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Molecular Role of Nitric Oxide in Secondary Products Production in *Ginkgo biloba* Cell Suspension Culture

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Abstract

Effects of sodium nitroprusside (SNP; nitric oxide donor) treatment on the enhancement of secondary metabolites production, oxidative stress mediators ($O_2^{\cdot-}$) accumulation and antioxidant defense enzymes of *Ginkgo biloba* callus culture was investigated. On one hand, the obtained data showed a highly metabolic modification of chemical constituents, PAL activity and various antioxidant defense enzymes (APX, SOD), which gradually increased in response to SNP treatments. On the other hands the high NO levels significantly increased the accumulation of various oxidative burst of $O_2^{\cdot-}$. MS basal medium supplemented with casein hydrolase (500 mg/L), NAA and BA at equal concentration (0.5 mg/L) recorded the highest number of regenerated shoots (4.81 cm) and shoot height (4.96 cm) as well as root number (2.25 cm) and root length (4.5 cm). The highest survival (40 %) was shown in acclimatization on the mixture containing sand, peat moss and vermiculite (1: 1: 1, v/v/v), which significantly confirmed and reflected the variation in survival percentage. Meanwhile, higher treatment (500 µM) of NO positively enhanced secondary products accumulation of total tannins, saponins, phenols and total flavonoids in *G. biloba* callus culture.

Keywords: antioxidant enzymes, *Ginkgo biloba*, nitric oxide, oxidative burst, secondary products, tissue culture

Abbreviations: NO: Nitric oxide; SNP: sodium nitroprusside; $O_2^{\cdot-}$: superoxide anion radical; MDA: malonaldehyde; H_2O_2: hydrogen peroxide; GSH: glutathione; AsA: Ascorbate; PAL: Phenyl alanine ammonia lyase; SOD: Superoxide dismutase; APX: ascorbate peroxidase; NAA: Naphthaleneacetic acid; BA: Benzyl amino purine; MS: Murashige and Skoog medium.

Introduction

Ginkgo biloba has been known as a popular remedy in traditional Chinese medicine for over 4000 years, and it has been a common herbal medicine in Europe since the 1730's. The *Ginkgo* tree, the only existing tree in the family *Ginkgoaceae*, is the world's oldest living tree and is thus sometimes referred to as a "living fossil". *Ginkgo* trees are now widely planted in China, Japan, Korea, France, Germany and the United States for both ornamental and medicinal purposes. Kuo *et al.* (2004) demonstrated that *G. biloba* extract competitively inhibited rat hepatic microsomal CYP1A-mediated enzyme activity, as determined by experiments performed on extracts containing terpene trilactones and ginkgo flavonol glycosides. Despite the fact that the plant appears to possess antioxidant activity, the mechanism of action of *G. biloba* is not known. Plant cells and tissue cultures have been used as alternative sources to the whole plant for the production of valuable phytochemicals such as flavors, fragrances, pharmaceuticals and nutraceuticals. Numerous strategies have been developed to improve the productivity of plant cell culture such as medium optimization, cell line selection, cell immobilization, precursor addition, elicitation, genetic transformation, organ or hairy root cultures, metabolic and integrated bioreactor engineering (Abdullah *et al.*, 2005; El-Beltagi *et al.,* 2011).

Nitric oxide as a bioactive molecule shows prooxidant as well as antioxidant properties in plants (Beligni and Lamattina, 1999; Delledonne *et al.,* 2001; Beligni and Lamattina, 2002; Delledonne *et al.,* 2002). The biosynthetic origin of NO during plant pathogen interactions involves L-arginine conversion into L citrulline. Nitric oxide induces a complementary set of plant defense genes, including two key enzymes of the phenylpropanoid pathway, so-called Phenyl alanine ammonia lyase (PAL) and chalcone synthase. Furthermore, NO-treated tobacco (*Nicotiana tabacum*) cells were shown to induce the accumulation of cyclic acid, a key molecule for the expression of systemic acquired resistance (Durner *et al.,* 1998; Crawford and Guo, 2005). The free radical NO has a half-life of just a few seconds and can also react with the free

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radical superoxide to form the reactive molecule peroxynitrite (ONOO), which can lead to the formation of NO_2 and the potent oxidant hydroxyl radical, which is a very strong oxidizing specie that can rapidly attack biological membranes and all types of bio-molecules such as DNA and proteins leading to irreparable damage, metabolic dysfunction and cell death (Del-Rio *et al.*, 2003). $ONOO^-$ is responsible for tyrosine nitration and oxidation of thiol (Stamler *et al.*, 1992; Tamir *et al.*, 1993; Lamattina *et al.*, 2003). Sodium nitroprusside (SNP) was used as the donor of nitric oxide (NO). SNP at high concentrations stimulated catharanthine formation of *Catharantuse roseus* cells, but inhibited the growth of the cells (Xu *et al.,* 2009). It stimulated production of hypericin by *Hypericum perforatum*. The cells were significantly increased after elicitor treatment by NO, achieving 4.2-fold higher over the control (Xu *et al.*, 2005).

Antioxidant defense enzymes such as superoxide distumase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR) are the systems designed to minimize the concentrations of superoxide and hydrogen peroxide. A demonstrated strong inhibitory effect of NO on the catalase (CAT) peroxidase (POD) activity of cytc/CL complex combined with its increased amount in mitochondria suggest that NO may regulate lipid peroxidation and apoptotic activity of CL membrane bound cytc during apoptosis (Vlasova *et al.,* 2006).

Recently, a function of nitric oxide (NO) in the protection of plants against oxidative stress under various adverse conditions was reported (Beligni and Lamattina, 2002; Shi *et al.*, 2005). Many previous studies had reported the presence of NO in the plant kingdom and its involvement in growth, development and defense responses (Beligni and Lamattina 1999). In addition, Tu *et al.* (2003) found that 0.1 mM SNP delayed the senescence of wheat leaves by inhibition of the degradation of chlorophyll and soluble proteins, especially Rubisco enzyme. Therefore, this study was conducted to investigate the molecular role of nitric oxide in secondary products production in *G. biloba* cell suspension culture.

Materials and methods

Plant materials and surface sterilization

All excised tissues of young stems of *G. biloba* were collected from Orman botanical garden, Giza governorate and stored temporarily in an antioxidant solution (150 mg/l citric acid and 100 mg/l ascorbic acid), prior to surface-sterilization in 1% chlorox for 20 min, 2 drops/ 100 ml solution of Tween 20 as wetting agent then rinsed three times with sterile distilled water.

Callus initiation and maintenance

Callus was initiated from the sterilized young stems of *G. biloba*. The callus was initiated in a MS-Medium (Murashige and Skoog, 1962) supplemented casein hydrolisate (500 mg/L) and different combinations of Naphthaleneacetic acid (NAA) and kinetin (kin) each with concentrations of 1, 2, 3 and 4 mg/L and sucrose (30 g/L) solidified with agar (6 g/L). Three jars (replicates) were used for each treatment. Cultures were incubated at 25±1 °C, and re-cultured 3 times in the same medium with four week intervals. Data of explants callus formation responses were recorded.

Growth dynamic

After several subcultures of callus (5 g F.W.) they were transferred into 250-ml flasks containing 50 ml of the same medium without agar and maintained on a rotary shaker at 100 rpm. Microscopic observations were carried out on cell suspension cultures during the growth cycle, which revealed the presence of single cells and small cell aggregates. The number of cell per unit can be determined using a hemocytometer. Cell growth was measured every two days for a total period of 12 days; starting, 2, 4, 6, 8, 10 and 12 days.

Cells viability

Cells viability was determined using Evan's blue staining test. A 2 mL sample from tank was incubated into 0.25% Evan's blue stain for 5 minutes and then 700 cells were counted. The viability was calculated taking into account the non-viable cells (Evans *et al.,* 2003).

Experimental design and cell culture treatment

G. biloba cell suspension cultures were treated with various concentrations of sodium nitroprusside (SNP, 50, 100, 250 and 500 μM) after sub-culturing and followed by cells harvest at time intervals. All parameters and enzymes activity were monitored after 0, 12, 48 and 72 h post NO donors treatments.

Determination of phytochemicals in *Ginkgo biloba*

Total tannins, saponins, flavonoids, and phenolic compounds were determined according to the methods described by: Balbaa (1974), Ebrahimzadeh and Niknam (1998), Zhuang *et al. (*1992), Swain and Hillis (1959).

The production rate of superoxide anion ($O_2^{\cdot-}$)

The production rate of superoxide anion ($O_2^{\cdot-}$) was measured by the modified method described by Elstner and Heupel (1976). Fresh mass (200 mg) from culture was homogenized in 1 ml of 50 mM phosphate buffer (pH 7.8), and the homogenate was centrifuged at 10,000 g for 10 min. Then 0.5 ml of the supernatant was added to 0.5 ml 50 mM phosphate buffer (pH 7.8) and 0.1 ml of 10 mM hydroxylamine hydrochloride. After 1 h reaction at 25 1C, 1 ml of 17 mM sulfanilamide and 1 ml 7 mM a-naphthylamine were added to the mixture at 25 1C, and after 20 min, the specific absorbance at 530 nm was determined. Sodium nitrite was used as standard solution to calculate the production rate of $O_2^{\cdot-}$.

Assay of total soluble protein

Soluble proteins were measured by the Bio-Rad micro assay modification of the Bradford (1976) procedure using crystalline bovine serum albumin as a reference.

Preparation of enzyme extracts and assay of protein content

The callus samples (1.0 g) were crushed into fine powder using liquid nitrogen. Soluble protein was extracted by homogenizing the powder in 5 mL 50 mM phosphate buffer (pH 7.8) containing ethylenediamine-N,N,N,N-tetraacetic acid (EDTA, 1 mM) and polyvinylpyrrilidone (PVP, 1%) in addition to ASC

(1mM) in the case of APX assay. The samples homogenate was centrifuged at 15,000 x g for 20 minutes and the supernatant was used for further enzyme activity assays.

Assay of superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Beauchamp and Fridovich (1971). APX activity was determined (13.7 mM^{-1} cm^{-1}) using the method of Nakano and Asada (1981).

Assay of phenylalanine ammonia lyase (PAL)

PAL activity was determined as the rate of conversion of L phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson *et al*. (1984).

Callus formation responses

It was determined according to the rate of scaling by Pottino (1981), which included: - No response, + Poor response, ++ Average response, +++ High response and ++++ very high response.

Callus regeneration and development

Callus was cultured on MS basic medium supplemented with casein hydrolisate (500 mg/L) and auxin (NAA) Naphthalene acetic acid in combination with cytokinins (BA) 6- Benzyl amino purine was used (mg/L); with different concentrations, as follows: 0.2, 0.5, 1.0 and 2.0 (mg/L) each. Incubation was carried out in a growth room at 25±1 °C with 16 h photoperiod (1500 lux). After 6 weeks, data including the number of regenerated shoots, shoot height (cm) as well as no. of roots and root length (cm) were recorded.

Acclimatization

In order to realize a proper acclimatization, plantlets were removed from rooting medium, rinsed under tap water and then, planted in plastic pots (5 cm) filled with a soil mixture as follows: sand, peat moss and vermiculite (1:1:1, v/v/v). Plants were covered with transparent polyethylene sheet and irrigated if needed. Potted plants were incubated for 30 days in phytotron at 25 ± 1 °C, relative humidity (80-90 %) and 16 h photoperiod with 3000 lux light intensity. Acclimatization was realized through gradual remove of the plastic sheet, every day, until it became removed after 30 days. Survival percent, the number of leaves/plant and leaf length (cm) were recorded.

Statistical analysis

All statistical analyses were carried out using SPSS 10 software. Mean and standard error were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples. All determinations have been done in triplicate, *p*-Values < 0.05 were considered significant.

Results and discussions

Establishment of Ginkgo callus culture

Callus formation occurred on young leaves excised from *G. biloba* tree. On one hand, explants were individually cultured on MS medium supplemented with different combinations of NAA (1, 2, 3 and 4 mg/l) and kinetin with concentrations of 1, 2, 3 and 4 mg/l. Data in Table 1 represents the effect of plant growth regulators (NAA and kin) combinations on callus formation responses. The highest response has been recorded with NAA and Kin with equal concentrations (3 mg/L) each followed by (2 mg/L) and (1 mg/L). On the other hand the application of NAA (1 mg/L) and kinetin (3 mg/L) did not represent any callus formation response of *Ginkgo* explants *in vitro*. Suspension culture of the cell line was initiated from *G. biloba* callus on a liquid MS medium supplemented with NAA (2mg/L), kinetin (2 mg/L) and sucrose 30 g/L. These data were in accordance with Wang *et al.* (2006), who found that hypocotyls and cotyledons of *Arctium lappa* were induced to form callus by culturing on MS medium supplemented with 2, 4-D (2.0 mg/L) and BA (0.5–2.0 mg /L).

Data in Table 2 showed that, the growth rate and biomass were gradually enhanced and reached their optimum on the 8th day of cultivation. These data are in agreement with Bulgakov *et al.* (2002), Chong *et al.* (2005) and Tanveer *et al.* (2012); they found a significant decrease in the culture growth.

Chemical analysis of different types of G. biloba explants

While data in Table 3 show that the percentage of tannins and saponins compounds were 2.02%, 0.59% and 1.15%, 0.79% based on the dry weight, respectively for the *G. biloba* leaves and cell suspension cultures. While the percentage of flavonoids and phenolic compounds were 1.75%, 1.09% and 2.95%, 1.68%. The highest concentration of tannins, saponins, flavonoids and phenolic compounds were found in the *G. biloba* leaves compared to its derived cell suspension culture.

As shown in Table 3, there were a high accumulation of total soluble phenols under various NO treatments. The

Table 1. Effect of plant growth regulators (NAA and kin) in combination with different concentrations on callus formation and growth of *Ginkgo biloba* explants grown *in vitro*

Treatments (mg/L)	Callus formation responses			
Kin / NAA	1	2	3	4
1	+	-	-	-
2	+	+++	++	++
3	-	++	++++	++
4	-	-	+	+

Rating Scale: - No response +Poor response ++Average response +++High response ++++very high response.

Table 2. Growth dynamic of suspension culture of *Ginkgo biloba* cultured for 12 days *in vitro*

Time (day)	Cells No./l
Starting	x10^5 564
2 days	x10^5 2288
4 days	x10^5 3521
6 days	x10^5 3800
8 days	x10^5 4287
10 days	x10^5 3987
12 days	x10^5 3870

Cells/ml = average count per square *dilution factor* 1000
Total cells = cells/ml * original volume of fluid from which the cells sample was taken.

Table 3. Effect of various NO treatments on total tannins, saponins, flavonoids and Phenolic compounds contents (% Dry weight) in *Ginkgo biloba* leaves and cell suspension culture

Treatments	Total % of			
	Tannins	Saponins	Flavonoids	Phenolic compounds
Ginkgo biloba Leaves	2.02±0.14^{c}	1.15±0.05^{c}	1.75±0.08^{b}	2.95±0.12^{c}
Ginkgo biloba Cell culture	0.59±0.03^{f}	0.79±0.02^{f}	1.09±0.05^{e}	1.68±0.04^{e}
SNP (50 μM)	0.95±0.08^{e}	0.90±0.04^{e}	1.26±0.11^{d}	2.45±0.06^{d}
SNP (100 μM)	1.87±0.10^{d}	1.01±0.06^{d}	1.43±0.07^{c}	2.92±0.08^{c}
SNP (250 μM)	2.49±0.13^{b}	1.45±0.12^{b}	1.79±0.10^{b}	3.36±0.14^{b}
SNP (500 μM)	2.61±0.14^{a}	1.74±0.13^{a}	1.93±0.03^{a}	3.59±0.11^{a}

- Means within the same column followed by different letters are significantly different at P< 0.05. Values are means of three replicates (±SE).

Table 4. Effect of administration of different SNP doses (0, 50, 100 and 250 µM) on superoxide anion ($O_2^{\cdot-}$) contents (nmol/h g FW) in *Ginkgo biloba* elicited suspension culture

SNP Treatments (μM)	Superoxide anion determination			
	0 h	12 h	48 h	72 h
Control (0)	0.36±0.02^{c}	1.03±0.04^{c}	1.25±0.05^{b}	1.75± 0.05^{e}
50	0.47±0.02^{c}	1.16±0.03^{b}	1.36±0.04^{ab}	1.99±0.05^{d}
100	0.76±0.02^{b}	1.21±0.03^{b}	1.57±0.02^{ab}	2.31±0.06^{c}
250	0.96±0.03^{ab}	1.25±0.02^{b}	1.80± 0.03^{ab}	2.46±0.04^{b}
500	1.10±0.10^{a}	1.44±0.01^{a}	2.31±0.03^{a}	2.57±0.05^{a}

- Means within the same column followed by different letters are significantly different at P< 0.05. Values are means of three replicates (±SE).

highest accumulation has been observed with SNP (500 µM). All NO treatments significantly increased the total phenolic compounds, tannins, flavonoids and total saponins contents. These data were in accordance with Goodman *et al.* (1967), who found a multifold increase of phenols in inoculated plant cells after challenging with SNP. In the present study the increase of phenols it can be due to the excess production of H_2O_2 in elicited plant cells through increased respiration (Farkas and Kiraly, 1962) or due to the activation of hexose-monophosphate pathway, acetate pathway and release of bound phenols by hydrolytic enzymes. Moreover, callus cultures can be employed and exploited for secondary metabolites production (Jedinák *et al.*, 2004; Radfar *et al.*, 2012). In tissue culture procedures, this used frequently for production of flavonoids. The results also demonstrate that NO signaling is involved in ROS induced secondary products production of *G. biloba* cell suspension culture. In addition to NO, many other signal molecules such as hydrogen peroxide (H_2O_2), jasmonicacid (JA), and salicylic acid (SA) have also been reported to play important roles in secondary metabolite production (Hahlbrock *et al.*, 2003). Whether these signal molecules are involved in NO-induced secondary metabolites production and their relationship and/or interaction with ROS in mediating the NO-stimulated secondary metabolite synthesis still needs further investigations. The role of nitric oxide in the biosynthesis of secondary metabolites in plant cells is not well established yet. Nitric oxide released from its donor sodium nitroprusside (SNP) enhanced the production of terpenoid indole alkaloids of *Catharanthus roseus* cells. Furthermore, one or more protein kinases were demonstrated to act downstream of nitric oxide and transduce the signal to indole alkaloid biosynthesis (Delledonne *et al.*, 2002).

Data in Table 4 show that, all NO treatments increased the $O_2^{\cdot-}$ content in a positive correlation with time duration. NO treatments increased the accumulation of defense related to enzymes such as PAL, APX, catalase and then increased the accumulation of phenols, secondary metabolites and reactive oxygen species (ROS). Data are in agreement with Lu *et al.* (2011), who found that Nitric oxide (NO) has recently emerged as an important signaling molecule in plants. It has been well documented that NO is widely involved in plant adaptive responses to biotic and abiotic stresses. Accumulating evidences indicate that this free radical, which was first identified as a second messenger in animals, plays an important role in stress-induced secondary metabolite accumulation in plants.

Data in Table 5 reveal that, the increase in phenylalanine ammonia lyase (PAL) activity has been triggered under all NO treatments. Furthermore, there was a positive correlation between administrated NO and affected PAL activity. In accordance to Goodman *et al.* (1967), Kobeasy *et al.* (2011), the administration of NO increase the PAL activity which might enhance the phenol content in challenge plant cells. In addition, NO treatments also increased the PAL activity which enhanced the phenolic compound and flavonoid accumulation (Durner and Klessig, 1999). These data indicate that many studies have implicated nitric oxide (NO) as a key regulator for many different physiological processes in plants.

Evaluation of elicitation induces antioxidant defense enzymes activity

Data in Table 5 show that, SOD and APX activities increased after elicitation of different SNP doses. Similar results were obtained by Qiao and Fan (2008) which confirms that, NO can protect oxidative stresses by decreasing the carbonyl group and H_2O_2 contents. The decrease of H_2O_2 concentration was probably produced by the direct NO and H_2O_2 interactions. Also, it is highly possible that the protective effect of NO may result from the increased expression of genes encoding active oxygen scavenging enzymes. Pretreatment of plants with NO also enhanced the antioxidant enzyme activities in concentrations (Mackernessa *et al.* 2001) compared with its corresponded control. In addition, the NO elicitation processes trigger a specific activation of peroxidase activity that interferes with the process of hypersensitive reactions against infection example: lignifications and antibacterial toxic compounds formation during and after the elicitation process. The application with 500 µM of SNP reduced the SOD and APX activities compared with its corresponded control. These data were in agreement with Zhu *et al.* (2009) and Cui *et al.* (2010) confirming that, NO treatment has led to inhibition in POD and SOD activity after fresh-cut of peach and copper toxicity of tomato plants. It has also been reported that NO inhibited the activity of haem-containing enzymes, such as catalase and peroxidases, the important H_2O_2-scavenging enzymes during pathogen attack (Clark *et al.*, 2000).

Regeneration and development

Data in Table 6 demonstrate that MS medium supplemented with NAA and BA with equal concentration (0.5 mg/L) recorded the highest number of regenerated shoots

Table 5. Effect of different SNP administrated doses (0, 50, 100, 250 and 500μM) on the activities (Unit /mg protein^{-1}/h) of phenylalanine ammonia lyase (PAL), ascorbate peroxidase (APX) and superoxide dismutase (SOD) in *Ginkgo biloba* suspension culture

SNP Treatments (μM)	PAL				APX				SOD			
	0	12	48	72	0	12	48	72	0	12	48	72
Control (0)	101.9±5.2^{e}	163.9±4.7^{e}	218.7±6.1^{b}	235.8±6.7^{d}	2.65±0.3^{e}	4.89±0.3^{e}	6.83±0.3^{d}	7.02±0.2^{d}	143.8±4.5^{e}	170.3±4.8^{d}	200.2±5.4^{e}	208.6±6.9^{d}
50	116.6±5.5^{d}	192.2±6.0^{d}	229.5±7.5^{b}	262.6±6.6^{c}	3.72±0.2^{d}	7.03±0.3^{b}	7.43±0.4^{c}	8.43±0.3^{c}	154.1±2.7^{b}	183.7±5.5^{cd}	217.3±6.7^{b}	231.6±5.4^{b}
100	125.5±5.8^{c}	228.3±7.1^{c}	253.0±6.9^{a}	271.6±7.1^{b}	6.09±0.4^{b}	7.04±0.2^{b}	8.43±0.2^{a}	8.88±0.5^{b}	162.1±3.1^{d}	190.5±3.7^{bc}	231.3 ±5.8^{ab}	245.8 ±3.6^{ab}
250	141.0±6.3^{b}	241.1±5.7^{b}	258.2±5.8^{a}	285.3±7.3^{a}	6.78±0.3^{a}	7.51±0.5^{a}	8.32±0.4^{a}	9.43±0.4^{a}	166.0±5.8^{a}	211.6±4.8^{ab}	237.7±6.2^{a}	260.2±5.9^{a}
500	163.0±5.8^{a}	253.6±4.3^{a}	267.8±4.4^{a}	290.7±4.9^{a}	5.87±0.2^{c}	7.00±0.4^{b}	8.00±0.5^{b}	8.84±0.3^{b}	150.3±0.9^{a}	201.9±1.0^{a}	221.8±0.9^{ab}	256.0±1.5^{a}

- Means within the same column followed by different letters are significantly different at P< 0.05. Values are means of three replicates (±SE).

Table 6. Effect of plant growth regulators (NAA and BA) in combination with different concentrations on regeneration response of *Gingko* callus grown for 6 weeks *in vitro*

Treatments (mg/L)		No. of regenerated shoots	Shoot height (cm)	No. of roots	Root Length (cm)
NAA	BA				
0.2	0.2	3.73^{a}	4.73^{a}	1.30^{a}	3.20^{a}
0.5	0.5	4.81^{a}	4.96^{a}	2.25^{a}	4.50^{a}
1.0	1.0	2.21^{b}	3.11^{b}	0.00^{b}	0.00^{b}
2.0	2.0	2.18^{b}	3.12^{b}	0.00^{b}	0.00^{b}

- Means within each column followed by the same letter are not significantly different at P< 0.05.

Table 7. Effect of NAA and IBA on in vitro rooting and their subsequent relation to plantlets survival %, after transplanted ex vitro for 3 months for acclimatization

Treatments (mg/L)		Soil mixture type treatment	Survival % in acclimatization
NAA	BA		
0.2	0.2	Sand + Peat moss + Vermiculite (1: 1: 1, v/v/v)	33.33^{b}
0.5	0.5		40.00^{a}
1.0	1.0		0.00^{c}
2.0	2.0		0.00^{c}

- Means within each column followed by the same letter are not significantly different at P< 0.05.

(4.81 cm) and shoot height (4.96 cm) as well as the number of roots (2.25 cm) and root length (4.5 cm) as compared to the other studied concentration treatments tested. In this concern, *Ginkgo* embryos culture initiated on MS medium with 2, 4-D plus NAA for 5 weeks produced shoots and roots when were transferred to media with 4.5 μM 2, 4-D alone for an additional 5 weeks. Plants were transferred from the 2, 4-D media to pots and maintained in the greenhouse (Camper *et al.* 1997).

High growth regulators (NAA and BA) with equal concentrations and combination (1.0 and 2.0 mg/L) gradually decreased the number of regenerated shoots and shoot height as well as the completely inhibited root formation.

Acclimatization

Data presented in Table 7 show that soil mixture containing sand, peat moss and vermiculite (1: 1: 1, v/v/v) significantly confirmed and reflected survival rate variation which is mainly ascribed to the strong relationship between plantlets and their received *in vitro* auxin concentrations during growth and development on rooting phase before *ex vitro* transfer. In this concern, Al-Salih *et al.* (1986) suggested that, success or failure of transferred plantlets to greenhouse is dependent primarily on the quality and type of materials produced in the previous stages of *in vitro* propagation.

Conclusion

This study shows that the NO treatments in *G. biloba* cell suspension cultures induced significant increase in phenolic compounds and glycosides accompanied with a significant increase in oxidative burst of $O_2^{\cdot-}$ which enhance specific enzymes activities of PAL and antioxidant defense enzymes SOD and APX. In addition, the optimal media for induced *G. biloba* embryo callus and subculture of callus were MS basaic medium supplemented with casein hydrolase (500 mg/L), NAA and BA with equal concentrations (0.5 mg/L).

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References

Abdullah R, Alizah Z, Wee YH, Leaw CL, Yeap CB, Yap WSP, Yeun LH, Rashdan MM, Joseph JL, Sitiazma J, Lee MP, Salwa AS (2005). Immature Embryo: A useful tool for oil palm genetic transformation studies. Electr J Biotechnol 8(1):24-34.

Al-Salih AA, Bader SM, Jarrah AZ, Al-Qadi MT (1986). A comparative morphological and anatomical study of seed and embryo culture-derived seedling of *Phoenix dactylifera* L. Date Palm J 4:153-161.

Balbaa SI (1974). Chemistry of crude drugs - laboratory manual. Al – Shaab Printing House. Cairo, Egypt, 194 p.

Beauchamp C, Fridovich I (1971). Superoxide dismutase: improved assays and assay applicable to acrylamide gels. Anal Biochem 44:276-287.

Beligni MV, Lamattina L (1999). Nitric oxide counteracts cytotoxic processes mediated by reactive oxygen species in plant tissues. Planta 208:337-344.

Beligni MV, Lamattina L (2002). Nitric oxide interferes with plant photo-oxidative stress by detoxifying reactive oxygen species. Plant Cell Environ 25:737-748.

Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Bulgakov VP, Tchernoded GK, Mischenko NP, Khodakovskaya MV, Glazunov VP, Radchenko SV, Zvereva EV, Fedoreyev

SA, Zhuravlev YN (2002). Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the rolB and rolC genes. J Biotechnol 97:213-221.

Camper ND, Coker PS, Wedge DE, Keese RJ (1997). In vitro culture of *Ginkgo*. *In Vitro* Cellular and Developmental Biology Plant 33:125-127.

Chong TM, Abdullah MA, Fadzillah NM, Lai OM, Lajis NH. (2005). Jasmonic acid elicitation of anthraquinones with some associated enzymic and non-enzymic antioxidant responses in *Morinda elliptica*. Enzyme Microb Technol 36:469-477.

Clark D, Durner J, Navarre DA, Klessig DF (2000). Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. Mol Plant Microbe Interact 13(12):1380-1384.

Crawford NM, Guo FQ (2005). New insights into nitric oxide metabolism and regulatory functions. Trends Plant Sci 4:195-200.

Cui XM, Zhang, YK, Wu XB, Liu CS (2010). The investigation of the alleviated effect of copper toxicity by exogenous nitric oxide in tomato plants. Plant Soil Environ 56(6):274-281.

Delledonne M, Zeier J, Marocco A, Lamb C (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. P Natl Acad Sci USA 23:13454-13459.

Delledonne M, Murgia I, Ederle D, Sbicego PF Biondani A, Polverari A, Lamb C (2002). Reactive oxygen intermediates modulate nitric oxide signaling in the plant hypersensitive disease-resistance response. Plant Physiol Biochem 40:605-610.

Del-Rio, LA, Corpas FJ, Sandalio LM, Palma JM, Barroso JB (2003). Plant peroxisomes, reactive oxygen metabolism and nitric oxide. IUBMB Life 2:71-81.

Dickerson DP, Pascholati SF, Hagerman AE, Butler LG, Nicholson RL (1984). Phenylalanine ammonia-lyase and hydroxyl cinnamate: CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. Physiol. Plant Pathol 25:111-123.

Durner J, Klessig DF (1999). Nitric oxide as a signal in plants. Current Opinion in Plant Biology 2(5):369-374.

Durner J, Wendehenne D, Klessig DF (1998). Defence gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. P Natl Acad Sci USA 95:10328-10333.

Ebrahimzadeh H, Niknam V (1998). A revised spectrophotometric method for determination of triterpenoid saponinis. Indian Drugs 35:379-381.

El-Beltagi HS, Ahmed OK, El-Desouky W (2011). Effect of low doses γ-irradiation on oxidative stress and secondary metabolites production of rosemary *(Rosmarinus officinalis* L.) callus culture. Radiat Phys Chem 80:968-976.

Evans DE, Coleman JOD, Kearns A (2003). Plant Cell Culture. BIOS Scientific Publ. Taylor and Francis, London.

Elstner EF, Heupel A (1976). Inhibition of nitrite formation from hydroxylaminonium- chloride: a simple assay for superoxide dismutase. Anal Biochem 70:616-620.

Farkas GL, Kiraly Z (1962). Role of phenolic compounds in the physiology of plant disease resistance. Phytopathol Z 44:105-150.

Goodman RN, Kiraly Z, Zaitlin M (1967). The Biochemistry and Physiology of Infectious Plant Disease. Van Nostrand Co Inc. Princeton, New Jersey 354 pp.

Hahlbrock K, Bednarek P, Ciolkowski I, Hamberger B, Heise A, Liedgens H, Logemann E, Nürnberger T, Schmelzer E, Somssich IE, Tan J (2003). Non-self-recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. P Natl Acad Sci USA 100:14569-14576.

Jedinák A, Faragó J, Pšenáková I, Maliar T (2004). Approaches to flavonoid production in plant tissue cultures. Biologia Bratislava 59:697-710.

Kobeasy MI, El-Beltagi HS, El-Shazly MA, Khattab EAH (2011). Induction of resistance in *Arachis hypogaea* L. against *Peanut Mottle Virus* by nitric oxide and salicylic acid. Physiol Mol Plant Pathol 76:112-118.

Kuo IF, Jie C, Thomas KH (2004). Chang Effect of *Ginkgo biloba* extract on rat hepatic microsomal CYP1A activity: role of ginkgolides, bilobalide, and flavonols. Can J Physiol Pharmacol 82:57-64.

Lamattina L, Garcia-Mata C, Graziano M, Pagnussat G (2003). Nitric oxide: the versatility of an extensive signal molecule. Ann Rev Plant Physiol 54:109-136.

Lu D, Dong J, Jin H, Sun L, Xu X, Zhou T, Zhu Y, Xu M (2011). Nitrate reductase-mediated nitric oxide generation is essential for fungal elicitor induced camptothec in accumulation of *Camptotheca acuminata* suspension cell cultures. Appl Microbiol Biotechnol 90:1073-1081.

Mackernessa SAH, John CF, Jordan B, Tomas B (2001). Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide. FEBS Lett 489:237-242.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497.

Nakano Y, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867-880.

Pottino BG (1981). Methods in Plant Tissue Culture. Dept Hort Agric. Maryland Univ, College Park, Maryland, USA p. 8-29.

Qiao W, Fan L (2008). Nitric oxide signaling in plant responses to abiotic stresses. J Integr Plant Biol 50(10):1238-1246.

Radfar M, Sudarshana MS, Niranjan MH (2012). Betalains from stem callus cultures of *Zaleya decandra* L. N. Burm. f. - A medicinal herb. Med Plants Res 6(12):2443-2447.

Shi SY, Wang G, Wang YD, Zhang LG, Zhang LX (2005). Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation. Nitric Oxide 13:1-9.

Stamler JS, Singel DJ, Loscalzo J (1992). Biochemistry of nitric

oxide and its redox-activated forms. Science 258:1898-1902.

Swain T, Hillis WE (1959). The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. J Sci Food Agric 10(1):63-68.

Tamir S, Lewis RS, De-Rojas WT, Deen WM, Wishnok JS, Tannenbaum SR (1993). The influence of delivery rate on the chemistry and biological effects of nitric oxide. Chem Res Toxicol 6:895-899.

Tanveer H, Ali S, Asi MR (2012). Appraisal of an important flavonoid, quercetin in callus cultures of *Citrullus colocynthis*. Int J Agric Biol 14:528-532.

Tu J, Shen WB, Xu LL (2003). Regulation of nitric acid on the aging process of wheat leaves. Acta Bot Sin 45:1055-1062.

Vlasova II, Tyurin VA, Kapralov AA, Kurnikov IV, Osipov AN, Potapovich MV, Stoyanovsky DA, Kagan VE (2006). Nitric Oxide Inhibits Peroxidase Activity of Cytochrome c - Cardiolipin Complex and Blocks Cardiolipin Oxidation. Journal of Biological Chemistry 281(21):14554-14562.

Wang CY, He WT, Hou SW (2006). Callus induction and high-frequency plant regeneration from hypocotyl and cotyledon explants of *Arctium lappa*. In Vitro Cellular and Developmental Biology - Plant 39(5):468-474.

Xu MJ, Dong JF, Zhu MY (2005). Nitric oxide mediates the fungal elicitor-induced hypericin production of *Hypericum perforatum* cell suspension cultures through a jasmonic-acid-dependent signal pathway. Plant Physiology 139(2):991-998.

Xu MJ, Dong J, Wang H, Huang L (2009). Complementary action of jasmonic acid on salicylic acid in mediating fungal elicitor-induced flavonol glycoside accumulation of *Ginkgo biloba* cells. Plant Cell Environ 32:960-967.

Zhu LQ, Zhou J, Zhu SH, Guo LH (2009). Inhibition of browning on the surface of peach slices by short-term exposure to nitric oxide and ascorbic acid. Food Chem 114:174-179.

Zhuang XP, Lu YY, Yang GS (1992). Extraction and determination of flavonoids in ginkgo. Chin Herbal Med 23:122-124.