences DNA replication, the cytokinin exerts control over the events that lead to mitosis (John *et al*., 1993 and Pasternak *et al*., 2000). Therefore, auxin can be seen as 'inducer' of the plant cell cycle while cytokinin may behave as its 'promoter' (Wood *et al*., 1990). The metabolism of the plant cell is altered and begins to divide under the stimulation of cytokinin and auxin added to the culture medium. During this process, cell specialization and differentiation are reversed, as the explants give a new mass of meristematic and undifferentiated cells called a 'callus' (Jha and Ghosh, 2005). Although 0.5 mg/L NAA + 0.5 m/L BA recorded the lowest value of callus frequency, it was more suitable for shoot formation when nodal explants were planted on it (Figure 1 g, h, i). Other reports have also proved the positive effect of the same combination of NAA and BA on *in vitro* shoot induction in other plants (Toaima *et al*., 2016). However, shoot survival and proliferation were failed when microshoots were re-cultured on the same medium (data not shown).

Effect of chitosan and light conditions on callus biomass production

Callus derived from leaf explants on MS medium fortified with 1.0 mg/L NAA and 2.0 mg/L BA under light incubation was subcultured on the same medium composition one time and incubated 4 weeks under the same conditions (Figure 1 f). Subculture of ginkgo callus is an important step to provide sufficient biomass necessary for further experiments without reducing the active substance content (Cheng *et al*., 2014). The biotic elicitors chitosan was applied to leaf-originated callus at five concentrations (0, 25, 50, 100 and 200 mg/L) to enhance the production of callus biomass and by-products in ginkgo under complete dark and 16/8 h light/dark cycle.

Data illustrated in Table 2 show that chitosan has a significant impact on callus growth parameters i.e., biomass fresh and dry weights, dry matter percentage, growth index, and relative growth rate. Callus cultures elicited with 100 mg/L of chitosan and grown under 16/8 h light/dark cycle exhibited the highest significant (P<0.05) values of callus biomass FW (2.83 g/explant; 283.07 g/L culture medium), callus biomass DW (0.206 g/explant; 20.63 g/L), growth index (4.16), and relative growth rate (0.408), which represent 1.62, 1.29, 1.38, and 1.18-fold increase than the corresponding controls. Increasing chitosan levels up to 100 mg/L promoted callus growth regarding the aforementioned parameters, while the continuous increase to 200 mg/L caused growth inhibition. Similarly, 100 mg/L of chitosan showed a stimulating effect on the biomass of *Agastache foeniculum* hairy roots while the fresh weight decreased at the concentration of 150 mg/L chitosan (Nourozi *et al*., 2014). Chitosan is a natural elicitor that can act as a growth stimulator in some plant species (Nourozi *et al*., 2014). The dry matter percent showed a different pattern in this regard as 25 and 200 mg/L of chitosan resulted in a higher dry matter under dark incubation (10.06 and 10.34%, respectively) with a little significant difference with control (8.84%), while callus tissue grown on medium augmented with 100 mg/L of chitosan and incubated in light recorded the lowest significant value (7.44%). In general,

incubation under 16 h photoperiod was more suitable for *G. biloba* callus cultures than the full completely dark. Lighting conditions (light intensity, type, and photoperiod) are among the factors that strongly influence the physiological and biochemical processes in the plant cell. Efficient production of secondary metabolites *via* tissue culture methods can be improved by optimizing the *in vitro* conditions including light photoperiod (Chen *et al*., 1997).

Effect of chitosan and light conditions on total flavonoids production

The major challenge in using tissue culture technology to produce bioactive phytochemicals from medicinal crops is the extremely low production of these components compared to field-grown plants. Therefore, after establishing aseptic *in vitro* cultures, further treatments are used to enhance the content of bioactive ingredients by increasing the biosynthetic cellular capacity through several mechanisms (Elateeq *et al*., 2020). Amongst different research approaches, elicitation had gained more attention due to its beneficial effects in increasing the production of many plant secondary metabolites. In the current study, chitosan was used as a biotic elicitor in callus media of *G. biloba* to enhance the accumulation of flavonoids and phenolics in light- or dark-grown cultures.

The effect of different chitosan concentrations (25, 50, 100, and 200 mg/L) with two time periods of incubation (light and dark) on the production of total flavonoids in *G. biloba* were studied and the obtained data are tabulated in Table 3. A significant difference was recorded between different levels of chitosan added to the callus culture medium placed in the light. In contrast, no significant difference was recorded between chitosan treatments for cultures grown under darkness. Our findings showed that chitosan concentrations are not the only factor controlling the biosynthesis and accumulation of flavonoids in the callus tissue of ginkgo, as their interference with the lighting conditions shows a different response. Callus cultures elicited with chitosan accumulated greater amounts of flavonoids when incubated under 16 h photoperiod compared to dark-grown cultures. This observation is in line with the results recorded with Chen *et al*., (1997) on leaf-derived callus of ginkgo who noticed that total flavonoid content in calli grown in light was significantly higher than that of the dark one. Moreover, Joshi, (2015) observed an increase in the total flavonoid content in *Helicteres isora* callus culture in response to light compared with dark incubation. The improvement of the content of other phytochemicals (ginkgolides) in ginkgo cell cultures was also observed in cultures incubated in light when compared to dark; however, bilobalide content decreased under light conditions (Park *et al*., 2004a). Moderate concentrations of chitosan (50 and 100 mg/L) accumulated the highest significant content (P<0.05) of total flavonoids (2.55 and 2.38 mg/g DW, respectively) in *G. biloba* callus under the light conditions which accounts as 1.62 and 1.52-fold increase than control (1.57 mg/g DW). Similarly, Udomsuk *et al*., (2011) found that applying chitosan at 100 and 150 mg/L to hairy root cultures of *Pueraria candollei* stimulated the production of total isoflavonoid by 1.7- and 2.8-fold in comparison with control. Substantially, evidence indicated that chitosan activates the genes-expression in the flavonoid biosynthetic pathway, specifically chalcone synthase (*CHS*) and flavonoid 3'-hydroxylase (*F3'H*) (Jiao *et al*., 2018) which could better explain the elevated content of flavonoid in chitosan-elicited callus.

The productivity (yield) of total flavonoids and total phenolics was calculated by multiplying the content expressed in mg/g DW and the callus biomass DW yield expressed in g/L medium. As the total flavonoid content followed the same pattern as callus biomass DW yield, therefore, the productivity of total flavonoids per liter of culture media had the same recorded trend for the total flavonoids content per gram dry matter. In this context, about 48.37 and 49.02 mg of total flavonoids/L of culture media were produced from cultures subjected to chitosan elicitation at 50 and 100 mg/L, which is higher than the control culture by 1.94 and 1.97-fold, respectively.

Effect of chitosan and light conditions on total phenolics production

Concerning the effect of chitosan on total phenolics production in callus cultures of *G. biloba*, significant differences were recorded between different levels of chitosan added to callus cultures incubated under light and dark (Table 3). All traits of chitosan significantly elicited the accumulation of total phenolics in callus tissue under light conditions more than that placed in the dark. Ali and Abbasi (2014) also found a significant increase in the content and productivity of total phenolics in *Artemisia absinthium* cell suspension placed in light more than dark conditions. Reports indicate that upon absorption of photons of light, the phytochromes are transformed into the active forms of far-red light-absorbing (Pfr), which

regulate gene expressions that lead to related phototactic responses, such as cell division and synthesis of phytoconstituents (Smith, 2000 and Liu *et al*., 2006).

Chitosan is a natural, non-toxic and low-cost product that can trigger the defensive responses of plant accompanied by an increase in phytochemical biosynthesis (Jiao *et al*., 2018). In the current study, chitosan at 100 and 200 mg/L resulted in the highest accumulation of total phenolics (5.39 and 4.99 mg/g DW, respectively) in callus incubated in the light conditions (represent as 3.06 and 2.84-fold increase than control, respectively) without significant differences between them. Likewise, production of withanolides in the adventitious root of *Withania somnifera* was enhanced with 100 mg/L of chitosan (Sivanandhan *et al*., 2012). Moreover, elicitation with 200 mg/L chitosan for 6 days in the hairy root of *Pueraria candollei* var. *mirifica* resulted in the highest content of deoxymiroestrol; 1.68-fold than control (Udomsin *et al*., 2019). The lowest abundance of total phenolics was observed in the control treatments as well as for low (25 mg/L) and high (200 mg/L) concentrations of chitosan under dark incubation.

The productivity of total phenolics was significantly affected only when callus cultures treated with chitosan were grown in the light. The highest significant value of total phenolics yield (111.03 mg/L medium; 3.96-fold increase than control) was obtained from a medium containing 100 mg/L chitosan under 16 h photoperiod. The rest concentrations of chitosan (25, 50 and 200 mg/L) also achieved significant increments in phenolic yield being higher than control by 1.54, 2.38 and 2.73-fold, respectively. Among biotic elicitors, yeast extract and chitosan have been employed in tissue culture systems due to their effect on triggering a variety of defense mechanisms in plant cells, leading to the accumulation of phytoalexins and secondary metabolites (Abraham *et al*., 2011; Hadwiger, 2013 and Toaima *et al*., 2017). Probably, chitosan enhanced flavonoids and phenolics biosynthesis in *G. biloba* callus by stimulating plant defense responses.

Effect of chitosan and light conditions on antioxidant activity

DPPH free radical scavenging activity was determined for callus extracts of cultures treated with chitosan. Overall, callus cultures grown under light conditions exhibited the greatest significant capacity of antioxidant activity compared to dark-grown cultures (Figure 2). All tested levels of chitosan (25-200 mg/L) under light incubation recorded the highest percentages of antioxidants activity (from 92.67 to 93.95%) versus control (79.58%). Under darkness, 50 and 100 mg/L of chitosan recorded the highest antioxidant activity percentage followed by 25 and 200 mg/L (93.73, 92.66, 87,10 and 83.32%, respectively). Higher percentages of antioxidant activity assayed for light-grown calli may be due to the higher significant contents of total flavonoids and total phenolics determined under light conditions. Similar to our data, Youssef *et al*., (2021) reported the enhancement of flavonoids, phenolics compounds, and antioxidant activity in callus cultures of *Antigonon leptopus* incubated under light conditions compared to the dark one. Additionally, cell cultures of ginkgo incubated in 16/8 h light/dark cycle accumulated more ginkgolide A and B than those in darkness (Park *et al*., 2004a), and thus, these terpene lactones may also share their antioxidant properties. Higher antioxidant activities were also reported by Abraham *et al*., (2011) and Jiao *et al*., (2018) in extracts from chitosan-elicited cultures of *Curcuma mangga* plantlets and *Isatis tinctoria* hairy roots, respectively.

CONCLUSIONS

Flavonoids and phenolics are valuable bioactive compounds accumulated in ginkgo leaves. Exploiting *in vitro* cultures to improve high-value compounds production is a promising approach to overcome various restrictions imposed by rare, endangered plants. For the industrial production of ginkgo medicinal compounds through tissue culture systems, it is necessary to establish promising *in vitro* cultures and to provide suitable elicitors that enable the sustainable production of such phytochemicals on a commercial scale. The findings of the current study revealed the possibility of using callus culture and chitosan elicitation as a biotechnological approach for the industrial production of flavonoids and phenolics from *G. biloba*, an endangered plant in Egypt. Light irradiation is a physical factor that affects cell growth and the biosynthesis of various phytomolecules. Herein, incubation of ginkgo callus cultures in lighting was significantly appropriate for the productivity of callus biomass having abundant of total flavonoids and phenolics. Furthermore, enhanced antioxidant activity was observed in the extracts of chitosan-elicited callus grown in light, which would broaden the application range in the pharmaceutical and nutraceutical

domains. However, in this work, we did not study the case of the unique active ingredients, ginkgolides and bilobalide, under chitosan elicitation. Therefore, future investigations should consider this point together with exploring the expression of genes involved in regulating the biosynthesis pathways of ginkgolides, bilobalide and flavonoids.

ACKNOWLEDGMENTS

The authors thank Dr. Mostafa M. Zarad and Dr. Mahmoud N.A. Soliman, Horticulture Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt for their great assistance during the implementation of the experiments.

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Table 1 Callus formation from leaf blade of *G. biloba* after 4 weeks of culture on MS medium contained different combinations of plant growth regulators under incubation in 16/8 h light/dark cycle or complete dark

Light conditions	Plant growth regulators (mg/L)	Explants producing callus (%)	Callus grade	Callus color	Callus texture
Light	Control	-	-	-	-
	1.0 2,4-D + 1.0 kin	69.33±8.02^{c}	+++	Yellowish white	Friable
	2.0 NAA + 1.0 kin	80.13±8.20^{b}	++++	Yellowish green	Semi friable
	0.5 NAA + 0.5 BA	9.67±2.52^{e}	+	Green	Compact
	1.0 NAA + 2.0 BA	95.00±2.65^{a}	+++++	Green	Compact
Dark	Control	-	-	-	-
	1.0 2,4-D + 1.0 kin	39.83±6.25^{d}	++	Yellowish white	Friable
	2.0 NAA + 1.0 kin	85.17±4.75^{b}	++++	White	Semi friable
	0.5 NAA + 0.5 BA	3.50±1.32^{e}	+	White	Compact
	1.0 NAA + 2.0 BA	94.43±2.89^{a}	+++++	White	Compact

Data are presented as mean ± SD, n=3. Mean values with different letters in the column are statistically different according to DMRT (P<0.05)

Table 2 Effect of chitosan on callus production in *G. biloba* after 4 weeks of incubation under 16/8 h light/dark cycle or complete dark

Light conditions	Chitosan (mg/L)	Callus biomass FW		Callus biomass DW		Dry matter (%)	Growth index	Relative growth rate
		(g/explant)	Yield (g/L)	(g/explant)	Yield (g/L)			
Light	Control	1.75±0.47^{bc}	175.40±47.15^{bc}	0.160±0.024^{bc}	16.03±2.40^{bc}	9.50±0.60^{ab}	3.01±0.60^{bc}	0.345±0.036^{abc}
	25	1.65±0.15^{bcd}	165.07±15.29^{bcd}	0.157±0.016^{bc}	15.73±1.63^{bc}	9.65±1.09^{ab}	2.93±0.41^{bc}	0.341±0.025^{abc}
	50	2.12±0.34^{b}	211.67±34.15^{b}	0.190±0.001^{ab}	19.00±0.10^{ab}	9.14±0.55^{ab}	3.75±0.03^{ab}	0.390±0.002^{ab}
	100	2.83±0.45^{a}	283.07±44.88^{a}	0.206±0.027^{a}	20.63±2.71^{a}	7.44±1.01^{c}	4.16±0.68^{a}	0.408±0.034^{a}
	200	1.85±0.15^{bc}	185.07±15.45^{bc}	0.152±0.051^{bc}	15.23±5.12^{bc}	8.12±1.23^{bc}	2.81±1.28^{bc}	0.326±0.079^{bc}
Dark	Control	0.92±0.18^{e}	91.97±18.42^{e}	0.081±0.012^{e}	8.07±1.18^{e}	8.84±0.62^{abc}	1.02±0.29^{e}	0.173±0.038^{e}
	25	0.93±0.16^{e}	92.70±16.45^{e}	0.093±0.020^{de}	9.33±2.04^{de}	10.06±0.78^{a}	1.33±0.51^{de}	0.207±0.059^{e}
	50	1.21±0.15^{de}	121.10±15.26^{de}	0.099±0.008^{de}	9.87±0.81^{de}	8.18±0.41^{bc}	1.47±0.20^{de}	0.225±0.021^{de}
	100	1.37±0.30^{cde}	136.77±29.65^{cde}	0.128±0.017^{cd}	12.83±1.66^{cd}	9.50±0.99^{ab}	2.21±0.41^{cd}	0.290±0.032^{cd}
	200	1.01±0.11^{e}	101.13±10.82^{e}	0.104±0.004^{de}	10.37±0.42^{de}	10.34±0.49^{a}	1.59±0.10^{de}	0.238±0.010d^{e}

Data are presented as mean ± SD, n=3. Mean values with different letters in the column are statistically different according to DMRT (P<0.05)

Table 3 Total flavonoids and total phenolics production in *G. biloba* callus cultures grown on MS medium with different levels of chitosan after 4 weeks of incubation under 16/8 h light/dark cycle or complete dark

Light conditions	Chitosan (mg/L)	Total flavonoids		Total phenolics	
		Content (mg/g DW)	Yield (mg/L)	Content (mg/g DW)	Yield (mg/L)
Light	Control	1.57±0.23^{c}	24.87±1.77^{c}	1.76±0.12^{e}	28.06±2.59^{cd}
	25	1.71±0.09^{c}	26.79±1.53^{bc}	2.76±0.15^{c}	43.26±2.39^{c}
	50	2.55±0.26^{a}	48.37±5.10^{a}	3.51±0.52^{b}	66.70±9.44^{b}
	100	2.38±0.07^{ab}	49.02±6.14^{a}	5.39±0.59^{a}	111.03±16.90^{a}
	200	2.27±0.13^{b}	34.16±9.54^{b}	4.99±0.19^{a}	76.56±28.47^{b}
Dark	Control	1.26±0.11^{d}	10.27±2.23^{d}	1.83±0.20^{e}	14.60±1.22^{d}
	25	1.22±0.15^{d}	11.55±3.54^{d}	2.22±0.12^{de}	20.83±5.31^{d}
	50	1.10±0.05^{d}	10.87±1.37^{d}	2.79±0.12^{c}	27.56±3.37^{cd}
	100	1.25±0.09^{d}	16.09±3.17^{d}	2.68±0.27^{cd}	34.13±1.06^{cd}
	200	1.16±0.02^{d}	12.02±0.49^{d}	1.75±0.08^{e}	18.17±1.24^{d}

Data are presented as mean ± SD, n=3. Mean values with different letters in the column are statistically different according to DMRT (P<0.05)

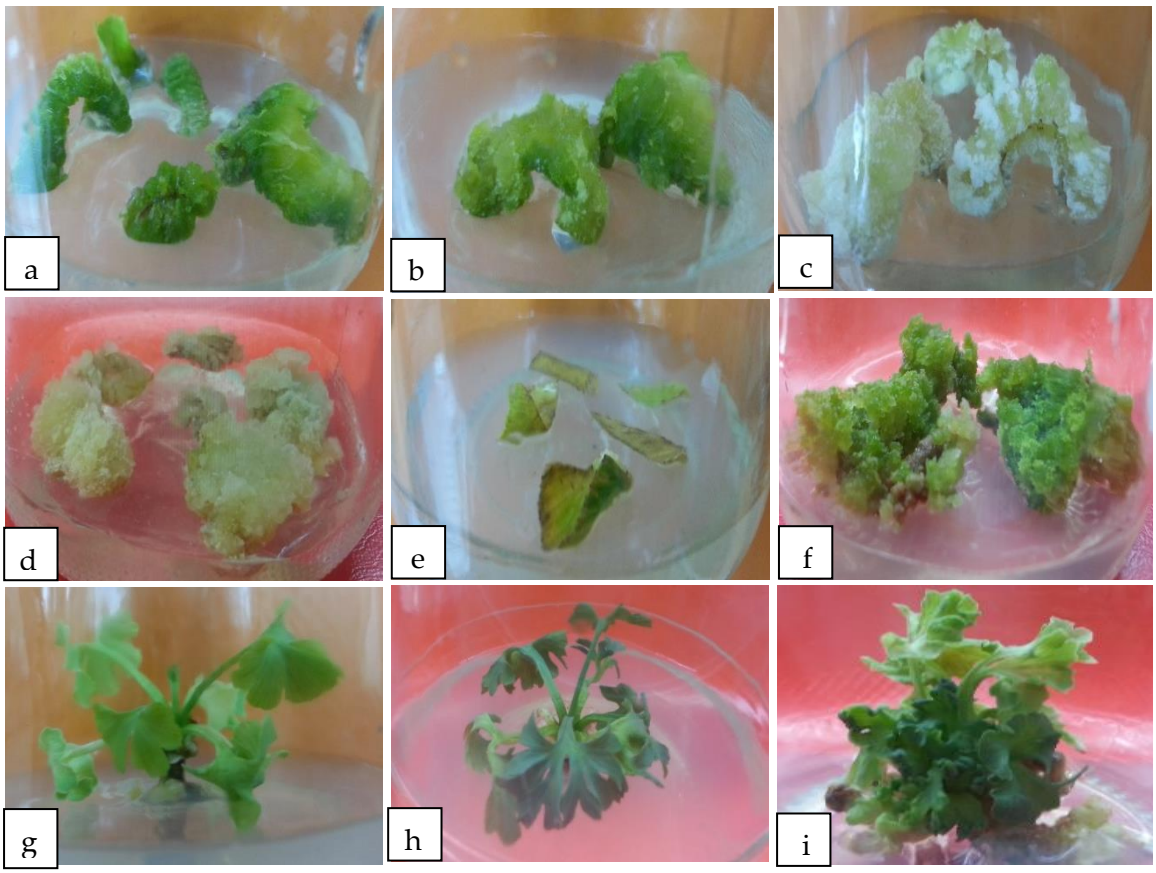

Figure 1. Callus induction in *G. biloba* from leaf explant after 4 weeks of culture on MS medium containing 100 mg/L myo-inositol, 0.5 g/L casein hydrolysate, 30 g/L sucrose, and supplemented with (a) 1.0 mg/L NAA + 2.0 mg/L BA, (b) 2.0 mg/L NAA + 1.0 mg/L kin, under 16/8 h light/dark cycle incubation, and (c) 1.0 mg/L NAA + 2.0 mg/L BA, (d) 1.0 mg/L 2,4-D + 1.0 mg/L kin under darkness. (e) hormone-free culture. (f) calli subcultured on MS with 1.0 mg/L NAA + 2.0 mg/L BA under 16/8 h light/dark cycle. (g, h, i) *in vitro* shoot induction of *G. biloba* from nodal explants grown on MS medium fortified with 0.5 mg/L NAA + 0.5 mg/L BA

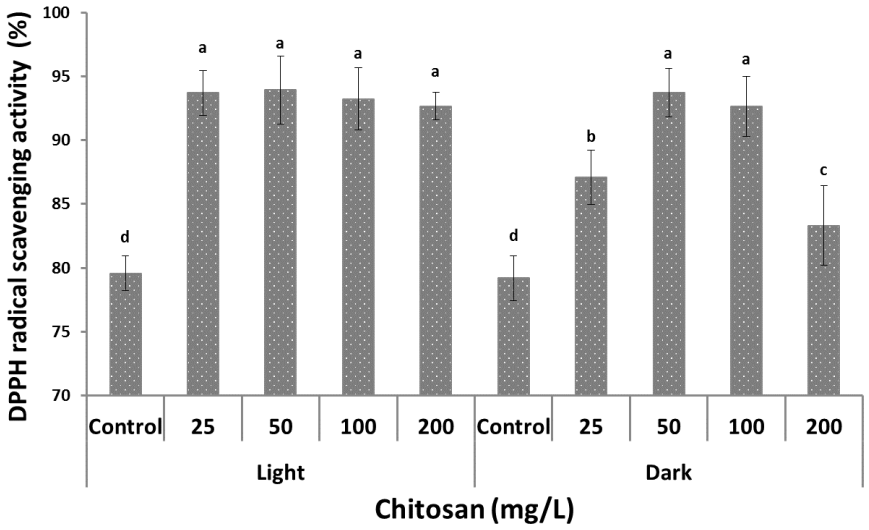

Figure 2. Effect of chitosan elicitation with concentration of 0 (control), 25, 50, 100 and 200 mg/L on DPPH free radical scavenging activity of *G. biloba* callus cultures grown on MS medium supplemented with 100 mg/L myo-inositol, 0.5 g/L casein hydrolysate, 30 g/L sucrose, and 1.0 mg/L NAA + 2.0 mg/L BA. Cultures were incubated under 16/8 h light/dark cycle or complete dark at 25±2°C for 4 weeks. Bars represent ±SD (n=3). Columns annotated with different letters are statistically different according to DMRT (P<0.05)

تأثير الشيتوزان وظروف الإضاءة على انتاج الكالس والفلافونيدات والفينولات الكلية في نبات الجنكو

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الملخص العربى

يعتبر نبات الجنكو من النباتات التي لها أهمية طبية كبيرة نظراً لإحتواء أوراقه على تربينات ثلاثية اللاكتون فريدة من نوعها، بالإضافة إلى مواد أخرى مثل الفلافونيدات والفينولات. ويعد نبات الجنكو من الأشجار المهددة بالإنقراض في مصر، وبالتالي يمكن إستخدام تقنية زراعة الأنسجة النباتية كطريقة بديلة لإنتاج المركبات الصيدلانية من الجنكو. في الدراسة الحالية تم إستخدام إستراتيجية الإستحثاث أو التحفيز من خلال المحفز الحيوي الشيتوزان، وذلك لتحسين إنتاجية مركبات الجنكو في الكالس الناشئ من الأوراق المحضنة في الإضاءة (16 ساعة ضوئية) أو فى الإظلام. أدى إضافة نفثالين حامض الخليك بتركيز 1 ملجم/لتر مع البنزيل أدينين بتركيز 2 ملجم/لتر لبيئة موراشيجي وسكوج إلى تسجيل أعلى نسبة تكوين للكالس تحت ظروف الإضاءة والإظلام (95 و94.43% على التوالى) حيث كان الكالس ذا قوام قوي متماسك ولكن مختلف في اللون. تم تسجيل إختلافات معنوية بين معاملات الشيتوزان فيما يتعلق بإنتاج الكالس والفينولات والفلافونيدات. تم تسجيل أعلى إنتاج من الكالس الطازج والجاف وأعلى مؤشر نمو ومعدل نمو نسبي لمزارع الأنسجة النامية في الإضاءة والتي تم معاملتها بـ 100 ملجم/لتر من الشيتوزان ثم تركيز 50 ملجم/لتر، وقد كانت نفس التركيزات أيضاً مفضلة في تحسين محتوى وإنتاجية الفينولات الكلية والفلافونيدات الكلية. كما أظهرت النتائج أن التحضين تحت ظروف الإضاءة افضل من التحضين تحت ظروف الإظلام فيما يتعلق بنمو الكالس والتخليق الحيوى للفينولات والفلافونيدت وكذلك نشاط مضادات الأكسدة. تشير النتائج المتحصل عليها من هذه الدراسة إلى إمكانية إستخدام مزارع الكالس كأداة تقنية حيوية للإنتاج الإصطناعى للمركبات الكيميائية الموجودة فى نبات الجنكو.

الكلمات الاسترشادية: نبات الجنكو، الشيتوزان، التحفيز الحيوى، الفلافونيدات، الفينولات، نشاط مضادات الأكسدة، مزارع الكالس، الفترة الضوئية