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An efficient protocol for in vitro organogenesis and antioxidant studies in Melia dubia Cav.

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Melia dubia Cav. (Meliaceae) is a multipurpose tree of tropical and subtropical regions mainly cultivated for its medicinal and industrial importance. Due to its versatile properties, it has been depleted in its natural environment. Moreover due to sluggish and poor seed germination, there is a threat of its gene pool exclusion from the natural habitat. The alternative method for conservation and efficient mass propagation is thus need of the hour. As per the extensive literature survey there is no report on efficient protocol for mass propagation of M. dubia through callus organogenesis. Therefore, the present work was aimed to develop in vitro organogenesis protocol for rapid and large scale production of planting material. From our results, maximum callus percentage, callus weight and fragile callus was observed on 1.0 mg/l benzylaminopurine (BA) in combination with 0.5 mg/l naphthalene acetic acid (NAA). The callus differentiation was achieved at different concentrations of BA and indole acetic acid (IAA). Multiple Shoot number per callus propagule 5.30 was observed on 0.5 mg/l BAB and 1 mg/l IAA concentration. The maximum rooting percentage (78.5%), root number per explant (4.33) and root length per explant (4.41 cm) was observed at 0.5 mg/l indol butyric acid (IBA) after 30 days of inoculation. Further the total flavonoid content, phenolic content and antioxidant properties of leaves of in-vitro regenerated plants were studied. Total flavonoids and phenolic content in leaves of in vitro Melia dubia was 0.56 ± 0.8 mg quercitin equivalent (QE) and 2.97 ± 0.17 mg gallic acid equivalent (GAE) respectively. The antioxidant property was further assed through measurement of DPPH radical scavenging activity. The in-vitro regeneration protocol can be exploited for commercial cultivation and fulfilling the growing demand for fresh explant material through mass propagation of M. dubia an economically important plant species.

Key words: Melia dubia, antioxidant, indole-3-butyric acid, flavonoids and phenolics.

INTRODUCTION

Melia dubia, a dicotyledonous multipurpose tree belonging to family Meliaceae, has huge commercial and industrial potential. The species is native to southern Asia (India-Pakistan-Iran) and has been introduced to South Africa, Middle East, America (Bermuda, Brazil and Argentina), Australia, Southeast (SE) Asia-Pacific Islands and

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southern Europe (Ram et al., 2014). Due to its multi-purpose uses like bioenergy production, paper and pulp manufacturing, furniture making, building constructions, making musical instruments etc., it has gained a great deal of attractiveness and elevated demand (Mandang and Aristien, 2003; Suprapti and Hudiansyah, 2004; Parthiban et al., 2009; Chinnaraj et al., 2011). Other than its industrial and commercial importance, the plant has proficient medicinal properties. The various extracts from different parts of the plant are known to have pharmacological importance which includes, antiviral, antibacterial, antifungal, antidiabetic, antineoplastic, antihelmintic and antileprosy properties (Kirikar and Basu, 1999; Pettit, 2002; Nagalakshmi et al., 2003; Vijayan et al., 2004; Gerige and Ramjaneyulu, 2007; Susheela et al., 2008; Sukumaram and Raj, 2010; Sharma and Arya, 2011). Due to its elevated demand for commercial, industrial and therapeutic basis, *M. dubia* trees growing naturally have been indiscriminately logged which resulted in significant decline in its population. Conventionally, *M. dubia* is propagated through seeds, which have very poor (14-34.3%) germination rates because of hard stony seed coat, which makes it difficult to germinate without any treatment (Nair et al., 2005; Manjunatha, 2007; Anand et al., 2012). Therefore, it is imperative to use an efficient plant regeneration system under in vitro conditions for large scale production of planting material of the species from superior genotypes for quick rejuvenation. For large scale production, efficiency of propagation method is imperative. The purpose of micropropagation is to develop physiologically stable plantlets which can be acclimatized in a reduced time period and at a lower cost. In this backdrop, we have established a well developed in vitro regeneration system for *M. dubia* for rapid and large scale production of planting material. There are many reports of in vitro regeneration of the plant through axially bud proliferation (Ram et al., 2014), but it is the first report of regeneration through in vitro callus organogenesis. The ethanolic extract of field grown plants has promising antioxidant activity with IC$_{50}$ (16.89 μg/ml) value (Valentina et al., 2013). In this direction, the present study was envisaged to evaluate the antioxidant activities of leaf tissue from in vitro regenerated plants of *M. dubia*. This activity can be ascribed to the phenolic and flavonoid compounds present in the species. Consequently the study was extended to determine total flavonoid and phenolic content of leaf tissue. The present protocol can serve as an important tool for commercial and industrial supply of bulk plant material of *M. dubia*. The tissue culture raised plants were further evaluated for their total phenolic and flavonoid content viz-a-viz its antioxidant property.

**MATERIALS AND METHODS**

**Explants collection**

Experiments were carried out in plant tissue culture laboratory of the Institute of Wood Science and Technology (IWSTR), Bangalore. Four Simple Randomized experiments were carried out. Explants of *M. dubia* were collected from established cultures grown under aseptic in vitro conditions. The leaf explants were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with different doses of N$^2$-Benzyladenine (BA; 0.25, 0.5 and 1.0, 2 mg/l) and α-Naphthalene acetic acid (NAA; 0.1, 0.25, 0.5 and 1.0 mg/l) for callus initiation in first experiment. In second experiment callus multiplication was observed on MS medium supplemented with BA and NAA at their best combination. In the third experiment, healthy shoots were developed on benzylaminopurine (BAP; 1.0, 1.25, 0.5 and 0.25 mg/l) and Indole-3-acetic acid IAA (0, 1.0 2.0 mg/l). In fourth experiment in vitro raised micro-shoots were transferred to MS/2 strength rooting medium supplemented with different doses of Indole-3-butyric acid (0.1, 0.25, 0.5 and 1.0 mg/l).

**Culture conditions**

The inorganic salts used for preparation of culture medium were obtained from Qualigens Pvt. Ltd., India and phytohormones and B vitamins from Sigma Chemicals Pvt. Ltd., India. The medium contained 3% (w/v) sucrose, 0.6% (w/v) agar (Hi-Media Chemical Ltd., India). The pH of the medium was adjusted to 6.0 before autoclaving for 15 min at 1.06 kg cm$^{-2}$ (121°C). Explants were cultured in a 150 ml Botel Borosi® Botails containing 40 ml semi-solid medium. For in vitro shoot multiplication and rooting experiment, the cultures were incubated at 25 ± 2°C under 16 h illuminations with fluorescent light (50 μmol Em$^{-2}$ s$^{-1}$).

**Hardening and transplantation**

Tissue culture raised microshoots of 3 to 4 cm in length with 2 to 3 nodes were tested with various concentrations of IBA, indol butyric acid (IAA) and NAA either alone or in combinations for in vitro rooting. The rooted plantlets of *M. dubia* were removed from agar-agar and washed with distilled water to remove the traces of agar. Plantlets were transferred to root trainers containing autoclaved potting mixture of vermiculite, sand and soil (1:2:1 v/v/v) and placed under 4 weeks in mist chamber at 30 ± 5°C temperature and > 60% relative humidity (RH). Later on the plants were kept in polythene bags containing autoclaved soil and then shifted to a shade net (50% shade) for another 2 weeks before placing it in open nursery. After successful acclimatization plants were finally transferred to natural condition.

**Quantification of total flavonoid content**

Total flavonoid content was quantified using spectrophotometer. Dried crude extract (prepared from 100 mg of dried plant material) dissolved in 500 μl of distilled water was mixed with 30 μl of a 5% NaNO$_2$ solution, followed by 5 min of incubation at room temperature. After the incubation, 300 μl of 10% AlCl$_3$-H$_2$O solution was added and the sample was further incubated for 6 min incubation. Finally 200 μl of 1 M NaOH and 200 μl of distilled water were added to the sample and absorbance was read at 510 nm. Total flavonoids were calculated using quercetin as standard (10 to 100 μg; R$^2 = 0.998$). The results were expressed as mg quercitin equivalent (mg QE) per gram dry weight of the plant material. The experiment was repetitively performed in triplicates.

**Quantification of total phenolic content**

Total phenolic content in leaves of *M. dubia* was measured using the Folin–Ciocalteu reagent method as described by Pinelo et al. (2004). The total phenolic content was expressed as mg of Gallic
Table 1. Effect of BA, NAA and their interaction on callus induction (%), callus weight and texture of callus induction in M. dubia at 30 days after inoculation. Values in the parentheses are arc sine transformation.

| Treatment numbers | BA and NAA (mg/l) | 30 Days after inoculation | Callus formation (%) | Fresh weight (g) | Disc (Callus texture) |
|-------------------|-------------------|--------------------------|----------------------|-----------------|----------------------|
| T₁                | BAP (0.25) + NAA (0.1) | 69(56.5) | 1.84±0.20 * | Compact |
| T₂                | BAP (0.5) + NAA (0.25) | 83(70.08) | 2.13±0.32 | Compact |
| T₃                | BAP (1.0) + NAA (0.5) | 100(85.84) | 2.44±0.36 | Fragile |
| T₄                | BAP (2.0) + NAA (1.0) | 66(54.33) | 1.72±0.19 | Compact |
| LSD (0.05)        |                   | 0.60 | 0.08±0.01 |                   |

*S.E; (LSD= Least Significant Difference).

Table 2. Effect of BAP and NAA on in vitro callus multiplication of M. dubia at 30 days after inoculation.

| Treatments | BA and NAA (mg/l) | 30 Days after inoculation | Fresh weight (g) |
|------------|-------------------|--------------------------|-----------------|
| T₁         | BAP               | 3.47±1.03                |                 |
| T₂         | BAP (0.25) + NAA (0.25) | 4.60±1.65               |                 |
| T₃         | BAP (1.5) + NAA (0.5) | 5.62±1.80               |                 |
| T₄         | BAP (2.5) + NAA (1.0) | 4.23±1.55               |                 |
| LSD(0.05)  |                   | 0.19±0.05               |                 |

*S.E (LSD= Least Significant Difference).

RESULTS

Callus induction

The experiment was aimed to investigate the different doses of BA and NAA (0.25, 0.5, 1.0, 2, 0.1, 0.25, 0.5, and 1.0 mg/l) on callus induction weight and callus texture at 30 days after incubation (Table 1). The effect of different doses of BA and NAA and their interaction on callus induction (%), weight and callus texture was found to be significant at the stage of sampling. Maximum callus induction, callus fresh weight and the best callus texture was observed on 1.0 mg/l BA and 0.5 mg/l NAA which was 57.99 and 41.86% in comparison to BAP 2.0 mg/l + NAA 1.0 mg/l. Fragile callus was observed on 1.0 mg/l BA and 0.5 mg/l NAA and compact callus was observed on other treatments.

Statistical analysis

Each experiment had three replicates for in vitro shoot multiplication and rooting. Each replicate had 10 propagules. The data were subjected to one way analysis of variance (ANOVA). All the experiments ascertained with "F" test for level of significance. The significance at p ≤ 0.05, LSD₀.₀₅ was computed for comparison of treatment means.

Callus multiplication

The experiment was carried out to investigate the different doses of BA and NAA (0, 0.25, 1.5, 2.5, 0, 0.25, 0.5, and 1.0 mg/l) on callus multiplication at 30 days after incubation (Table 2). Various doses of BA and NAA significantly influenced callus multiplication at the stages of sampling Figure 1a. Fresh weight of callus was found to be maximum on 1.5 mg/l BA and 0.5 mg/l NAA which was 51.6% more in comparison to BAP 2.0 mg/l + NAA 1.0 mg/l. Fragile callus was observed on 1.0 mg/l BA and 0.5 mg/l NAA and compact callus was observed on other treatments.

Callus differentiation

Various doses of BA and IAA (1.0, 1.25, 0.5, 0.25, 0, 1.0 and 2.0 mg/l) significantly influenced callus differentiation
at 30 days after inoculation Figure 1b. The highest shoot number per callus propagule was observed on 0.5 mg/l BA and 1 mg/l IAA. Shoot number per callus propagule was 133% more in comparison to treatment 1 Figure 1c. Shoot length per callus propagule was recorded at 30 days after inoculation. Shoot length per callus propagule was found to be maximum at 0.5 mg/l BA and 1 mg/l IAA which was 175% more in comparison to treatment 1 (Table 3).

**In vitro rooting**

The experiment was carried out to study the effect of four doses of IBA (0.1, 0.25, 0.5 and 1.0 mg/l) with MS/2 medium and their all possible interactions on *in vitro* rooting (%) and root number per explant at 30 days after inoculation (Table 4). Various doses of IBA significantly influenced rooting (%) at the stage of sampling Figure 1d-f. The effect of 0.5 mg/l IBA provide significantly maximum rooting % which was 71% more in comparison to 0.25 mg/l IBA.

**Root number per explant**

The effect of four doses of IBA significantly enhanced root number per explant at 30 days after inoculation. 0.5 mg/l IBA provide significantly maximum on root number per explant which was 270% more in comparison to 0.25 mg/l IBA.
Table 3. Effect of BAP and IAA on callus differentiation in *M. dubia* at 30 days after inoculation.

| Treatments | BAP+IAA (mg/l) | Shoot number/explant | Shoot length/explant (cm) |
|------------|----------------|----------------------|---------------------------|
| T<sub>1</sub> | BAP (1.0) | 2.27±0.32* | 1.27±0.22* |
| T<sub>2</sub> | BAP (1.25) | 3.13±0.54 | 1.37±0.27 |
| T<sub>3</sub> | BAP (0.5) + IAA (1.0) | 5.30±1.23 | 3.53±0.64 |
| T<sub>4</sub> | BAP (0.25) + IAA (2.0) | 4.27±1.10 | 3.27±0.61 |
| LSD (0.05) | | 0.28±0.03 | 0.28±0.02 |

*SE (LSD= Least Significant Difference).

Table 4. Effect of different concentrations of IBA and MS/2 medium on *in vitro* rooting %, root number and root length in *M. dubia* at 30 days after inoculation. Values in the parentheses are arc sine transformation.

| Treatment numbers | IBA (mg/l) | Rooting (%) | Root number per explant | Root length per explant (cm) |
|-------------------|------------|-------------|------------------------|-----------------------------|
| T<sub>1</sub> | IBA (0.1) | 56 (49.2) | 1.27±0.26* | 1.37±0.32* |
| T<sub>2</sub> | IBA (0.25) | 53 (48) | 3.22±1.25 | 3.23±1.30 |
| T<sub>3</sub> | IBA (0.5) | 96 (78.5) | 4.33±1.54 | 4.41±1.61 |
| T<sub>4</sub> | IBA (1.0) | 60 (51.4) | 1.17±0.21 | 1.13±0.30 |
| LSD (0.05) | | 20.21 | 0.19±0.02 | 0.13±0.02 |

*SE (LSD= Least Significant Difference).

Table 5. Measurement of total flavonoids, phenolics contents and antioxidant activity in extracts prepared from leaves of tissue culture raised plant of *Melia dubia*.

| Material | Total flavonoid content (mg QE) | Total phenolic content (mg GAE) | IC<sub>50</sub> (µg/ml) |
|----------|--------------------------------|--------------------------------|------------------------|
| Leaves   | 0.56 ± 0.08*                   | 2.97 ± 0.17*                   | 242.88 ± 12.55*        |

*S.E, P-value < 0.05. IC<sub>50</sub>: the concentration of extracts (µg/ml) causing 50% inhibition of DPPH radical.

mg/l IBA.

Root length per explant

Administration of different doses of IBA significantly enhanced root length per explant at 30 days after inoculation. 0.5 mg/l IBA provide significantly maximum for root length per explant which was 290% more in comparison to 0.25 mg/l IBA.

Hardening

Following hardening procedures, 6-weeks old hardened plants (Figure 1g-i) were successfully transferred to field conditions. The survival rate of *in vitro* raised plants under hardening conditions showed prominent growth with about 94% success. This gives the suitability of protocol.

Total phenolic and flavonoid content

Flavonoids significantly contribute to the total antioxidant property of the plants (Luo et al. 2002). Total phenolic content were found to be high in *M. dubia* in comparison to total flavonoids content (Table 5).

DPPH radical-scavenging activity

DPPH method is a simple, rapid and reproducible assay used for measuring the antioxidant activity of plant extracts (Mishra et al., 2012). The IC<sub>50</sub> value for methanolic extract of leaves of *M. dubia* was found to be 242.88 ± 12.55 µg/ml (Table 5). The antioxidant activity (DPPH radical-scavenging activity) of all the extracts was lower than that of ascorbic acid (IC<sub>50</sub>, 6.1 ± 0.32 µg/ml), used as positive control; a lower IC<sub>50</sub> value corresponds to a higher antioxidant activity of sample.
DISCUSSION

A culture medium is defined as a formulation of inorganic salts and organic compounds (apart from major carbohydrate sources and plant growth regulators) used for the nutrition of plant cultures (George, 1993). It usually consists of a balanced mixture of macro-and-micro elements together with vitamins and other organic nutrients, including a carbon source. Nutritional requirements for optimal growth of a tissue, in vitro, may vary with the species and parts of a plant. When starting with a new system, it is essential to work out a medium that will fulfill the specific requirements of the tissues (Bhojwani and Razdan, 1996; Smith, 2000). During the present investigations on M. dubia MS nutrient medium was used. There are many reports comparing different media for their effect on in vitro shoot multiplication (Rugini, 1984; Mehta et al., 2000; Lu, 2005; Jain et al., 2009). Most of the workers have routinely used MS medium for shoot multiplication (Reddy et al., 1998; Komalavalli and Rao, 2000; Devi and Srinivasan, 2008). Sha Valli Khan et al. (2002), reported the multiplication of white friable callus on MS medium with NAA (1.0 μM) in combination with BAP (1.0 μM) in Bixa orellana L. Nirmalakumari et al. (1993) reported 6-7 shoots from one month old callus of leaf and stem on MS medium supplemented with BAP (2.0 mg/l) and IAA (0.5 mg/l) in Azadirachta indica. Chaicharoen et al. (1996) observed maximum 9 shoot from callus, when medium was supplemented with 1.0 mg/l BA in Melia azedarach. Chaturvedi et al. (2003) revealed that 5 μM BAP alone favored shoot induction from anther derived callus on MS medium. Srivastava et al. (2009) observed maximum (78%) shoot regeneration when callus was sub cultured on MS medium containing 5 μM BAP alone in A. indica. Contrary to the above observation, Islam et al. (1993) reported that shoot organogenesis was the best from cotyledons origin callus on MS medium with 2.0 mg/l BA and 0.2 mg/l NAA used in A. indica. Vila et al. (2003) in M. azedarach reported successful regeneration of plantlets from leaf derived callus by using BA (4.4 μM) and NAA (0.46 μM) in MS medium. Sharry et al. (2006) reported induction of multiple shoots from callus in MS medium with NAA (0.5 mg/l) and BAP (1.0 mg/l). In the present study, IBA was found to be the most excellent auxin for rooting in terms of number of roots and root length. In accordance to our result, IBA have been used extensively for rooting in a wide range of plant species of Meliaceae family such as; M. azedarach (Thakur et al., 1998; Sen et al., 2010), IBA 1.0 mg/l in MS/2 medium favoured the best root induction 83 to 90% respectively. Chemically MS medium shows variation in concentration of different components as compared to other media. It has been observed that IAA, IBA and NAA enriched medium was also responsible for root induction in B. tulda (Saxena, 1990) and D. strictus (Mascarenhas and Murlidharan, 1989). In case of M. dubia IBA is best which play a key role in root induction. The procedure offers an efficient and rapid method which can be adopted commercially for mass multiplication of M. dubia. MS medium supplemented with NAA 0.5 mg/l + BAP1.0 mg/l on callus multiplication, BAP 0.5 mg/l + IAA 2 mg/l for shoot regeneration and MS/2 with IBA 0.5 mg/l for in vitro rooting and has been selected for efficient and rapid multiplication of M. dubia 30 days after inoculation. Also the technology developed may be used to get sustained supply of M. dubia, which requires raising them on a mass scale for plantations and forestation purposes. Commercial exploitation of this protocol for multiplication of this economically important species is possible as demand for fresh explant can meet easily.

Plants produce a diverse number of secondary metabolites in response to changing oxidative environment. As such plant extracts are expected to contain a diversity of molecules with different structures and functions. M. dubia is being previously reported for the presence of flavonoids and phenolic compound (Murugesan et al., 2013; Gopal and Manju, 2015). In the present study total flavonoids content, phenolic content and antioxidant potential were determined from the leaves of tissue culture raised plant of M. dubia. In this study, flavonoid content was found to be 0.56 ± 0.08 mg quercitin equivalent (mg QE) per gram dry weight of the plant material, while total phenolic content was 2.97 ± 0.17 mg gallic acid equivalent (mg GAE) per gram dry weight of the plant material. Flavonoid and phenolic content reported in other species of meliaceae family such as; A. indica was 0.2 to 1.07 mg/g of plant material (phenolic content) and 0.61 to 5.29 mg/g of plant material (flavonoids content). These report are comparable with the total flavonoids and phenolic content of M. dubia (Khamis Al-Jadidi and Hossain, 2015).

Conclusion

As such there is no report on efficient protocol for mass propagation of M. dubia. Therefore, the present work was aimed to develop in vitro propagation protocol for rapid and large scale production of planting material. The results demonstrate that the leaves may act as excellent source for isolation of potential antioxidants with significant amount of flavonoids and phenolics. They may serve as natural antioxidants in pharmaceutical preparations.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations

BAP, 6-Benzylaminopurine; NAA, α-naphthalene acetic acid; IAA, indole-3- acetic acid; IBA, indole-3-butyric acid; DPPH, 2, 2-diphenyl-1-picrylhydrazly; GAE, gallic acid equivalent; QE, quercetin equivalent.

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