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Direct and Indirect Micropropagation of *Paulownia tomentosa* and Genetic Stability of Produced Plantlets

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Abstract: Establish a micropropagation protocol of *Paulownia tomentosa* in Egypt and estimate the genetic similarity between the regenerated plants and the mother plant is the aim of this investigation. Direct multiplication and growth parameters of *P. tomentosa* were significantly affected by MS strength, concentrations of either BA or Kn as well as cobalt forms (Co^{2+} ions or CoNPS) and concentrations. The highest shoots number was obtained from 3/4 MS + 2.0 mg/l BA in presence of low concentrations of Co^{2+} or CoNPS, which enhanced growth parameters and delayed the physiological disorders. The indirect micropropagation was implemented through callus induction and differentiation. Two explants (leaf and inter node) in combined with NAA concentrations were examined. Inter nodes combined with 0.3 mg/l NAA gave the highest callus induction percent (100% CIP) as well as the highest callus differentiation (60% differentiation). The highest roots number, length, plantlet fresh weight and the highest number of success of acclimatization were observed from 3/4 MS medium supplemented with 6.0 mg/l IAA. Genetic similarity percent between mother plant and micropropagated plants based on combination between RAPD and ISSR was 75.45%. Results proved the efficiency of RAPD and ISSR to discriminate the genetic characterization and distinguished the genetic similarity between the examined samples.

Key words: *Paulownia* · Direct micropropagation · Indirect micropropagation · cytokinin · MS strength · Co^{2+} · CoNPS · RAPD · ISSR

INTRODUCTION

Paulownia tree is an important economically genus belongs to the family Scrophulariaceae (Paulowniaceae) [1]. It is a multi-purpose species, bioenergy, fast-growing tree, producing a high superior timber and high adaptable [2, 3]. The trees reach to marketable size in 5-8 years [4]. *Paulownia tomentosa* is native from China. *Paulownia* is naturalized in United States of America, Japan, Brazil, Australia and Europe [5, 6]. *Paulownia* has a multiple wood uses, because of its important properties like resistance to cracking, bending, moisture damage and rotting [7, 8]. *Also, Paulownia* can be used as energy production, paper pulp and building materials [9]. Paulownia is a low demand water plant because of deep soil growing of its root system [2]. The tree has high ability to uptake nitrates heavy metals which considered land contamination, so it is an eco-friendly [10, 11]. The valuable advantage of *Paulownia* is a desired plant because it can extend from warm to tropical climates and tolerant of poor soil conditions [12].

Paulownia can be propagated through seeds, stem and root explants, but the using seeds is un reliable because of slow rate and poor germination which takes a long time for development when comparison to plants growing from stem or root explants, beside the disease and pest problem [13]. So, vegetative propagation is favor for *Paulownia*, micropropagation, as a vegetative propagation, offers a rapid mean of propagation, with maintain the genetic gain [14, 15]. Direct and indirect micropropagation may be utilized in Paulownia. Several explants; shoot tip, leaf, petiole, node and root were implemented in *Paulownia* micropropagation. Nodes explants considered an excellent explant source to direct organogenesis induction. Among two types of explants, leaf segment produced the highest number of shoots per

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explant (12 ± 0.4) when they were cultured on MS supplemented with 3.0 mg/l Kn and 0.5 mg/l NAA [16]. Murashige and Skoog medium [17] (MS) and woody plant medium (WPM) Lloyd and McCown [18] widely used for *Paulownia* micropropagation with valuable success. Benzyl amino purine (BA); among various tested growth regulators, was the most effective in *Paulownia* proliferation [16, 19, 20]. Kinetin, reduced shoot induction percent, shoots number and shoot length [3]. On other report, BA was significantly induced shoots proliferation of *Paulownia tomentosa*, the recommended BA concentration was 2.0 mg/l, while, kinetin significantly improved growth and decreased the necrosis parameters when compared with other cytokinins [21-23]. While other research stated that the best *in vitro* proliferation was obtained from MS medium substituted with 1 mg/l BA [24, 25]. Contrast results reported that MS basal salt composition increased multiplication coefficient of *Paulownia* [26].

Also, MS salt strength affected micropropagation and growth parameters of *Paulownia*, half MS strength significantly induced shoot length, greening and number of roots parameters compared with other medium strengths. While, medium at full strength enhanced the shoot necrosis comparing with the other MS strength [23]. The half MS strength supplemented with 2.0 mg/l NAA augmented the root formation within 12 - 15 days [27]. MS medium enriched with 0.1 to 1.0 mg/l IBA resulted in 96 to 100% rooting. Rooted plantlets were successfully acclimatized [21, 25, 26]. Auxin in high concentration promoted root induction 100% in *Paulownia tomentosa* within 4 weeks. Roots average length was 4.9 cm [28]. On the other hand, Ozaslan *et al.* [19] conducted that rooting was *in vitro* enhanced on hormone free media. Successful acclimatization was achieved when the mixture of cultivation soil was 1:1 sand and compost [3]. During acclimatization, *Paulownia elongate* negatively affected because of sudden changes in temperature and humidity. So, in their acclimatization, it is recommended to use glass jars for plants covering for the first 5 days to avoid the death of plantlets [15].

Cobalt is a trace element in the most media of plant tissue culture [29]. The low concentrations of cobalt (7-10 mM) showed varies effects according to the species, [30]. An inhibitory effect on plantlets growth and production can occur [31, 32]. Outgrowth was almost normal on a threshold metal concentration and no specific cytological variation was observed. Slightly increasing in metal concentration led to growth completely inhibited [33], this inhibition may occur as a result of oxidative stress which proved by presence of oxidative stress markers; eg: malondialdehyde (MDA) [34]. Promoting reactive oxygen species (ROS) formation, associated with stress conditions, leads to decrease the biomass accumulation which caused the damaging role of ROS on biomolecules (eg: nucleic acids, proteins and lipids). These damages lead to inhibition or delay of cell proliferation and synthesis of protein, decreased the enzymatic activities, alteration in cell membrane characteristics, finally the scenario end with cell death [35]. Positive correlations were determined between both cobalt Forms (Co^{2+} and CoNPs) and both gene expression and the activities of antioxidant enzymes (SOD and APX). Results revealed the higher capability of Co^{2+}, compared with CoNPs, in enhancing metabolites accumulation [36].

Genetic stability (true-to-type or clonal fidelity) is one of the most important pre-requisites in the crop species micropropagation. The genetic defects which appear as a result of somaclonal variation in the proliferated shoots can limit the unlimited utility of the micropropagation technique [37]. Molecular markers are an effective method for genetic characterization of the representatives of different genotypes of the genus *Paulownia*. RAPD and ISSR are useful in determine the genetic stability of *in vitro*-propagated plantlets in many crop species [38, 39]. RAPD and ISSR markers are very simple, fast, low- cost, effective, highly discriminative and reliable [40]. RAPD and ISSR are able to discriminate the genetic differences among the closely related genotypes at the DNA level and can identify the polymorphism between repeated sequences [41, 42].

The true to type nature of the micropropagated clones was confirmed using DNA-based markers. No variants were detected among the produced plantlets. Based on RAPD and ISSR analysis, all DNA fragments profiles of micropropagated plants were monomorphic and similar to the mother plant [41, 43-47].

This investigation aims to establish a micropropagation protocol of *Paulownia tomentosa* in Egypt and estimate the genetic characterization of the regenerated plants compared with mother plant.

MATERIALS AND METHODS

Plant Materials: Shoot tips were collected from one-year old tree cultivated in South El-Tahrir Horticultural Reearch Station, Agriculture Research Center, Ministry of Agriculture and Reclamation Land, in early Spring.

Culture Medium: Murashige and Skoog [17] nutrient culture medium (MS) supplemented with 30 g/l sucrose, 2 g/l gelrite was implemented in the investigation. The pH of the MS medium was adjusted at 5.7 and distributed in 350 ml culture glass jars; where each jar contained 50 ml culture medium, before the autoclaving under temperature 121°C and pressure 15.2 Kg/cm^2 for 20 min.

Sterilization of Plant Materials: Shoot tips were washed with detergent under tap water, then sterilized using 1% Sodium hypochlorite (NaOCl) for 7 min. Shoot tips were then washed twice with sterilized distilled water and cultivated on basal Murashige and Skoog [17] nutrient culture medium (MS). The vital and free contaminated shoots were used as explants in the investigations.

Culture Incubation Conditions: Cultures were incubated at 26 ± 2 °C, photoperiod 16/8 dark/light and light intensity 2000 lux provide through white fluorescent tubes.

Cobalt Nanoparticles (CoNPs) Preparation and Characterization: Cobalt nanoparticles (CoNPs) were obtained from Nanotech, Egypt. CoNPs are roughly spherical about average size 20-50 nm. For preparing Nano-stock, 100 mg of CoNPs were dissolved in 100 ml deionized water by sonicator at 100 W and 30 kHz for about 45 min.

Direct Shoot Multiplication: Effect of MS strength and different concentrations of either Benzyl adenine (BA) or Kinetin (Kn) on direct shoot multiplication and growth parameters of *P. tomentosa*.

MS at full, 3/4 or 1/2 salt strength combined with different concentration (0.0, 1.0, 2.0 and 3.0 mg/l) of either BA or Kn were examined. *In vitro* shoot tips were used as explants, two shoot tips for each jar and five jars for each treatment. After three weeks shoots number/shoot tip, shoot length (cm), nodes number and growth vigor were recorded as described by Pottino [48], where: 1 is slight growth vigor, 2 is moderate growth vigor, 3 is good growth vigor, 4 is very good and 5 is excellent growth vigor.

Effect of cobalt ions (Co^{2+}) and cobalt nanoparticles (CoNPs) on shoot multiplication and growth parameters MS medium (3/4 salt strength) supplemented with 30 g/l sucrose, 2 g/l gelrite and various concentrations {control(0.0), T1 (2.5mg/l), T2 (5.0 mg/l), T3 (7.5 mg/l) and T4 (10.0 mg/l)} of either Co^{2+} or CoNPs was examined. The media were distributed into 350 ml glass jars; where each jar contained 50 ml nutrient medium and autoclaved. Five jars for each treatment and four shoot tips were cultivated in each jar and the cultivation were followed up for two subcultures on the same medium. After six weeks the number of shoots, the shoot length (cm), the number of nodes/shoot as well as the stress injury as affected by treatments were recorded.

In Direct Multiplication of Shoots

Callus Induction and Differentiation: *In vitro* leaves and inter nodes were cultured on MS supplemented with different Naphthalene acetic acid (NAA) concentrations (0.0, 0.1, 0.2 and 0.3 mg/l) to induced callus. After six weeks, the response of the two explants combined with NAA concentrations were expressed as number of explants which induced to form callus (CIN), the percent of explant which formed callus (CIP). After eight weeks, differentiated shoots or roots on the same medium were recorded as differentiated percent of the induced callus.

Root Formation and Acclimatization: The generated shoots were transferred to MS medium contained different concentrations of Indole acetic acid (IAA) (0.0, 1.0, 2.0, 3.0 mg/l). After four weeks, roots number/shoot, root length (cm), plantlet height and plantlet fresh weight were recorded. The plantlets were removed from the rooting media, washed to remove the medium and soaked in Rezolex (2 g/l) for 5 min to avoid fungal infection and transplanted into 5 cm pots contained peat moss, sand and perlite at equal volume as culture medium. To provide a suitable humidity around the plantlets, they were covered with transparent polyethylene bags which gradually removed after one month. The acclimatization success was recorded as number of acclimatized plantlets and the acclimatization success percent was calculated.

Statistical Analysis: This investigation was designed in complete randomized design, including one or two factorials. Each treatment contained five replicates. Data were analyzed by using MSTAT software ver. 2.2. Differences among observed data were compared using the least significant difference (LSD) at 5% level according to Steel *et al*. [49].

Comparison Between Genetic Characterization of *Paulownia tomentosa* Mother Plant and the *In vitro* Propagated Plantlets: Genetic characterization of mother plants and *in vitro* propagated plantlets were distinguished based on PCR based techniques, i.e., random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR).

Preparation of Plant Samples: Meristem tissues were collected and well-grounded using liquid Nitrogen. About 200 mg of the fin powder samples were used for DNA extraction using the Mini Kit of i-genomic Plant, iNtRON Biotechnology Co. the concentrations of isolated DNAs were 50 ng/°l (as described by protocol). DNAs quality were illustrated through electrophoresis separation (5 V/cm) in 1% agarose gel.

RAPD and ISSR Techniques: Eleven RAPD primers and Thirteen ISSR presented in Table 1 were obtained from Bio Basic Inc. used in PCR reaction, which contained DNA template (100 ng) of, master mix solution (12.5°l) (i- TaqTM, iNtRON Biotechnology), ISSR primer for (2 °l) and PCR buffer with 1.5mM of $MgCl_2$ (4 °l) to reach 25°l as a final volume. PCR program conditions were implemented as described by Hamza [47].

Electrophoresis of DNA: Charge dependent separation (5V/cm) were implemented for the PCR products in 1.5% agarose gel for both RAPD and ISSR primers. Ethidium bromide was used to stain the DNA fragments as described by Sambrook and Russel [50]. One-Kb plus blue DNA Ladder, Gene One. Co was used to illustrate the DNA fragments molecular weights. The UV transilluminator was used for agarose gel photo-record.

DNA Electrophoresis Analysis: The DNA amplified fragment was recorded as present (1) or absent (0) fragment for each RAPD or ISSR primer. Data were analyzed according to Rohlf [51]. The genetic relationship was determined by unweighted pair group's method arithmetic (UPGMA) with Jaccard similar coefficient.

RESULTS

Direct Shoot Multiplication: There are multiple factors affected the direct shoot proliferation. The medium strength and composition; especially cytokinin type and concentrations, are limited factor to reach to a sufficient micropropagation protocol. In this investigation, these factors were examined beside studying the effect of adding various concentrations of the cobalt in two different forms; cobalt ions (Co2+) and cobalt nanoparticles (CoNPS).

Effect of MS Strength and BA Concentrations: Number of *Paulownia tomentosa* shoots proliferation was not significantly affected by MS strength, full, 3/4 and 1/2 MS strength produced 2.25, 2.08 and 2.25 shoots/shoot tip (Table 1). While BA concentrations positively affected the number of proliferated shoots, the maximum number of shoots (2.89 shoots/shoot tip) was obtained from 3.0 mg/l BA. The interaction between MS strength and BA concentrations revealed that BA concentrations at both 2.0 and 3.0 mg/l in combination with all MS strength (full, 3/4 and 1/2 MS) significantly induced shoot proliferation number which ranged from 2.67 to 3.00 shoots/shoot tip. On contrast, MS strength negatively related with shoot length (Fig. 1), while BA concentrations significantly enhanced shoot length (5.08 and 5.56 cm) at 1.0 and 2.0 mg/l, respectively. The analysis of interaction among treatments proved that 3/4 MS +1.0 or 0.2 mg/l BA maximized the shoot length (7.00 and 7.33 cm, respectively). Slight effect of MS strength was observed on number of nodes. While, BA at 1.0 mg/l significantly augmented nodes number (3.78 nodes/shoot). Interaction among MS strength and BA concentrations showed superiority effect of 3/4 MS + 1.0 mg/l BA on nodes number (4.64 nodes/shoot). Growth vigor showed variation as affected by MS strength, but the variation did not significant. Presence of all BA concentrations positively increased growth vigor when compared with control. Inter action between MS strength and BA concentrations highly affected growth vigor, the maximum growth vigor was obtained from 3/4 MS supplemented with 1.0 or 2.0 mg/l BA.

Effect of MS Strength and Kn Concentrations: Shoot number of *Paulownia tomentosa* significantly affected by MS strength, the highest shoots number (1.67 shoot/shoot tip) was obtained from 3/4 MS salt strength (Table 2). The high concentration of Kn increased the number of shoots proliferation. Interaction among MS strength and Kn concentrations showed that 3/4 MS with presence of 1.0, 2.0 or 3.0 mg/l Kn significantly induced the shoots proliferation (2.00, 2.00 and 1.67 shoots/shoot tip), also, full and 1/2 MS + 3.0 mg/l Kn increased shoots proliferation number (2.00 shoots/ shoot tip). MS strength did not affect shoot length of *P. Tomentosa*. There was a positive relationship between Kn concentrations and shoot length, 2.0 mg/l Kn possessed the highest shoot length (8.67 cm) (Fig. 2). Interaction between MS strength and Kn concentrations revealed that 3/4 MS strength combined with 2.0 or 3.0 mg/l Kn gave the highest shoot length (10.00 cm for each) followed by full MS + 2.0 mg/l Kn and 1/2 MS without Kn (8.83 and 8.27 cm, respectively) with no significant difference between these combinations. The nodes number showed not significant difference as affected by MS strength, while, Kn concentration at 2.0 mg/l significantly promoted nodes number followed by

Table 1: Response of *Paulownia tomentosa* shoot proliferation and growth parameters as affected by MS strength and BA concentrations

	MS strength (A)				MS strength (A)				MS strength (A)				MS strength (A)			
	Full	3/4	1/2		Full	3/4	1/2		Full	3/4	1/2l		Full	3/4	1/2	
BA conc. (mg/l)(B)	Shoot No/shoot tip			Mean (B)	Shoot length (cm)			Mean (B)	Nodes number/shoot			Mean (B)	Growth vigor			Mean (B)
0.0	1.00	1.00	1.00	1.00	2.67	3.00	3.27	4.64	3.00	2.33	3.00	2.78	2.83	2.33	2.89	2.45
1.0	2.33	1.67	2.33	2.11	4.57	7.00	3.67	5.08	3.67	4.00	3.67	3.78	2.83	4.67	3.67	3.72
2.0	2.67	3.00	2.67	2.78	4.67	7.33	4.67	5.56	3.67	3.00	3.67	3.44	3.67	4.17	3.83	3.86
3.0	3.00	2.67	3.00	2.89	4.33	5.67	4.17	4.72	3.00	2.67	3.00	2.89	3.33	3.33	3.83	3.45
Mean (A)	2.25	2.08	2.25	----	4.06	5.75	5.19	----	3.33	3.00	3.33	----	3.07	3.40	3.55	----
LSD at5%	A: NS B: 0.99 AxB; 1.72			----	A: 0.68 B: 0.79 AxB:1.36			----	A: 0.32 B: 0.37 AxB: 0.64			----	A: NS B: 0.54 AxB: 0.94			----

Table 2: Response of *Paulownia tomentosa* shoot proliferation and growth parameters as affected by MS strength and Kn concentrations

	MS strength (A)				MS strength (A)				MS strength (A)				MS strength (A)			
	Full	3/4	1/2		Full	3/4	1/2		Full	3/4	1/2		Full	3/4	1/2	
Kn conc. (mg/l) (B)	Shoots NO/shoot tip			Mean (B)	Shoot length (cm)			Mean (B)	Nodes number/shoot			Mean (B)	Growth vigor			Mean (B)
0.0	1.00	1.00	1.00	1.00	3.00	3.00	8.27	4.76	2.83	3.00	3.00	2.94	3.00	2.33	3.17	2.83
1.0	1.00	2.00	1.00	1.33	5.83	7.67	5.83	6.44	3.00	4.67	3.00	3.22	3.00	3.33	2.67	3.00
2.0	1.00	2.00	1.00	1.33	8.83	10.00	7.17	8.67	3.67	4.67	3.67	4.00	3.67	5.00	3.67	4.11
3.0	2.00	1.67	2.00	1.89	7.67	10.00	7.17	8.28	2.33	3.00	3.00	3.78	3.67	4.67	3.00	3.78
Mean (A)	1.25	1.67	1.25	----	6.33	7.67	7.10	----	3.29	3.84	3.17	----	3.33	3.83	3.13	----
LSD at 5%	A: 0.34 B: 0.40 AxB: 0.69			----	A: NS B: 1.64 AxB: 2.83			----	A: NS B: 0.66 AxB: 1.15			----	A: 0.47 B: 0.54 AxB: 0.93			----

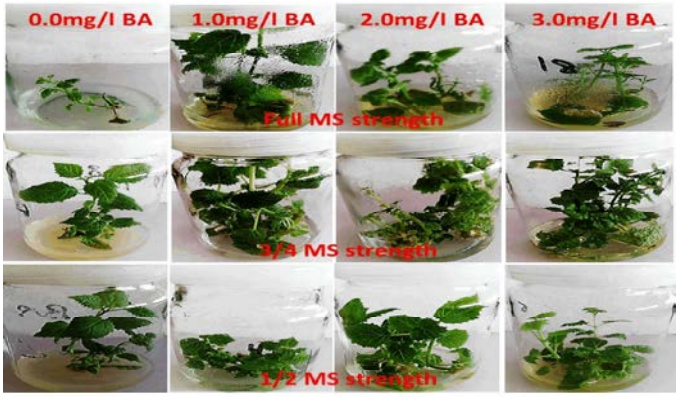

Fig. 1: Response of *Paulownia tomentosa* shoot proliferation and growth parameters as affected by MS strength and BA concentrations

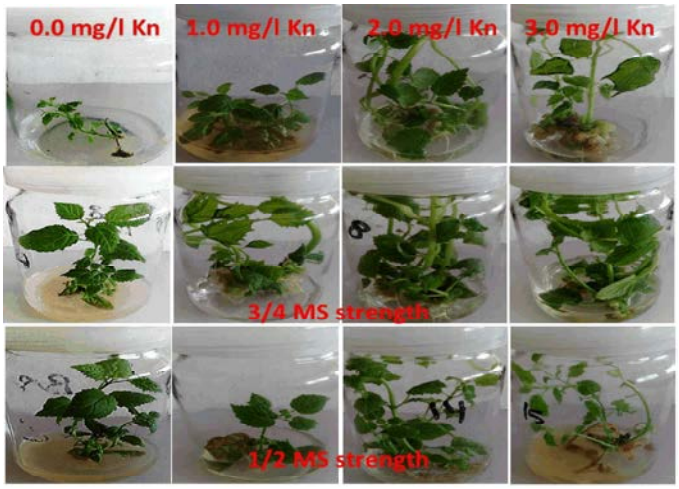

Fig. 2: Response of *Paulownia tomentosa* shoot proliferation and growth parameters as affected by MS strength and Kn concentrations

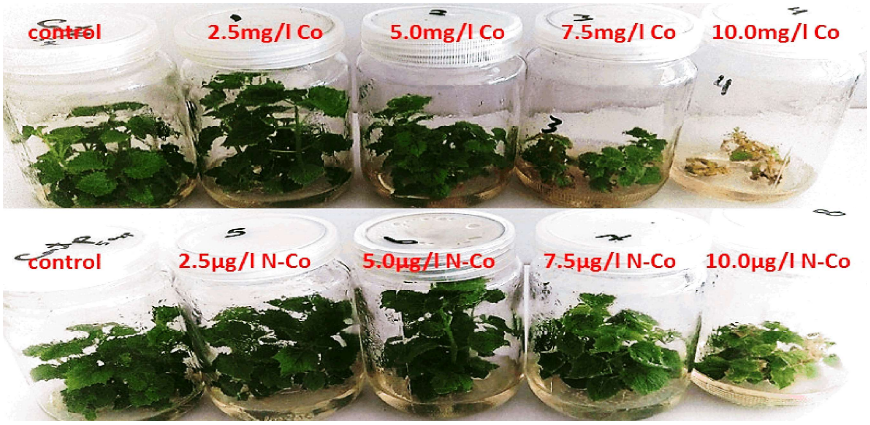

Fig. 3: Response of Paulownia tomentosa to various concentrations of cobalt ions (Co2+) and cobalt nanoparticles (CoNPs)

3.0 and 2.0 mg/l Kn (4.00, 3.78 and 3.22, respectively). Inter action showed that 3/4 MS + 1.0 or 2.0 mg/l Kn gave the highest nodes number (4.67 cm for each). Growth vigor of the *in vitro* shoots were significantly increased with 3/4 MS. Kn concentrations enhanced growth vigor, 2.0 mg/l Kn maximize the growth vigor (4.11). Interaction between M strength and Kn concentrations indicated that 3/4 M +2.0 or 3.0 mg/l Kn significantly augmented shoot growth vigor of *P. tomentosa* (5.00 and 4.67, respectively).

Effect of Cobalt Ions (Co^{2+}) and Cobalt Nanoparticles (CoNPs): Cobalt ions (Co^{2+}) and cobalt nanoparticles (CoNPs) affected the multiplication and growth parameters of *P. tomentosa*. Shoot number recorded 3.33 shoot/shoot tip as a response of control treatment while shoot number increased to 5.2 shoot/shoot tip when shoot tips were treated with either Co^{2+} or CoNPs with n significant differences between the two treatments. Concentrations of Co^{2+} and CoNPs significantly affected shoot number, T2 possessed the highest shoot number (7.00 shoots/hoot tip). The interaction between the metal form and the concentrations revealed that both metal forms in all concentrations were superior when compared with control. The highest shoot number was observed from Co^{2+} at T2 and T3 concentrations (8.00 and 6.00 shoots/shoot tip, respectively) as well as CoNPs at T2 (6 shoots/shoot tip). Shoot length significantly affected by the metal form, CoNPs showed taller shoots than Co^{2+} ((7.9 and 5.6 cm, respectively). Concentrations of both metal forms affected shoot length. The interaction between the metal form and their concentrations revealed a significant increasing, CoNPs at T1, T2 and T3 highly promoted shoot length (9.7, 10.0 and 8.0 cm, respectively). Metal form affected number of nodes, but the effect was not significant. Also, concentration of the metal significantly affected nodes number, T1, T2 and T3 possessed the highest nodes number (5.3, 5.5 and 4.7 nodes/shoot, respectively). Anyway, both Co^{2+} and CoNPs at T1 and T2 enhanced nodes number with no significant difference between them. Also, the form of cobalt affected the degree of plant injury, Co^{2+} showed plant injury more than CoNPs. Anyway, the injury effects appeared in the high concentrations of both cobalt forms but reach to plant death in the case of Co^{2+} at T4 but the same concentration of the CoNPs form led to burn the edges of leaves.

In Direct Multiplication

Callus Induction and Differentiation: The indirect micropropagation means organogenesis or embryogenesis proliferation through callus induction, where callus may differentiate to organ (shoot or root) or embryo. Callus induction was expressed as number of explants which response to induce callus. Type of explants significantly affected the callus induction number, inter nodes showed high number of callus induction when compared with leaf explant (8.5 and 7.3 induced explant, respectively). Also, concentrations of NAA positively affected callus induction number (CIN). NAA at concentration 0.2 and 0.3 possessed the highest callus induction number (10.0 explant for each (100% callus induction). The interaction between the explant type and the NAA concentration revealed that all examined explants at 0.2 or 0.3 mg/l NAA induced callus (10.0 I nduced explants for each). The percent of the explant which formed callus expressed as callus induction percent (CIP). Explant type significantly affected CIP, internodes showed the highest callus

Table 3: Response of Paulownia tomentosa to various concentrations of cobalt ions (Co^{2+}) and cobalt nanoparticles (CoNPs)

	Cobalt form			Cobalt form			Cobalt form		
	Co^{2+}	CoNPs		Co^{2+}	CoNPs		Co^{2+}	CoNPs	
Concentrations(mg/l) (B)	Shoot number/shoot		Mean(B)	Shoot length (cm)		Mean (B)	Nodes number/ shoot		Mean (B)
Control	3.33	3.33	3.33	6.2	6.2	6.2	3.0	3.0	3.0
T1	4.67	5.67	5.17	5.5	9.7	7.6	5.0	5.7	5.3
T2	8.00	6.00	7.00	6.7	10.0	8.3	6.0	5.0	5.5
T3	6.00	5.33	5.67	5.7	8.0	6.8	5.0	4.3	4.7
T4	4.00	5.67	4.83	4.0	5.7	4.8	3.0	2.3	2.7
Mean (A)	5.2	5.2		5.6	7.9		4.4	4.1	
LSD	NS	1.26	1.79	0.9	1.4	2.0	NS	1.0	1.3

Table 4: Callus induction and differentiation as affected by type of explant and NAA concentrations

	Explant type (B)			Explant type (B)			Explant type (B)		
	Leaf	Inter node		Leaf	Inter node		Leaf	Inter node	
NAA Con. (mg/l) (A)	Callus induction number		Mean (A)	Callus induction percent		Mean (A)	Differentiation %		Mean (A)
0.0	5	6	5.5	20	30	25.0	10	15	12.5
0.1	7	8	7.5	40	50	45.0	20	30	25.0
0.2	10	10	10.0	50	70	60.0	23	50	36.5
0.3	10	10	10.0	55	100	77.5	25	60	42.5
Mean (B)	7.3	8.5	---	41.5	62.5	---	19.5	38.3	---
LSD at 5%	A: 1.5 B: 0.9 AxB: 2.0			A: 5.3 B: 6.2 AxB: 8.2			A: 5.7 B: 5.3 AxB: 7.2		

CIN means the callus induction number of the total initial explants

CIP means callus induction percent of the explant

Table 5: Root formation and acclimatization of Paulownia tomentosa as affected by IAA concentrations

IAA conc. (mg/l)	Root NO/plantlet	Root length (cm)	Plantlet height (cm)	Fresh weight (g)	No. of plantlets success in acclimatization
0.0	8.33	3.69	4.67	0.97	6.33
2.0	11.33	5.43	5.57	1.77	7.33
4.0	13.67	7.37	9.17	2.93	8.33
6.0	14.67	6.80	8.27	3.17	8.67
LSD at 5%	2.03	0.38	1.33	0.74	1.29

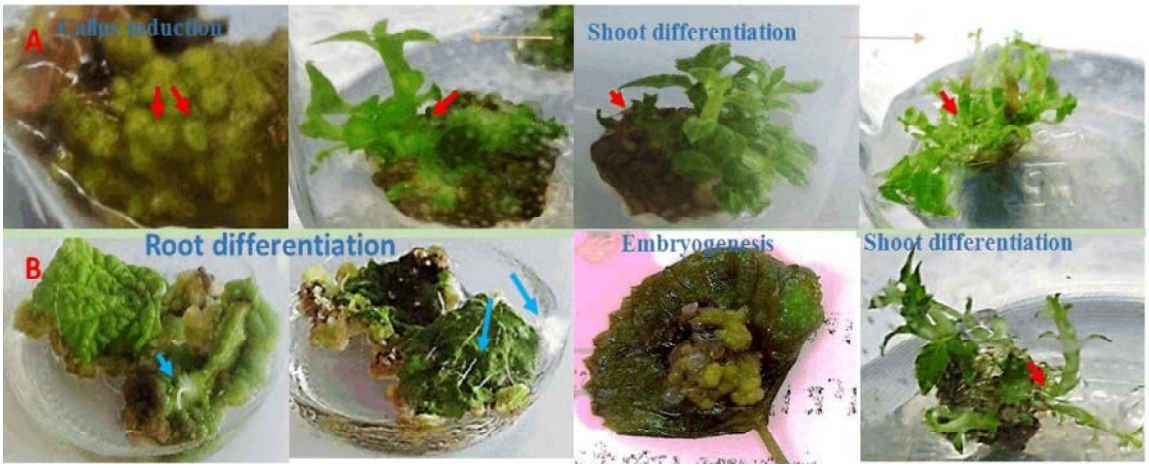

Fig. 4: Callus induction and differentiation as affected by types of explant and NAA concentrations, A: callus induction and differentiation which initiated from inter node explants, B: callus induction and differentiation initiated from leaves explant

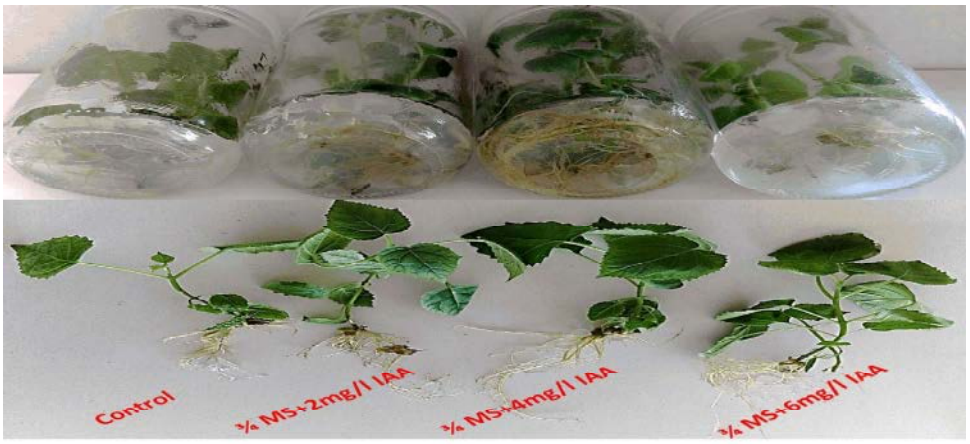

Fig. 5: Root formation of Paulownia tomentosa as affected by IAA concentrations

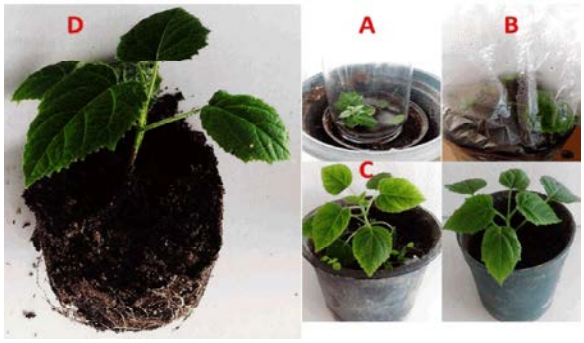

Fig. 6: Acclimatization of Paulownia tomentosa. A: At the beginning of acclimatization, the plantlets covered with glass jar to provide a suitable relative humidity and decrease the plantlets shock. B: After five days of acclimatization, the glass cover replaced with polyethylene bags. C and D: The acclimatized plantlets after acclimatization

induction percentage (62.5%). CIP was enhanced by NAA concentration, the highest NAA concentration (0.3 mg/l) significantly induced callus induction percentage (77.5%). The interaction between explant type and NAA concentration revealed that inter nodes combined with 0.3 mg/l NAA gave the highest CIP (100%). There was a relationship between explant type and callus differentiation percent, internodes resulted in high differentiation (38.3%). Also, differentiation percentage increased with increasing NAA concentration. The interaction between treatments showed that internodes with 0.3 or 0.2 g/l NAA gave the highest differentiation.

Root Formation and Acclimatization: Proliferated shoots about 6 to 8 cm in length were transplanted in to rooting 3/4 MS medium contained different concentrations of IAA. The concentrations of IAA significantly positive promoted formation of roots, the highest roots number was observed from 3/4 MS medium supplemented with 6.0 mg/l IAA followed by MS supplemented with 4.0 mg/l IAA (14.67 and 13.67 root/shoot, respectively, with no significant differences between the two concentrations. Also, root length was affected with IAA concentrations, the tallest root length (7.37 cm) was obtained from 4.0mg/l IAA. Also, the high concentrations of IAA (4.0 and 6.0 mg/l) resulted in the highest fresh weight (2.93 and 3.17 g, respectively) and the highest number of success of acclimatization (8.33 and 8.67), with no significant differences between the two concentration (Fig. 6).

Comparison Between Genetic Characterization of *Paulownia tomentosa* Mother Plant and the *In vitro* Propagated Plantlets:

Based on RAPD Technique: Eleven RAPD primers was implemented to define the genetic characterization of *Paulownia tomentosa* mother plants (MP) and micropropagated ones (Pr) (Table 6 and Fig. 7). The number of amplified DNA fragments varied according to the used primer as well as the plant sample. OPA-09 and OPA-20 produced the highest number of amplified fragments; 6, 6 AF for mother plant and 6, 7 AF for micropropagated plants. Some primers, i.e., OPA-14, OPA-19, OPE-01 and OPE-20 showed an observed variation between mother plant and propagated ones with polymorphic percent ranged between 17 to 50 %. The total number of amplified DNA fragments differed according to the DNA source; MR produced 61 AF while Pr produced 51 AF. Tha analysis of multiplied DNA fragments based on RAPD technique revealed that the similarity percent between mother plant and micropropagated plants was 65.75%.

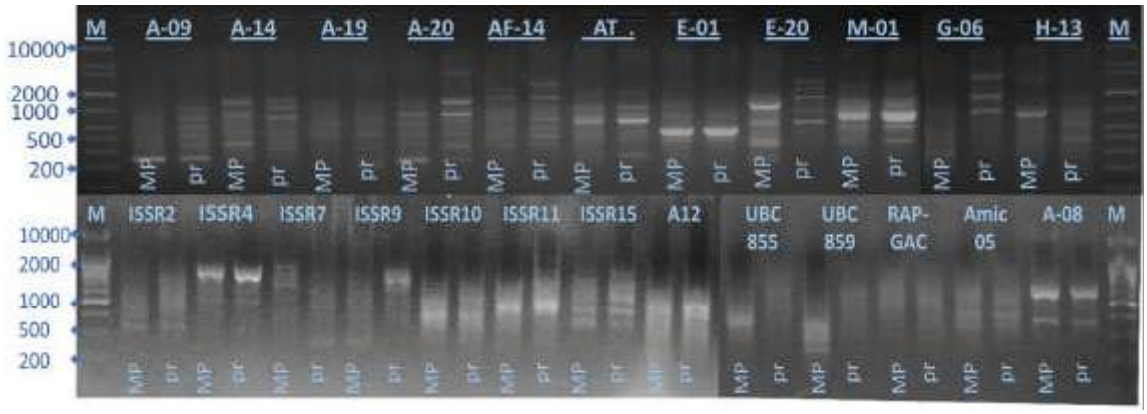

Fig. 7: DAN electrophoreses of *PCR products of P. tomentosa* mother plant and micropropagated ones on 1.5% agarose. where: MP is mother plant, Pr is propagated plant, M is the I Kb Lader.

Table 6: Genetic characterizations of *P. tomentosa* based on RAPD and ISSR PCR based techniques

			N.A. F.*					N.A. F.*		
No.	Name of RAPD Primer	Sequences	MP	Pr	P%	Name of ISSR primer	Sequences	MP	Pr	P%
1	OPA-09	GGGTAACGCC	6	6	0	ISSR-2	(AC)8T	3	3	0
2	OPA-14	TCTGTGCTGG	5	8	38	ISSR-4	(GA)8 T	3	3	0
3	OPA-19	CAAACGTCGG	3	6	50	ISSR-7	(TC)8 C	3	2	33
4	OPA-20	GTTGCGATGC	6	7	17	ISSR-9	(TG)8 A	2	3	33
5	OPAF-14	GGTGCGCACT	5	5	0	ISSR-10	(CTC)6	3	3	0
6	OPAT	CAGTGGTTCC	5	3	40	ISSR-11	(AGG)5 CC	2	2	0
7	OPE-01	CCCAAGGTCC	2	4	50	ISSR15	(AC)8GA	3	3	0
8	OPE-20	AACGGTGACC	4	2	50	A12	(GA) 6 CC	2	2	0
9	OPM-01	GTTGGTGGCT	5	5	0	UBC855	(AC)8CT	1	0	100
10	OP-G6	GTGCCTAACC	6	4	33	UBC859	(TG)8GC	3	3	0
11	OPH-13	GACGCCACAC	4	3	25	RAMP-GAC	G(AC)9	1	1	0
12			61	51		Amic-05	CGGC (AC)6 A	4	4	0
13						A08	(AGC)4 GC	3	3	0
Total number of amplified fragments*						Total number of amplified fragments*		33	32	
Similarity percentage			65.75%			Similarity percentage		96.50%		

Where: NAF is number of amplified fragments P% is polymorphic percent MP is mother plant Pr is regenerated plant

Based on ISSR Technique: Thirteen ISSR primers were used in DNA multiplication in PCR reaction (Table 6 and Fig. 7). Eleven primers showed monomorphic fragments and produced the same number of amplified DNA fragment for the both examined plants. While, only ISSR7, ISSR9 and UBC855 produced various number of amplified DNA fragments (3, 2 and1, 0, for MR and Pr, respectively) and polymorphic percent 33, 33 and 100%, respectively. The total number of amplified fragments for mother plant and micropropagated plant were 33 and 32 AF, respectively. The similarity percent between mother plant and micropropagated plant was 96.50%. Combination analysis based on both RAPD and ISSR revealed that the similarity percent between the two examined samples was 75. 45%.

DISCUSSION

Multiplication and growth parameters of *P. tomentosa* significantly affected by MS strength and BA concentrations. BA concentration at 2.0 mg/l in combination with 3/4 MS strength significantly induced shoots proliferation number and shoot length (3.00 shoots/shoot tip, 7.33 cm, respectively). Superior nodes number (4.64 nodes/shoot) and growth vigor resulted from 3/4 MS + 1.0 mg/l BA. Similar to our results, Nguyen *et al.* [16]; Ozaslan *et al*. [19]; Barhi and Bettaieb [25] and Ghatas, [23] reported that MS salt strength affected micropropagation and growth parameters of *Paulownia*, among various tested growth regulators, BA is the most effective for *Paulownia* proliferation. Contrast

results obtained by Chunchukov and Yancheva [26] who stated that MS basal salt composition increased multiplication coefficient of *Paulownia*, the number of shoots ranged from 1.8 to 3.9 shoots/explant, this contrast may be attributed to the various responses of different species.

On other hand, full, 3/4 and 1/2 MS with presence of 3.0 mg/l Kn significantly induced shoots proliferation number and shoot length. While, 3/4 MS + 2.0 mg/l Kn significantly augmented nodes number and shoot growth vigor. Results were supported with the finding of Rahman *et al*., [3] who stated that half MS strength and Kn at concentration 3.0 mg/l Kn + 0.5 mg/l NAA, enhanced shoot induction percent, shoot number and shoot length to 76%, 6.5 shoots and 5.02 cm, respectively.

Callus induction, callus induction percent and callus differentiation were stimulated by both explant types and NAA concentrations. All examined explants; inter nodes and leaves, at 0.2, 0.3 mg/l NAA high induced callus (10.0 induced explants for each). Inter nodes combined with 0.3 mg/l NAA gave the highest callus induction percent (100% CIP) as well as the highest callus differentiation (60% differentiation). Results agree with Ozaslan *et al*. [19] who stated that direct and indirect micropropagation may be utilized in Paulownia.

Results indicated that the two forms of cobalt; Co^{2+} ions and CoNPs, as well as the used concentrations positively affected shoot multiplication and growth parameters of *P. tomentosa* in the most concentrations compared with control. The highest shoot number possessed from Co^{2+} ions and CoNPs at T2 (8.00 and 6.00 shoots/shoot tip, respectively). While CoNPs at concentration T2 and T1 resulted in the highest shoots (10.0, 9.7 cm, respectively). The both cobalt forms at T1 and T2 (low concentrations) maximized the nodes number. On the other hand, the high concentration of Co^{2+} ions lead to totally death of shoots. While the high concentration of CoNPs showed dwarf shoots with deep burn of the edges of leaves. These results may be due to the promoting effect of the low concentrations of Co^{2+} ions and CoNPs on gene expressions which enhance the activity of antioxidant enzymes and resulted in promotion the growth parameters. While the high concentrations may lead to accumulation of the ions and/or CoNPs in the plant cells which resulted in cells toxicity. These results supported with the finding of Fouad and Hafez [36] who stated that Co^{2+} has higher capability compared with CoNPs, which enhancing metabolites accumulation which positive correlation with both gene expression and the activities of antioxidant enzymes (SOD and APX).

Root parameters and plant acclimatization were affected by IAA concentrations, the highest roots number, length, plantlet fresh weight and the highest number of success of acclimatization were observed from 3/4 MS medium supplemented with 6.0 mg/l IAA followed by MS supplemented with 4.0 mg/l IAA, with no significant differences between the two concentrations. Results appeared harmony with Abd El-Kader [21]; Barhi and Bettaieb [25]; Shtereva *et al*. [28] and Chunchukov and Yancheva [26] who illustrated that auxin in high concentration promoted root induction 100% in *Paulownia tomentosa* within 4 weeks and rooted plantlets were successfully acclimatized. On the other hand, Ozaslan *et al.* [19] conducted that rooting was *in vitro* enhanced on hormone free media. The later result may be correct the root could enhance on basal medium, but the produced roots are poor and not enough to reach to plantlet acclimatization.

Genetic characterization revealed that based on RAPD, the total number of amplified DNA fragments differed according to the DNA source; MR produced 61 AF while Pr produced 51 AF. That analysis of multiplied DNA fragments based on RAPD technique revealed that the similarity percent between mother plant and micropropagated plants was 65.75%. Also, based on ISSR, the total number of amplified fragments for mother plant and micropropagated plant were 33 and 32 AF, respectively. The similarity percent between mother plant and micropropagated plant was 96.50%. Combination analysis based on both RAPD and ISSR PCR products revealed that the similarity percent between the two examined samples was 75. 45%. Results proved the efficiency of RAPD and ISSR to discriminate the genetic characterization and distinguished the genetic similarity between the examined samples. Results agree with Vanova *et al*. [41] and Hamza *et al*. [42] who concluded that RAPD and ISSR are able to discriminate the genetic differences among the closely related genotypes at the DNA level and can identify the polymorphism between repeated sequences.

CONCLUSION

Paulownia tomentosa micropropagation could implemented through direct and indirect methods. MS strength is an effective factor in micropropagation as well as cytokinin types and concentrations. MS at 3/4 strength combined with 2.0 mg/l BA augmented the direct shoots proliferation. BA is the most effective cytokinin in *Paulownia* proliferation. Kn enhanced shoots

proliferation but its efficiency did not like BA. The low concentrations of Co^{2+} ions and CoNPs promoted shoot multiplication and growth parameters. For the indirect micropropagation, inter nodes combined with 0.3 mg/l NAA gave the highest callus induction percent (100% CIP) as well as the highest callus differentiation (60% differentiation). The use of RAPD and ISSR showed an efficient ability to discriminate genetic similarity between the mother plant and propagated ones.

REFERENCES

1. Barton, I.L., I.D. Nicholas and C.E. Ecroyd, 2007. *Paulownia*. The Forest Research Bull., 231: 5-68.
2. Lu, J., 2006. Energy balance and economic benefits of two agroforestry systems in northern and southern China. Agr Ecosyst Environ, 116: 255-262.
3. Rahman, Md. A., F. Rahman, M. Rahmatullah, 2013. *In vitro* regeneration of *Paulownia tomentosa* Steud. plants through the induction of adventitious shoots in explants derived from selected mature trees, by studying the effect of different plant growth regulators. Am.Eurasian J. Sustain. Agric., 7(4): 259-268.
4. Kiaei, M., 2012. Physical and mechanical properties of *Paulownia* wood (*Paulownia fortunei*) in North Iran. Middle-East Journal of Research, 11(7): 964-968.
5. Zho, Z.H., C.J. Chao, X.Y. Lu and Y.G. Xiong, 1986. *Paulownia* in China: cultivation and utilization. Asian Network of Biological Sciences, Singapore and International Development Research Center (Canada), Singapore, pp: 1-65.
6. Kalmukov, K., 1995. Influence of primary density upon the process of growth and development of some fast-growing tree species used for biomass. In: Brezin, V.N. (ed.) 70 Years Forestry education in Bulgaria. Jubilee Sciences Session, 7-9.VI. 1995. Sofia, v. I, 129-137.
7. Yang, J.C., C.K. Ho, Z.Z. Chen and S.H. Chang, 1996. *Paulownia* x *taiwaniana* (Taiwan Paulownia). Biotechnol. Agric. Forest., 35: 269-290.
8. Ipekci, Z., A. Altinkut, K. Kazan, K. Bajrovıc and N. Gozukirmizi, 2001. High frequency plant regeneration from nodal explants of *Paulownia elongata*. Plant Biol., 3: 113-115.
9. Park, Y.S. and J.M. Bonga, 1992. Conifer micropropagation: its function in tree improvement programs. In: Ahuja, M.R. (ed.) Micropropagation of woody plants. Kluwer Academic, Dordrecht, pp: 457-470.
10. Caparros, S., M.J. Diaz, J. Ariza, F. Lopez and L. Jimenez, 2008. New perspectives for *Paulownia fortunei* L. valorization of the auto hydrolysis and pulping processes. Bioresource Technol., 99: 741-749.
11. Wang, J., W. Li, C. Zhang and S. Ke, 2010. Physiological responses and detoxific mechanisms to Pb, Zn, Cu and Cd in young seedlings of *Paulownia fortunei*. J. Environ. Sci. (China), 22: 1916-1922.
12. Rao, C.D., C.J. Goh and P.P. Kumar, 1996. High frequency plant regeneration from excised leaves of *Paulownia spp.* cultered *in vitro*. Plant Cell Rep., 16: 204-209.
13. Ipekci, Z. and N. Gozukirmizi, 2003. Direct somatic embryogenesis and synthetic seed production from *Paulownia elongata*. Plant Cell Rep., 22: 16-24.
14. Bergmann, B.A. and H.K. Moon, 1997. *In vitro* adventitious shoot production in *Paulownia*. Plant Cell Rep., 16: 315-318.
15. Salkić, B., A. Salkić, H. Keran, S. ećajević, E. Salkić and E. Imširović, 2018. Production of Seedlings of Fast -Growth Tree of Paulownia elongata S. Y. Hu. International Journal of Plant & Soil Science, 25(1): 1-8.
16. Nguyen, M.U., X.D. Thai and V.L. Bui, 2005. Effects of Plant Growth Regulators on Callus Induction and Shoot Regeneration of *Paulownia fortune*. Tap Chi Congnghe Sinh Hoc., 3(4): 479-485.
17. Murashige, T. and F. Skoog, 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures, Physiologia Plantarum, 15(3): 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x
18. Lloyd, G. and B. McCown, 1981. Commercially Feasible Mi- cropropagation of Mountain laurel, *Kalmia latifolia*, by Use of Shoot Tip Culture," Proceedings of the Interna- tional Plant Propagators Society, 30: 421-427.
19. Ozaslan, M., C. Can and T. Aytekin, 2005. Effect of explant source on *in vitro* propagation of *Paulownia tomentosa* Steud. Biotechnol. & Biotechnol., 19(3): 20-26.
20. Clapa, D., A. Fira, M. Simu, L.B. Vasu and D. Buduroi, 2014. Improved *in vitro* propagation of *Paulownia elongata, P. fortune*. Bulletin UASVM Horticulture, 71(1): 6-14.
21. Abd El-Kader, S.F., 2004. Studies on propagation and growth of some trees. M.Sc. Thesis Hort. Dept. Fac. of Agric. Moshtohor, Zagazig Univ.

22. Zayova, E., M. Petrova, R. Vasilevska-Ivanova, D. Stoeva and B. Krapchev, 2013. A tissue culture technique for propagation of *Paulownia elongata* tree. Biological Diversity and Conservation, 6(3): 1-5.
23. Ghatas, Y.A.A., 2016. Employment of tissue culture techniques in improvement propagation of *Paulownia tomentosa* plant. J. Plant Production, Mansoura Univ., 7(6): 619-625.
24. Taha, L.S., M.M.S. Ibrahim and M.M. Farahat, 2008. A Micropropagation Protocol of *Paulownia kowakamii* through in vitro culture technique. Australian Journal of Basic and Applied Sciences, 2(3): 594-600.
25. Barhi, N.B. and T. Bettaieb, 2013. *In vitro* propagation of a forest tree *Paulownia tomentosa* (Thunb.) Steud. A valuable medicinal tree species. Albanian J. Agric. Sci., 12(1): 37-42.
26. Chunchukov, A. and S. Yancheva, 2015. Micropropagation of *Paulownia species* and hybrids. First National Conference of Biotechnology, Sofia 2014. Faculte de Biologie, 100(4): 223-230.
27. Roy, P.K., 2015. *In vitro* plant regeneration of *Paulownia tomentosa* (Thunb.) Seud. From shoot tip and segment. Bangladesh J. Bot., 44(3): 459-463.
28. Shtereva, L., R. Vassilevska-Ivanova, T. Karceva and B. Kraptchev, 2014. Micropropagation of six *Paulownia* genotypes through tissue culture. Journal of Central European Agriculture, 15(4): 147-156.
29. George, E.F., D.J.M. Puttock and H.J. George, 1988. In: Plant culture media (vol 2), Exegetics Co, Ltd., Westbury, 1988.
30. Hilmy, L.M. and N. Gad, 2002. Effect of cobalt fertilization on the yield, quality and the essential oil composition of parsley leaves. Arab Univ. J. Agric. Sci., 10: 803-29.
31. Bartolo, W.C.F. and M.J.K. Macey, 1989. Cobalt requirement in tissue culture of three species: *Brassica oleracea* L., *Passiflora mollissima* Bailey and *Saintpaulia ionantha*Wendl. Journal of Horticultural Science, 64(6): 643-647.
32. Javed, S.B. and M. Anis, 2015. Cobalt induced augmentation of *in vitro* morphogenic potential in *Erythrina variegata* L.: a multipurpose tree legume. Plant Cell Tissue Organ Cult; 120: 463-74.
33. Heath, J.C., 1954. The effect of cobalt on mitosis in tissue culture. Experimental Cell Research, 6(2): 311-320. On line from 2004, https://doi.org/10.1016/0014-4827(54)90178-0.
34. Karuppanapandian, T. and W. Kim, 2013. Cobalt-induced oxidative stress causes growth inhibition associated with enhanced lipid peroxidation and activates antioxidant responses in Indian mustard (*Brassica juncea* L.) leaves. Acta Physiol Plant, 35: 2429-43.
35. Sharma, P., A.B. Jha, R.S. Dubey and M. Pessarakli, 2012. Reactive oxygen species, oxidative damage and antioxidative defense mechanism in plants under stressful conditions. J. Bot., 2012.
36. Fouad, A.S. and R.M. Hafez, 2018. Effect of cobalt nanoparticles and cobalt ions on alkaloids production and expression of CrMPK3 gene in *Catharanthus roseus* suspension cultures. Cell Mol. Biol., 64(12): 62-69.
37. Salvi, N.D., L. George and S. Eapen, 2001. Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. Plant Cell Tiss. Organ Cult., 66: 113-119.
38. Lakshmanan, V., S.R. Venkatar-amareddy and B. Neelwarne, 2007. Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. Electron J. Biotech., 10: 1-8.
39. Joshi, P. and V. Dhawan, 2007. Assessment of genetic fidelity of micro propagated *Swertia chirayita* plant-lets by ISSR marker assay. Biol. Plant, 51: 22-26.
40. Hamza, E.M., 2013. Genetic diversity of some citrus varieties based on microsatellite and RAPD molecular markers in Egypt. World J. Agric. Sci., 9(4): 316-324.
41. Vanova, B., S. Yancheva and B. Bojinov, 2012. Molecular differentiation of *Paulownia species* and hybrids. Journal of Central European Agriculture, 13(1): 73-84.
42. Hamza, E.M., I.A. Ibrahim, A.A. Nower, N.A. Awd and G.E. Hazzaa, 2017. Horticultural and genetical evaluation of Le-Conte pear and seven induced mutants through RAPD, ISSR and microsatellite. World Applied Sciences Journal, 35(11): 2445-2455.
43. Varshney, A., M. Lakshmikumaran, P.S. Srivastava and V. Dhawan, 2001. Establishment of genetic fidelity of *in vitro*-raised Lilium bulblets through RAPD markers. *In vitro* Cell Dev. Biol. Plant, 37: 227-231.
44. Martin, M., D. Sarmento and M.M. Oliveira, 2004. Genetic stability of micropropagated almond plant-lets, as assessed by RAPD and ISSR markers. Plant Cell Rep., 23: 492-496.

45. Sreedhar, R.V., L. Venkatachalam and N. Bhagyalakshmi, 2007. Genetic fidelity of long-term micropropagated shoot cultures of vanilla (*Vanilla planifolia* Andrews) as assessed by molecular markers. Biotechnol. J., 2: 1007-1013.
46. Abdel Razik, A.B., 2012. The genetic stability of *in vitro* propagated *Paulownia tomentosa* using DNA-based markers. Egypt. J. Genet. Cytol., 41: 151-161.
47. Hamza, E.M., 2019. Short-term preservation of *Nepeta septemcrenata via* production of synthetic seeds. American-Eurasian J. Agric. & Environ. Sci., 19(1): 54-63.
48. Pottino, B.G., 1981. Methods in Plant Tissue Culture. Dept. of Hort., Agric., Maryland Univ., College Park, Maryland, USA, pp: 8-29.
49. Steel, R.G.D., J.H. Torrie and M.A. Boston, 1997. Principles and procedures of statistics. 2nd edition, McGraw-Hill Book Co. Inc., USA., pp: 633.
50. Sambrook, J. and D.W. Russel, 2001. Molecular cloning: A laboratory manual. 3rd ed, Vol. 3 Cold Spring Harbour Laboratory Press, New York
51. Rohlf, F.J., 2005. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System Version 2.1. Exeter Publications, Setauket, New York, 2: 02.