Micropropagation of *Koelreuteria bipinnata* Using Juvenile and Mature Explants

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Abstract

An efficient micropropagation protocol for *Koelreuteria bipinnata*, an ornamental tree, from mature and juvenile phase tissues was described. Nodal explants from one-year-old branches of a field-grown mature tree (mature phase) and from *in vitro* growing seedlings (juvenile phase) were used. The nodal explants from mature explants cultured on Murashig and Skoog (MS) medium supplemented with or without growth regulators did not show any axillary shoot development. However, 6-benzylaminopurine (BAP) at 1.5 mg/l induced axillary shoots from all juvenile explants (100%). It also produced the highest number of shoots (2.5 shoots per explant), the highest rooting percentage (100%), the maximum number of roots (2.80 roots/shoot) and the longest roots (5.87 cm) when it was added as a supplement to the half-strength MS medium culture that included 15 g/l sucrose. Plantlets were successfully acclimatized and transferred to the field with 60% survival rate.

Keywords: BAP, Chinese flame tree, Juvenile nodal explants, mature nodal explants, sucrose, woody plants
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1. Introduction

Koelreuteria is a genus of small to medium-sized ornamental trees, indigenous to East Asia. The members of the genus *Koelreuteria* have been widely cultivated as ornamental trees because of their large showy inflorescences of yellow flowers [1]. *Koelreuteria bipinnata* (Figure 1), also known as the Chinese flame tree, the Bougainvillea golden rain tree, and the Chinese golden rain tree, belongs to the family Sapindaceae [2]. It is often used as a tree along streets and highways, and in parking lots. It is grown to provide shade in gardens and is a fine display tree. The trees bloom in summer from July to August, and in some regions in Europe, they flower in September. The flowers are small with four yellow petals which have a touch of red color at the base. The flowers can yield a yellow dye. The three-sided seed pods of *K. bipinnata* look like red Chinese paper lanterns that hang all over the tree in the late autumn forming a spectacular picture. So, the trees are commonly used as a focal point in landscape design in regions where they flourish [3].

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Figure 1. *Koelreuteria bipinnata* tree growing in the nursery of ornamental plants, Faculty of Agriculture, Assiut University, Assiut, Egypt

The conventional propagation method for the golden rain tree (*Koelreuteria* spp.) is usually via seeds. However, seeds germination is slow because seeds have physical dormancy [4, 5]. Seeds can germinate best if the seed coats are scarified for about one hour in sulfuric acid, followed by stratification for about 3 months at 2 to 4°C, which overcomes embryo dormancy [6]. For *K. bipinnata*, the seed germination percentage was 55% (unpublished data). Moreover, no reports on using other methods for propagation of *K. bipinnata* could be found and it seems that the tree cannot be propagated by conventional vegetative propagation methods such as making stem cuttings. These problems could possibly be overcome by the application of plant tissue culture techniques such as micropropagation, and thus a protocol for in the vitro propagation of these ornamental trees should be established. However, most of the previous investigations of woody plants utilized seeds and juvenile tissues were more responsive to *in vitro* manipulations than tissues from mature plants [7, 8]. Moreover, the degree of maturity affects the efficiency and production of a forest tree species.

There is scarce information available in the literature about the use of tissue culture technique for *Koelreuteria bipinnata* [9]. Thus, the aim of the study was to establish a simple procedure for *in vitro* propagation of *K. bipinnata* tree from juvenile and mature explants.

2. Materials and Methods

2.1 Explant collection, disinfection and culture establishment

Seeds and mature nodal explants of *Koelreuteria bipinnata* were obtained from an approximately 20 year-old tree growing in the nursery of ornamental plants, Faculty of Agriculture, Assiut University, Egypt. The healthy seeds were thoroughly cleaned in running tap water for 30 min and disinfected by submerging them in 70% (v/v) ethanol for 3 min. Then, the seeds were rinsed three times using sterile distilled water and cultured in 250 ml jars containing 30 ml of Murashig and Skoog (MS) medium [10] supplemented with 3% (w/v) sucrose, 0.75% (w/v) agar and without growth regulators for seed germination. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121ºC and 1.5 kg/cm^2 for 30 min. The cultures of all experiments were incubated at 25 ±2°C under 16 h light from cool white fluorescent tubes.

After 5 weeks of seed germination, nodal explants were excised from *in vitro* grown seedlings (juvenile explants) and used for further experiments. Mature nodal explants were taken from one-year-old branches collected from the same tree grown in the nursery. The branches were cleaned under running tap water for 15 min, cut into pieces with 1-2 nodes, and the surfaces were disinfected for 20 min in 30 % (v/v) commercial bleach (~5% sodium hypochlorite).

Finally, in a laminar air flow hood, the nodes were rinsed three times using sterile distilled water and 1.5-2 cm nodal explants were cultured in jars (250 ml) that contained 30 ml of plant growth regulators (PGRs)-free basal MS medium which had been supplemented with 3% (w/v) sucrose and 0.75% (w/v) agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121º C and 1.5 kg/cm^2 for 30 min. The cultures of all experiments were incubated at 25 ±2°C under 16 h light from cool white fluorescent tubes.

2.2 Effect of 6-benzylaminopurine (BAP) concentration on shoot multiplication

For the juvenile phase, nodal segments (~1cm containing 2 nodes) were taken from *in vitro*-growing seedlings. For the mature phase, nodal segments (~1.5-2 cm with 1-2 nodes) were excised from recent branches of the tree and the following experiment was conducted to study the effect of BAP on both types of explants.

Mature and juvenile explants were cultured in 250 ml baby food jars containing 30 ml MS basal salt medium. The medium was supplemented with 6-benzylaminopurine (BAP) at 0, 0.5, 1, 1.5 and 2 mg/l. A completely randomized design (CRD) with three replicates was used. The data were analyzed according to the multiobservation approach where each treatment per replicate contained five jars. The following data were recorded after 3 weeks culturing on media: percentage of axillary shoots induction (%), average number of shoots per explant, average length of shoots (cm), and number of leaves/shoot

2.3 Effect of sucrose concentration on root formation

Proliferated axillary shoots were excised and transferred to half-strength MS medium without PGRs to allow rooting, and the following experiment was conducted to study the effect of sucrose on root formation.

Uniform individual shoots (each ~3-4 cm long) derived from the multiplication stage were transferred to PGRs-free half-strength MS medium supplemented with four concentrations of sucrose (15, 20, 25 and 30 g/l).

A completely randomized design (CRD) was used with three replicates and each treatment per replicate contained five jars. Data were recorded after two weeks for rooting percentage (%), number of roots per explant, and root length (cm).

2.4 Acclimatization stage

After four weeks, the rooted plantlets (~3- 4 cm) were gently removed from the medium, and the roots were rinsed using tap water to remove the excess medium. Finally, the plantlets were transplanted into small plastic pots (10-cm dia.) that contained a 1:1 mixture of peat moss and perlite. Data on the survival rate was calculated after four weeks.

2.5 Statistical analysis

The data from all experiments were subjected to analysis of variance (ANOVA) and mean comparison was performed using the least significant difference (LSD) method with a significant level of 5%, which was adapted from Gomez and Gomez [11].

3. Results and Discussion

3.1 Establishment stage (initiation stage)

All the nodal explants (1.5 -2 cm) taken from the mature phase did not respond and turned brown when cultured on PGRs-free MS medium. Under *in vitro* conditions, the explant proliferation depends on the specific balance of PGR's, auxins and cytokinins [12]. However, the establishment of explants for micropropagation of woody plants and the induction of adventitious shoots and roots is greatly influenced by the maturation status of the tissue used for the primary explant [8]. Young plant material from mature woody plants can be obtained in two ways, the first being the use of new growth parts of the mature plants, and the second way being the renovation of the mature parts of the woody plants themselves. In this work, seeds of *K. bipinnata* that were cultured on plant growth regulators (PGRs)-free MS basal medium germinated after four weeks of culture. The nodal explants, which were approximately 1 cm in length, had two nodes, and which had been taken from the *in vitro* growing seedlings (juvenile phase), were used for the multiplication stage.

3.2. Axillary shoots multiplications

3.2.1 Effect of BAP on mature nodal segments

All nodal explants (1.5-2 cm) taken from the mature phase did not respond and turned brown when cultured on MS medium supplemented with different concentrations of BAP (0, 0.5, 1.0, 1.5, and 2 mg/l). Browning is a common and often severe problem in plant tissue culture that is caused by the accumulation and oxidation of phenolic compounds and is frequently observed in tissue cultures of woody plants [13-15]. This may be due to high oxidative stress during culture and strong wound reactions accompanied by increase in phenolic compounds [16].

Most of the studies carried out on woody plants have utilized seeds and juvenile tissues that were more responsive to *in vitro* manipulations than tissues from mature plants [6, 7, 14, 15]. It is well known that 6-benzylaminopurine (BAP) and/or kinetin (6-furfurylaminopurine, Kin) are often used as cytokinins [17-19] in plant tissue culture proliferation. However, they both have a

limited success in woody plant regeneration [20]. Many reports stated that Thidiazuron (TDZ) induces regeneration via axillary shoot proliferation, adventitious shoot organogenesis, and somatic embryogenesis in many woody plant species [21-25].

3.2.2 Effect of BAP on juvenile nodal segments

The data presented in Table 1 and Figure 2 (E) showed that increasing BAP concentration up to 1.5 mg/l increased all the measurement parameters of nodal explants taken from the germinated seedlings (juvenile explant). Nodal explants significantly responded to all concentrations of BAP in comparison to the control treatment (BAP-free medium). Nodal segments gave the highest shoot number (2.5 shoots /explant), shoot length (4 cm) and leaves number (5 leaves/shoot) when cultured on MS medium supplemented with 1.5 mg/l BAP. The promoting effect of BAP on shoot proliferation of *Koelreuteria* spp and other members of family Sapindaceae was reported by several investigators [8, 9, 25, 26]. Groach *et al.* [26] reported that using BAP at 1.5 mg/l in the multiplication stage of *Koelreuteria elegans* gave the highest shoot regeneration (70%) and the highest number of shoots (4.35 shoots) from calli explants.

Table 1. Effect of different concentrations of BAP on axillary shoots induction from nodal explants (juvenile phase) of *Koelreuteria bipinnata*.

Treatment	**Measurements**			
BAP mg/l	**Axillary shoots induction (%)***	**No. of axillary shoots/ explant**	**Shoot length (cm)**	**Leaves number/ shoot**
0.0	50.00	1.00	2.10	2.00
0.5	90.00	1.25	2.30	2.23
1.0	90.00	2.00	2.70	3.33
1.5	100.00	2.50	4.00	5.00
2.0	70.00	1.90	3.80	3.68
LSD at 5%*	11.50	0.11	0.62	0.74

***Means were compared using Least Significant Difference (LSD) test at 5% level of probability

3.3 *In vitro* rooting

In the present study, shoots were easily rooted (100%) after two weeks when cultured on half-strength MS medium supplemented with sucrose at either 15 or 20 g/l (Table 2). A similar result was found by Yang *et al.* [27], who reported that the greatest number of roots (3.8 roots per explant) was formed when shoot tips of the *Melaleuca* tree were cultured on MS medium supplemented with 20 g /l sucrose. As shown in Table 2, using 15 and 20 g sucrose/l gave the highest root number (2.80 and 1.90 roots /explant) and root length (5.87 and 5.30 cm) respectively. Increasing sucrose concentration up to 30g/l significantly decreased rooting percentage, root number/shoot and root length. The results also demonstrated that the addition of auxins is

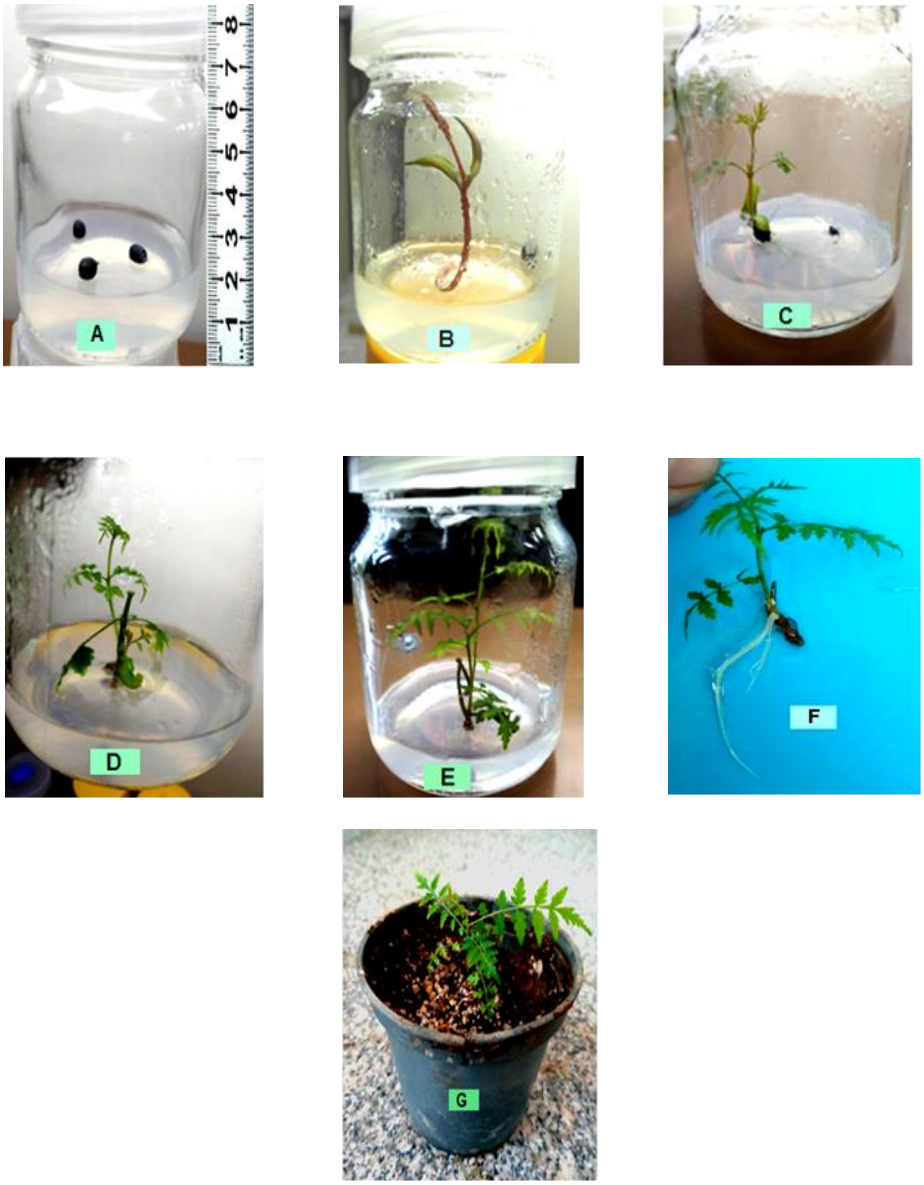

Figure 2. Micropropagation of *Koelreuteria bipinnata* from *in vitro* germinated seeds (juvenile phase). (A) aseptically cultured seeds on PGRs-free MS medium; (B and C) germinated seeds after 4 weeks; (D) cultured nodal explants on PGRs-free MS medium for axillary shoot induction; (E) shoot multiplication on MS medium with 1.5 mg/l BAP after 3 weeks; (F) Rooting of isolated single shoot on 1/2 MS medium with 15 g sucrose/l after 2 weeks; (G) Acclimatized plantlets transferred to plastic pots with peat moss and perlite (1:1) after 4 weeks

unnecessary. Similar results were reported by Tawfik [28] on *Melaleuca* where the explants formed roots in auxin-free medium. Also, Tawfik *et al*. [29] reported that the best interaction effect on rooting percentage (61.00%), highest number of roots/shoot (5.12) and root length (3.33 cm) were observed when the shoots of *Rosa* spp cv Eiffel Tower were cultured on hormone-free MS medium and pH level was adjusted to 5.5.

Table 2. Effect of different concentrations of sucrose on rooting percentage, root number and root length of *Koelreuteria bipinnata*

Sucrose g/l	**Measurements**		
	Rooting %*	**Roots number/ shoot**	**Root Length (cm)**
15	100.00	2.80	5.87
20	100.00	1.90	5.30
25	90.00	1.00	4.83
30	90.00	1.00	4.35
LSD at 5%*	9.41	0.27	0.68

***Means were compared using Least Significant Difference (LSD) test at 5% level of probability

3.4 Acclimatization stage

Acclimatization of plantlets is a vital step in the micropropagation of any plant species. It involves the transfer of plantlets from tissue culture containers to *ex vitro* conditions. In the present study, a plantlet of *K. bipinnata* is shown in Figure 2 (G). Acclimatized plantlets were transferred to plastic pots with peat moss and perlite (1:1) after four weeks. Plantlets were successfully acclimatized and transferred to the field with 60% survival rate.

4. Conclusions

Nodal segments of *Koelreuteria bipinnata* from *in vitro* growing seedlings (juvenile phase) were used for mass production of good-looking trees. The best medium for axillary shoot multiplication was MS supplemented with BAP at 1.5 mg/l. The highest rooting percentage (100%) was obtained on half MS medium supplemented with 15 g/l sucrose. Plantlets were successfully acclimatized on peat moss and perlite (1:1) with a survival rate of 60%.

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