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# An efficient micropropagation protocol for direct organogenesis from leaf explants of an economically valuable plant, drumstick (*Moringa oleifera* Lam.)

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ABSTRACT

Drumstick is a tree with a range of potential commercial uses: the vegetative organs have high nutritional value and the seeds have high oil content. In the present study, an efficient regeneration protocol for this multipurpose tree has been developed using leaf segments as explants. In addition, the effects of genotype, developmental stage and inoculation orientation of the explants, and the combination of plant growth regulators (PGRs) in the culture medium have been investigated. It was found that Murashige and Skoog (MS) basal elements supplemented with 0.8 mg/L 6-benzyladenine (BA), 0.2 mg/L kinetin (KT) and 0.05 mg/L a-naphthaleneacetic acid (NAA) provided the most suitable medium for shoot-bud regeneration, delivering a maximum regeneration frequency of 93.33% and a mean of 4.40 shoots per explant. In addition, orienting the distal ends of the explants down towards the medium was much better than them facing upwards. We were able to root approximately 90% of the regenerated shoots on MS medium with 0.1 mg/L NAA. Even though success of this protocol varied according to genotype, it still has high applicability at a large scale and could be used in the production of high quality plantlets to meet large scale cultivation needs. In addition, it may be useful for genetic transformation studies.


## 1. Introduction

*Moringa oleifera* Lam., commonly known as drumstick (Ramachandran et al., 1980), belongs to the monogeneric family *Moringaceae* and is naturally distributed in the sub-Himalayan areas of India and various tropical African countries (Leone et al., 2015; Popoola and Obembe, 2013). Because of its high commercial value, it is now widely planted in the tropics and sub-tropics, both as a native and as an exotic species. Drumstick has a number of medicinal properties (Anwar et al., 2007; Fahey, 2005) and high nutritional value (Sánchez-Machado et al., 2010), and the seeds and leaves are a good source of anti-oxidants (Govardhan-Singh et al., 2013; Shih et al., 2011), attracting the interest of many cosmetic and nutraceutical industries. In addition, it is valuable as a source of natural coagulants, forestry products, fertilizer, and for living fencing, alley cropping and fuel (Popoola and Obembe, 2013).

Drumstick is generally propagated by seed but the viability of the seeds declines quickly (Fotouo-M et al., 2015). Moreover, propagation by seeds is not recommended due to the heterogeneous nature of the seedlings. Thus, using somatic tissues as explants for propagation is particularly valuable in drumstick production. At present, genetic engineering is useful for studying gene function and improving aspects of the performance of plant species. The absence of highly efficient regeneration protocols for drumstick has hampered the species' genetic transformation. Thus, establishing a highly stable and efficient regeneration system for drumstick would be especially valuable. To date, several studies on propagating drumstick have been published. The techniques described have included: shoot-bud proliferation of nodal explants taken from young seedlings on medium supplemented with 0.5–1.0 mg/L BA,

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producing a maximum average of 1.65–4.0 axillary shoots (Förster et al., 2013; Gayathri et al., 2015; Marfori, 2010; Mathur et al., 2014; Saini et al., 2012) and from mature plants on medium containing 1.0 mg/L BA (Islam et al., 2005); proliferation from the cotyledonary node without using plant growth regulators (Steinitz et al., 2009); and regeneration from hypocotyl explants cultured on MS medium supplemented with 0.1–0.5 mg/L KT (Shahzad et al., 2014). However, the regeneration frequency is still low if the aim is to develop an efficient propagation system and, in addition, the starting materials (nodal, cotyledonary node, hypocotyl) are relatively limited. Only one study started with leaves, and the researchers were only able to produce callus when they eventually cultivated the leaves on a medium with IAA and TDZ (Förster et al., 2013). Leaves are abundant compared with other plant parts and they have low genetic variation, but so far there is no adequate regeneration protocol that uses them. The purpose of this study was to establish a highly efficient and sustainable regeneration system from the leaves of drumstick, which will be useful for micropropagation and genetic transformation. We also investigated factors affecting direct shoot regeneration, such as PGRs, developmental stage, orientation, and genotype of explants in shoot-bud induction, proliferation, elongation and rooting.

## 2. Materials and methods

### 2.1. Plant materials and explant source

Seeds were harvested from trees of the PKM-1 cultivar at the drumstick germplasm conservation farm of the South China Agricultural University. The seed coat was removed, and the seeds were soaked in tap water overnight. After surface-sterilization with 75% (v/v) ethanol for 50 s and with 0.1% (w/v) mercuric chloride solution for 15 min, the seeds were rinsed five times with sterile distilled water and inoculated on PGR-free, 4.5 g/L agar-solidified MS medium (Murashige and Skoog, 1962) containing 30 g/L sucrose. Two weeks later, when the seedlings growing from the seeds had reached a height of about 5 cm, the leaves of the seedlings were used as a source of explants for the experiments.

### 2.2. Culture media and growth conditions

All the media used were adjusted to pH 5.8–6.0 with 1*N* NaOH or 1*N* HCl solution, set using 0.45% (w/v) agar and autoclaved at 121 °C for 15 min. Cylindrical, transparent polypropylene bottles (10 cm height and 7 cm diameter) with an internal volume of 280 mL were used for all the cultures. Each bottle was filled with 40 mL medium and covered with a polycarbonate screw cap to be almost air-tight. All the cultures were kept under cool-white light (about 50 μmol/m$^2$/s) with a 12 h photoperiod and at a temperature of 25–27 °C.

### 2.3. Induction and growth of shoot-buds

MS basal medium, containing 3% (w/v) sucrose and various concentrations and combinations of the PGRs BA (0.4–1.6 mg/L), KT (0.05–0.8 mg/L), and NAA (0.01–0.2 mg/L) were used to compare the PGR factors influencing the culture responses. Stage 2 leaves (Fig. 1Ac) were used in all experiments, with their distal end in contact with the medium, except in the experiments investigating the influence of developmental stages and orientation. Five trees with different genetic backgrounds, coded M-2, M-5, M-6, M-17 and M-18, were used to examine the influence of genotype on culture responses. Results for shoot-bud induction are given in terms of the proportion of successful explants on day 20. For growth, the results represent the number of shoots per planted explant on day 40.

**Table 1**
Effect of different BA concentrations on shoot-buds induction.

| BA (mg/L) | % Explants regenerated shoots | Shoots per explant |
|---|---|---|
| 0.0 | 0 d | 0 d |
| 0.4 | 33.33 ± 12.60 bc | 1.40 ± 0.54 bc |
| 0.8 | 73.33 ± 11.82 a | 3.0 ± 0.56 a |
| 1.2 | 60.0 ± 13.09 ab | 2.47 ± 0.56 ab |
| 1.6 | 20.0 ± 10.69 cd | 0.53 ± 0.31 cd |

Each value represents the mean ± standard error (SE) of three replicates, each with 10 explants. All the media contained 0.2 mg/L KT and 0.05 mg/L NAA. Means followed by the same letter in the same column are not significantly different from each other at $P \leq 0.05$ level, according to Duncan's multiple range test.

### 2.4. Rooting of the regenerated shoots

The regenerated shoots were cut from the mother tissue and transferred onto MS basal medium containing various NAA concentrations (0.1–0.4 mg/L) to facilitate the initiation of roots and further growth of intact plantlets. The culture conditions were the same as mentioned above for shoot-bud induction and the results were recorded at the end of the experiment, 25 days later.

### 2.5. Acclimatization of the rooted plantlets

Plantlets with well-developed roots were removed carefully from the culture bottles and the roots were washed gently under running tap water to remove any remaining medium. Subsequently, the plantlets were placed in plastic pots filled with autoclaved planting substrates. To ensure high humidity, the pots were covered with transparent polythene bags and the bags were gradually opened 7 days later. After two weeks of acclimatization, the plantlets were ready to be transferred to the field.

### 2.6. Data collection and analysis

All the experiments were arranged in a Randomized Complete Block Design and each treatment consisted of 10 explants and was repeated three times. The number of regenerated, usable buds (about 3 cm in height) from each explant was counted in the bud regeneration cultures, and the number and the length of the roots were measured in the rooting cultures of the isolated buds. Statistical analysis was carried out using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA), Duncan's multiple range test was used to detect differences among means. A *P*-value <0.05 was considered significant.

## 3. Results and discussion

### 3.1. Effect of PGRs on shoot-bud regeneration

Two-week-old seedlings (Fig. 1Aa) were removed aseptically from the culture bottles, and only stage 2 leaves (Fig. 1Ac) were used for the preparation of explants in this experiment. These leaves were excised and cut into pieces measuring about 5 × 5 mm, then placed on MS medium containing cytokinin BA and KT, alone or in combination with NAA, for the induction of organogenesis. Results of the experiments are shown in Tables 1–3 . It is clear that cytokinin was necessary for the regeneration of adventitious buds, even though BA was more effective than KT and similar results were obtained by Khateeb et al. (2013); these hormones could not work effectively alone and the regeneration efficiency was greatly improved when they were combined (Tables 1 and 2). Meanwhile, the role of NAA was just as important as KT in direct shoot induc-