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A Micropropagation Protocol of *Paulownia kowakamii* through *in vitro* culture technique

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Abstract: The goal of this study was to establish an *in vitro* propagation method of *Paulownia kowakamii* trees. Shoot proliferation was induced on microcutting explants cultured on MS (Murashige and Skoog) medium containing different concentrations of BA. During multiplication stage, specific treatments were tested to enhance the rate of shoot multiplication by using different light intensities (1300, 1900, 2800 or 4100 Lux). *In vitro* – grown shoots were cultured to root on half strength MS medium containing either of 3 auxins IAA, IBA or NAA at concentration of 1.0 or 0.5 mg/L. The results showed that supplementation of the culture medium by 1.0 mg/L of BA and medium light intensity (1900 Lux) increased the number, length of shootlets and number of leaves per shootlet as well as pigment contents (chlorophyll-a, -b and carotenoids) in shootlet tissues. However, increasing BA concentration to 6.0 mg/L and high light intensity (4100 Lux) produced the highest amounts of indoles and total soluble phenols. Concerning rooting potentiality, addition of 1.0 mg/L IAA to half strength MS medium gave 100% rooting percentage. Successful transplanting was obtained when rooted plantlets were transferred to a mixture of peat moss and sand (1 : 1).

Key words: Paulowonia kawa kamii, *in vitro* and micropropgation

INTRODUCTION

Paulownia is a genus of between 6-17 species of plants in the family Paulowniaceae (Scrophulariaceae). They are native to much of China. They are fast growing deciduous trees, with a large leaves arranged in opposite pairs on the stem. The trees are used for reforestration, roadside and as ornamental tree. It grows well in a wide variety of soil types, notably poor ones(Zhu *et al.* 1988).

Paulownia timber is a pale whitish coloured wood with a straight grain. Its characteristics of rot resistance and a very high ignition point ensures the timber's popularity in the world market.

The wood and bark were reported to have astringent properties. The charcoal made from this wood is used in high class fire work and in the preparation of gun powder (Anonymous, 1988). It is propagated through seed or by cuttings. Conventional methods of propagation through seed are unreliable because of disease and pest problem, poor germination and also slow growth than cuttings (Bergmann and Moon, 1997). Therefore, *in vitro* technique was investigated in order to provide mass production of shootlets in a short period of time and all over the year to cover the demand of the new settlements.

In vitro propagation of *Paulownia tomentosa* was achieved through shoot bud regeneration directly from leaf explants or via the callus phase (Marcotrigiano and Stimart, 1983; Rao *et al.,* 1996). Mass multiplication of *Poulownia elongata* through nodal culture has also been reported (Ipekci *et al.,* 2001). The success procedures of consecutive micropropagotion of many woody plants could be influenced by various factors from which plant growth regulators, physical conditions and growing media are the most important ones. The effect of benzyladenine (BA) at different concentrations on shoot multiplication rate was recorded on *Eucalyputs tereticornis* (Rao, 1988; Das and Mitra, 1990), Johonson and Walker (1990) on *Quercus lobata*, Bunn and Dixon (1992) on *Stlingia latifolia*, Abou Dahab *et al.*(2005) on *Ruscus hypoglossum* and Sayed and Gabr (2007) on *Deutzia scabra* .

The effect of light intensity on *Saintpaulia ionantha* was indicated by Lercari *et al.* (1986). They demonstrated that light was necessary for shoot differentiation. Castillo *et al.* (1997) on *Carica papaya*

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stated that highest multiplication rates of shootlets per explant were achieved with increasing light intensity. The influence of different types and concentrations of plant growth regulators on rhizogensis behaviour of shootlets *in vitro* was studied by Romano *et al.* (1992) on *Quercus ruber.* They indicated that rooting was achieved on *in vitro* regenerated shoots in an IBA concentrated solution compared with other auxins treated (IAA or NAA). Ibrahim *et al.* (1992) on *Ficus bengamina* observed that rooting of shoots was obtained in MS medium without the addition of growth regulators. Ostrolucka and Bezo (1994) cultured the explant of *Quercus species* (*Q.ruber, Q.cerris and Q.rubra)* on woody plant medium with IBA or NAA at various concentrations. They noticed that the highest rhizogensis rates were obtained in case of using low concentration of IBA or NAA. Regarding growing media, Nower (1998)in study on *Syngonium podophylum* mentioned that using a soil mixture of peat moss and sand at ratio 3:1 resulted in best growing after 1, 2 and 3 months of planting compared to soil mixtures of peat moss and sand 2:1 and 1:1 or peat moss only. Wherease, Venkateswarlu *et al.* (2001) showed that *in vitro* grown shoots of *Paulownia fortuneii* could be successfully *ex vitro* in a vermiculite+ cocopeat mixture under 95% humidity, followed by gradual reduction. Abou Dahab *et al.* (2005) on *Ruscus hypoglossum* and Sayed and Gabr (2007) on *Deutzia scabra* pointed out that, transfer of rooted shootlet to different kind of media supstrate to greenhouse showed different effects on the survival of plants. Therefore, the present experiment is achieved on *Paulownia* kawakamii to study the effect of BA concentration and light intensity on the shoot multiplication rate, various types of auxins (IAA, IBA and NAA) on rhizogensis behaviour of shootlets *in vitro* as well as various growing media after transplanting plantlets to greenhouse.

MATERIALS AND METHODS

These experiments were carried out at Tissue Culture and Germplasm Conservation Researches Laboratory, Hort. Res. Inst., Agric. Res. Center, Giza and Department of Ornamental Plants and Woody Trees, National Research Center during the years 2006 and 2007 to study the consecutive micropropagation behaviour (multiplication, rooting and acclimatization stages) of *Paulowina kawakamii* in order to improve plantlets production quantitatively and qualitatively.

Plant Material and Explant Source:

Shoot apexes from axillary buds *of Paulowina kawa kamii* (3- to 4 year - old) grown at Research and Production station, Nubaria, National Research Center, were used as a source of vegetative materials.

The shoot tips (15-20 mm length) were firstly soaked for 30 Sec. in 70% ethanol solution then surface sterilized for 15 min in 0.2% mercuric chloride with few drops of tween-20 (polyoxyethylene sorbiton monolaurate) and finally rinsed several times in sterile distilled water.

Culture Medium:

Murashige and Skoog, 1962 (MS) basal medium supplemented with 25g/l sucrose, and solidified with 0.7% agar at pH 5.7 the phytohormons were added as follows:

For Multiplication Stage:

Effect of BA Concentration:

Microcutting explants (5±2 mm length) were excised from *in vitro* plantlets and cultured on MS medium supplemented with different concentrations of 6-Benzyladenine (BA) (0.0, 1.0, 2.0, 4.0 and 6.0 mg/l).

Effect of Light Intensity:

The cultures coming from the concentration of 1.0 mg/l of BA were used for this experiment and incubated under different light intensities (1300, 1900, 2800 and 4100Lux.).

In multiplication stage, each treatment consists of 25 replicates, four – five weeks after culturing, the following data were recorded.

- Number of formed shootlts per explant.
- Shootlet length (mm).
- Number of leaves per shootlet.
- Chemical constituents (Pigment, total soluble sugars(TSS), indoles and total soluble phenols) of shootlets tissues.

For Rooting Stage:

Shootlets producing from multiplication stage were transferred to half strength of basal salts of MS – medium supplemented with Indole acetic acid (IAA), Indole-butyric acid (IBA) or Naphthalenacetic acid (NAA) at concentrations of 1.0 and 0.5 mg/l for each. Each treatment consists of 25 shootlets. Data, as Percentage of root formation (%) and number of roots / shootlet were recorded after 5-6 weeks.

Acclimatization:

Plantlets obtained *in vitro* were removed from culture vessels and washed with tap water, then disinfected by immersion in Benlate Solution (1.0g/l) as a fungicide for 5 min. The plantlets were transferred to plastic pots (6 cm width) containing Peat moss, peat moss + sand (1:1) or peat moss + silt (1:1). Pots were covered with transparent polyethylene pages for two weeks and gradually they removed in the greenhouse before subsequent transfer to the field. Data were recorded after 8 weeks as a survival percentage of plantlets (%), Stem length of plantlets (mm), number of leaves, roots length (mm),fresh and dry weights of stems and roots of plantlets (g).

Culture Condition:

Culture media were adjusted to PH 5.7 before autoclaving and autoclaved for 20 min at 121˚C. The cultures of the different experiments were incubated under 16h photoperiod under light intensity 1900 Lux provided from white fluorescent lamp. The light intensity was measured at the top of the cultures by Lux-meter (Tburton lx -101)instrument. All cultures were maintained at 25 ±2˚c.

Extraction and Determination:

Photosynthetic pigments (chlorophyll a and b) as well as carotenoids were determined in shootlets tissues as mg / 100g fresh weight, according to the procedure achieved by Saric *et al.* (1997). Total soluble sugars were determined in the methanolic extract by using the phenol – sulfuric method according to Dubois *et al.* (1966). The total indoles were determined by using " Erlic's reagent" according to Larsen *et al.* (1962). While, total soluble phenols were calorimetrically determined using Folin Ciocaltea reagent (A.O.A.C. 1985).

Statistical Analysis:

The average of three series of experiment was calculated and among them, the data were statistically analyzed using LSD test for comparison among means according to methods of Steel and Torrie (1980).

RESULTS AND DISCUSSION

Shootlets Multiplication:

Effect of BA on In vitro Multiplication:

The effect of different concentrations of BA (0.0,1.0,2.0,4.0 and 6.0 mg/L) on the *in vitro* shootlets formation of *Paulownia kawakamii* is shown in Table (1). Data indicated that, supplementation of the culture medium by 1.0 mg/L of BA favoured shootlets initiation compared with control. It can be mentioned that the concentration of BA at 1.0 mg/L gave the best results of shootlets number per explant (2.60), length of shootlets (35.37 mm) and number of leaves formed per shootlets (18.71). Also, it is clear that the addition of BA with 1.0mg/L to the medium increased the shootlets and enhanced their vigorously. Whereas, increasing BA concentration from 1.0 to the other concentrations (2.0, 4.0 or 6.0) mg/L, generally had a depressive effect on those morphogenesis characteristics of *Paulwonia kawakamii* . These results are in agreement with those obtained by Takayama *et al.*, (1991). They noticed that BA was more effective for Lilium bulblets development. However, high BA had an inhibiting effect on further bulblet growth. These results could be explained by that cytokinins have important physiological effects, as they have been shown to stimulate cell division as well as cell elongation, to activate RNA synthesis and to stimulate protein synthesis and enzyme activity, as was reviewed by Kulaeva(1980). The use of high cytokinin levels was one of the most effective methods to reduce shoot and leaf growth and promote the formation of meristematic clusters (Ziv, 1992).

Pigment Content:

According to the data illustrated in Table (2), using MS medium supplemented with BA at 1.0 mg/L significantly augmented chlorophyll-a,b and carotenoids content (157.12,66.72 and 139.18 mg/100g FW, respectively) in the *in vitro* shootlets tissues compared with control. In this share, Marino and Bertazzo (1990)

on *Actinidia deliciosa* reported that , chlorophyll –a and –b were affected by using BA in culture media. This phenomenon may be due to the enhancement of pigments accumulation in the shootlets tissues.

Table 1: Effect of benzyladenine (BA) concentrations on *in vitro* multiplication of *Paulownia kawakamii.*

BA (mg/L)	No.shootlets per explant	Shootlet length (mm)	No. leaves per shootlet
0.0	1.37	25.27	9.13
1.	2.6	35.37	18.71
2.0	0.97	22.64	7.82
4.0	1.23	21.77	8.63
6.0	0.43	17.7	11.5
LSD at 0.05	0.29	2.74	1.77

Table 2: Effect of benzyladenine (BA) concentrations on chemical constituents (mg/100g FW) of *Paulownia kawakamii.*

BA (mg/L)	Chl. –a	Chl. –b	Carotenoids	TSS	Total indoles	T.S. phenols
0.0	72.69	47.08	116.23	224.72	135.39	65.47
1.0	157.12	66.72	139.18	220.61	122.3	87.50
2.0	74.40	33.74	102.27	266.42	114.49	86.08
4.0	36.56	56.10	59.17	380.46	152.17	120.12
6.0	31.10	49.78	49.3	466.04	159.3	123.38
LSD at 0.05	8.64	8.09	8.68	15.9	11.76	9.31

Total Soluble Sugars:

Data in Table (2) revealed that total soluble sugars significantly increased in shootlets tissues (466.04 mg/100g FW) with increasing BA concentration to 6.0 mg/L as comparing with control.

Total Indoles and Total Soluble Phenols:

Results in Table (2) showed that the highest amounts of total indoles and total soluble phenols (159.30 and 123.38 mg/100 g FW, respectively) resulted from BA at 6.0 mg/L. Both treatments (BA at 4.0 and 6.0 mg/L) increased significantly total indoles and total soluble phenols as compared to other treatments and control. These results are in line with those obtained by Youssef (1994) on *Acacia salicina*, who indicated that using BA at high concentration (5.0 mg/L) remarkably augmented the endogenous level of total indoles.

Effect of Light Intensity on *In vitro* Multiplication:

It is evident for the obtained data that number of shootlets formed per explant, length of shootlets and number of leaves per shootlets were affected significantly by light intensity (Table 3). Data indicated that using 1900 Lux light intensity gave the highest shootlets number (1.60), shootlets length (30.67 mm) and number of leaves (12.67) compared with other light intensities. The same results were found by Lee *et al.,*(1985) who pointed out that plants grown in the presence of high photosynthetic photon flux(PPF) (314 µ mol $m^{-2}s^{-1}$) had a lower saturation photosynthesis than plants grown under medium PPF (115 µ mol $m^{-2}s^{-1}$) . This decrease in photosynthesis was attributed to specific damage to the light harvesting pigments. Also, Ansari *et al*., (2003) on *Eucalyptus tereticornis* mentioned that low light intensity significantly promote multiplication of shootlets.

Table 3: Effect of light intensity on *in vitro* multiplication of *Paulownia kawakamii.*

Light intensity (Lux)	No. shootlets per explant	Shootlet length (mm)	No. leaves per shootlet
1300	1.40	21.00	9.67
1900	1.60	30.67	12.67
2800	1.30	28.00	8.40
4100	1.07	22.67	9.03
LSD at 0.05	0.24	2.430	1.09

Pigment Content:

As shown in Table (4), the various light intensities had a significant influence on chlorophyll –a, b and carotenoids. Data indicated that incubating the cultures under light intensity of 1900 Lux resulted in the highest formation rates of chlorophyll –a, b and carotenoids in tissues of the *in vitro* shootlets (1051.00, 473.64 and 796.12 mg/100g FW, respectively) with no significant difference between the values of chlorophyll –a which formed (1054.57) when the light intensity increased to 2800 Lux. While increasing light intensity to 4100 Lux caused decreasing chlorophyll –a, -b and carotenoids to the lowest values (522.03, 265.36 and 302.88 mg/100gFW, respectively). This might be ascribed to that anabolism processes of chlorophyll –a, -b and carotenoids were in higher activity in case of using medium light intensity (1900 Lux). In this regard, Lee *et al.,* (1985) on *Liquidambar styrociflua* observed that the chlorophyll content was significantly higher in low-light treated plants. Webster *et al.,* (1995) demonstrated that xanthophyll in leaves of *Digitalis purpurea* increased to a maximum at a light intensity of 400 µ mol $m^{-2}s^{-1}$, then decreased with further increases

in light intensity.

Table 4: Effect of light intensity on chemical constituents (mg/100g FW) of *Paulownia kawakamii.*

Light intensity (Lux)	Chl. –a	Chl. –b	Carotenoids	TSS	Total indoles	T.S. phenols
1300	789.56	323.64	457.25	41.28	200.59	266.84
1900	1051.00	473.64	796.12	83.54	461.65	800.68
2800	1054.57	340.9	659.83	61.53	386.08	718.27
4100	522.03	265.36	302.88	66.26	591.29	894.82
LSD at 0.05	41.16	27.57	23.04	8.77	78.37	76.76

Total Soluble Sugars:

According to data illustrated in Table (4), the highest amount of total soluble sugars (83.54 mg/ 100g FW) was determined in shootlets obtained from explants subjected to light intensity of 1900 Lux, whereas the lowest value (41.28 mg/100g FW) was found in case of using low light intensity. These results were in confirmation with those obtained by Leaky (1983) on *Triplachiton scleroxylon,* who cited that stock plants grown at low irradiance seemed to be had low starch contents.

Total Indoles and Total Soluble Phenols:

The amounts of both total indoles and total soluble phenols were significantly differed according to data presented in Table (4). The tissues of shootlets incubated under high light intensity (4100 Lux) resulted in the highest level of indoles (591.24 mg/g FW) and total soluble phenols (894.82 mg/g FW). The results were in agreement with Arezki *et al.*, (2001) on *Eucalyptus camaldulensis*, who discovered that light promoted an increase in phenolic compounds .

Rooting Potentiality:

Microshoot derived from multiplication stage were separated and transferred to a rooting medium having half- strength basal MS medium supplemented with two concentrations (1.0 and 0.5 mg/l) of IAA, IBA or NAA.

The percentages of rooting as well as the number of roots per shootlet were recorded after 5 weeks of culture. The results presented in Table (5) show that the addition of IAA (1 mg/l) led to the highest percentage of rooting (100 %) compared with culture medium without growth regulators and that contained low concentration of IAA (0.5 mg/l) which gave the lowest percentage of rooting (53.33 % and 46.67 %, respectively). Some investigators came to the same results with other plants as Zaghloul *et al.* (1992) on *Eucalyptus citriodora* . However , the highest number of roots formed per shootlet (5.53) were observed with culture media contained (1.0 mg/l) NAA . It is noticed from data in Table (5) that both culture medium without growth regulators and using IAA at 0.5 mg/l produced the lowest number of roots (3.03 and 3.33, respectively). These results agree with that obtained by Chua *et al.* (1981). They found that more roots of dracaena were obtained by subculturing on medium supplement with 1.0 mg/l of NAA. Dragan (1989) found that, *in vitro* rooting of dracaena was stimulated by addition of IBA or NAA . The mean number of roots per shoot increased with increasing IBA concentration.

Table 5: Effect of auxins on rooting potentiality of *Paulownia kawakamii* .

Auxins (mg / L)		Mean percentage of rooting	Number of roots /shootlet
Control	0.0	53.33	3.03
IAA	1.0	100.00	4.93
	0.5	46.67	3.33
IBA	1.0	66.67	3.57
	0.5	60.00	3.93
NAA	1.0	73.33	5.53
	0.5	93.33	5.17
LSD at 0.05		34.18	1.44

Acclimatization Behavior:

Rooted plantlets of *Paulowina kawakamii* were transferred from the aseptic culture environment *(in-vitro*) to soil of Peat moss, mixture of peat moss and sand at the ratio 1:1 or mixture of peat moss and silt at 1:1. These processes were established in the greenhouse. After six weeks, the results were tabulated. The data in Table (6) clearly show that the highest percentage of survival (90.00 %) was obtained by using a soil mixture of Peat moss and sand. While, no significant differences between neither stem lengths nor leaves number of

the acclimatized rooted plants *ex vitro* were due to different growing media tested. Since, the root length was in highest value (93.33 mm) when Paulowina plantlets were cultured in mixture of peat moss and sand (1:1). The data in Table (6) disclosed that stem fresh and dry weights of acclimatized plantlets were in highest values

Table 6: Effect of growing media on acclimatization behaviour of *Paulownia kawakamii*.

Characters	Peat moss	Pea-moss+ sand(1:1)	Peat moss+silt (1:1)	LSD at 0.05
Survival %	70.00	90.00	40.00	19.98
Stem length (mm)	62.33	56.00	60.00	N.S.
Leaves No.	10.67	8.67	10.67	N.S.
Roots length (mm)	60.00	93.33	48.33	21.32
Stem fresh weight (g)	0.51	0.27	0.45	0.11
Roots fresh weight (g)	0.17	0.13	0.24	0.06
Stem dry weight (g)	0.048	0.037	0.056	0.012
Roots dry weight (g)	0.016	0.017	0.03	0.008

(0.51, 0.048 and 0.45, 0.056g, respectively) when peat moss only or soil mixture of peat moss and silt (1:1) were used. The highest roots fresh and dry weights (0.24 and 0.03 g, respectively) were resulted with growing culture media of peat moss and silt (1:1). In this regard, Nower (1998) in study *on Syngonium podophylum* mentioned that using a soil mixture of Peat moss and sand at the ratio (3:1) resulted in best result after 1,2 and 3 months of planting compared to soil mixture of peat moss and sand (2:1) and (1:1) or peatmoss only. However, Sayed and Gabr (2007) on *Deutzia scabra* indicated that different growing media had no significant effect on the survival percentage of plant during acclimatization. The tallest plants and greatest number of leaves produced with peat moss growing medium as comparing with peat moss + sand (1:1), peat moss + vermiculite (1:1) or peat moss + sand + sand (1:1:1).

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