



Efficient micropropagation protocol and genome size estimation of an important cover crop, *Mucuna bracteata* DC. ex Kurz

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Abstract

Mucuna bracteata DC. ex Kurz is an important cover crop in plantations across the tropics. However, low germination rate and poor viability of *Mucuna* seeds pose significant challenges of using the seeds as starting material. To address these limitations, we have optimized seed germination conditions (such as scarification period, surface sterilization protocols and imbibition period) and in vitro propagation protocols for *M. bracteata*. We found that seeds treated with sulphuric acid for 30 min, imbibed for 6 h and incubated in dark conditions on a wet cotton roll (10 mL of sterile distilled water) supplemented with 0.1% activated charcoal produced the highest percentage of seed germination (44%) and seed vigor index. In vitro-derived cotyledonary nodes showed the highest number of shoots per explant (5.60) and rooting response (92.9%) when cultured on Murashige and Skoog medium containing 4.44 μM 6-benzylaminopurine and 10.7 μM 1-naphthaleneacetic acid, respectively. Of the 100 rooted plantlets acclimatized, 89.0% survived after 4 weeks of transplanting. Single sequence repeat and flow cytometry analysis were performed to confirm the genetic fidelity of the plants. Our protocol offers, for the first time, a simple and effective seed germination and scalable propagation procedures for *M. bracteata*. Furthermore, we have also estimated the genome size (1448 ± 9 Mb) and DNA content (1.48 ± 0.01 pg) for *M. bracteata* that can be used for future cytogenetic studies on genetic diversity and gene exchange.

Keywords Leguminous plants · Hydropriming · Osmopriming · Plant propagation · Regeneration · Seed germination · Genome size

Introduction

Mucuna bracteata, a fast-growing perennial leguminous creeper, is an ideal cover crop for rubber and oil palm plantation industries, especially in South India and Southeast Asia (Mathews 1998; Mendham et al. 2004; Ng et al. 2005), due to its high tolerance towards drought and shade conditions. It belongs to the family of Fabaceae (Leguminoceae), which is indigenous to North India and has been found in sub-tropical

climate region in China, Hainan, Laos, Myanmar, Thailand, Vietnam and Andaman Island.

M. bracteata has been studied for its impact on yield (Mathews and Saw 2007; Shaharudin and Jamaluddin 2007), nutrient return (Chiu and Madsun 2006), biomass production and weed management for oil palm (ChinTui et al. 2005; Samedani et al. 2014), indicating its important role as a cover crop. *M. bracteata* may grow to approximately 0.75–1.0 m/week and the mature tap roots can grow up to 2–4 m depth in soil (Mathews 1998; Chiu 2007). Its vigorous growth and ability to tolerate a wide range of climatic conditions made *M. bracteata* a suitable cover crop over conventional cover crops, such as *Pueraria phaseoloides* and *Calapogonium mucunoides*. This is because most conventional cover crops tend to die-back after several years of establishment due to the shading effect from the growing oil palm canopies (Mathews and Saw 2007).

Besides being a cover crop, *M. bracteata* is also popular in Ayurvedic medicine for constipation, oedema, fever, delirium, and dysmenorrhea (Sangvikar et al. 2016).

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Recent studies found that the extracts isolated from young and mature leaves of *M. bracteata* showed antiproliferative activity against nasopharyngeal carcinoma cells (Mai et al. 2009), whereas seed extracts demonstrated potential antimicrobial properties (Kumar et al. 2009).

Despite its importance as a cover crop in plantations and its medicinal prospects, no report has been found on propagating *M. bracteata* through plant tissue culture technique. *Mucuna* is a seed-grown annual herbaceous plant (Chattopadhyay et al. 1995). The presence of needle-like hair trichomes around the seed pods that causes great irritation when touched poses significant challenges of using seeds as starting material (Faisal et al. 2006). Moreover, low germination rate and poor viability of the seeds have limited its supply to plantation companies. Most importantly, seed production is only available to a certain climatic region in North India (Chiu 2007) as *M. bracteata* rarely produces seeds outside of this region although it may flower occasionally. Therefore, plantation companies rely heavily on seed producing companies for their constant supply of *Mucuna* seeds. This dependency is exacerbated with stringent regulatory procedure that may vary between countries for importation of this 'foreign' seeds (Chee 2007). Although some plantation companies have developed their propagation method through stem cutting, the method is cumbersome and the success rate is very much depends on the skill of the laborers and the age of the mother stem (Lee et al. 2007).

In this study, we aimed to optimize the seed germination protocol and to develop an effective and scalable micropropagation protocol for *M. bracteata*.

Materials and methods

Plant material

Seeds of *M. bracteata* DC. ex Kurz procured from a local seed supplier in Subang Jaya, Selangor, Malaysia, were cleaned thoroughly by rinsing twice with tap water, each time for at least 2 min, followed by distilled water for 10 min and dried at room temperature for two nights.

Effect of sulfuric acid treatments on seed germination

Sixty-five dry seeds were immersed in 100 mL of concentrated sulfuric acid (98%), agitated at 100 rpm for 10, 30 or 60 min and rinsed at least five times with sdH₂O. Seeds without immersion in concentrated sulfuric acid were considered as control.

Effect of different disinfection protocols on seed germination

Three surface sterilization protocols were evaluated on seeds that have been scarified for 30 min in sulfuric acid prior to imbibition in sdH₂O at a ratio of 1:20 (seed weight:solution volume) (approximately 5.34 g/100 mL) for 6 h. In the first treatment, scarified seeds were immersed in 95% (v/v) ethanol for 1 min followed by rinsing three times with sdH₂O. In the second treatment, scarified seeds were immersed in 50% (v/v) Clorox (commercial bleach with 5.25% w/v sodium hypochlorite) containing 7 drops of Tween 20 per 100 mL and agitated at 100 rpm for 10 min. The seeds were then rinsed five times with sdH₂O. In the third treatment, scarified seeds were immersed in 95% (v/v) ethanol for 1 min, rinsed three times with sdH₂O, disinfected in 50% (v/v) Clorox for 10 min and washed five times with sdH₂O. All disinfected seeds were rinsed once with sdH₂O before culturing on semi-solid Murashige and Skoog (MS) (1962) medium supplemented with 3% (w/v) sucrose. The media were adjusted to pH 5.8 and autoclaved at 121 °C for 15 min. The cultures were maintained in the dark at room temperature.

Effect of culture methods and photoperiods on seed germination

To determine the effect of culture methods and photoperiods on seed germination, new batch of scarified, disinfected and imbibed seeds that underwent the above optimized protocol was cultured on either semi-solid MS medium or double layers of cotton roll moistened with 10 mL of sdH₂O. All cultures were maintained at room temperature either in the dark or under 16 h light regime with a light intensity of 31.4 μmol m⁻² s⁻¹ provided by cool fluorescent lamps.

Effect of hydropriming conditions on seed germination

Seeds that have been scarified for 30 min in sulfuric acid were imbibed in sdH₂O for different durations (0–48 h) and rinsed once with sdH₂O before culturing on double layers of cotton roll moistened with different volumes of sdH₂O (0–40 mL).

Effect of osmopriming and antioxidant treatments on seed germination

The suitable imbibition period and volume of sdH₂O selected from the hydropriming experiment was used for osmopriming treatment. In this experiment, seeds that have been scarified for 30 min in sulfuric acid were primed in

either 10% (w/v) polyethylene glycol (PEG) 6000 solution or sdH₂O for 6 h. Seeds primed in 10% PEG 6000 solution were rinsed at least five times with sdH₂O before culturing on double layers of cotton roll moistened with 10 mL of sdH₂O, whereas seeds primed in sdH₂O were cultured on double layer cotton roll moistened with 10 mL of sdH₂O, 10 mL of 2.27 mM ascorbic acid (AA) or 10 mL of 0.1% (w/v) activated charcoal (AC).

Effect of different types and concentrations of cytokinins on shoot induction and multiplication

Nodal segments, leaf discs and cotyledonary nodes were used as explants for shoot multiplication. Nodal segments and leaf discs (50 mm diameter) were excised from fully germinated in vitro seedlings (1 month-old), whereas cotyledonary nodal explants were prepared from 5 day-old seed cultures by removing the plumules and radicles. All explants were cultured on semi-solid MS medium containing different concentrations of 6-benzylaminopurine (BAP) (4.44–17.76 μM) and kinetin (4.65–18.59 μM). All cultures were maintained under 16 h light regime at room temperature. The shoot height and number of shoots per nodal segment and cotyledonary node were determined after 1 month of culture. The percentage of callus formation was recorded in leaf discs after 2 months of culture since there was no shoot formation. Each treatment consisted of 30 explants and the experiment was repeated three times.

Effect of different types and concentrations of auxins on root induction

Elongated shoots (5–20 cm in length) were excised from shoot clumps and transferred to semi-solid MS medium supplemented with different concentrations of 1-naphthylacetic acid (NAA) (2.69–16.1 μM). Each treatment consisted of 15 shoots and the experiment was repeated three times. All cultures were maintained under 16 h light regime at room temperature. The percentage of shoots with roots, number of roots per shoot and root length were recorded after 1 month of culture. Rooted shoots were washed with distilled water before transferring to polybags containing garden soil. The plants were grown in a greenhouse at the University of Malaya, Malaysia, and fully covered with perforated transparent polybags before gradually removed after 2 weeks of culture. The plants were watered twice a day.

Estimation of DNA content and genome size via flow cytometry analysis

A young reddish-purple leaf (1 week-old) randomly selected from in vitro-derived or seed-germinated plants was chopped with razor blades in a Petri dish containing 0.9 mL of LB01

lysis buffer that was supplemented with 50 μg/mL RNase A and 50 μg/mL propidium iodide to release nuclei (Doležel and Bartos 2005). The suspension was filtered through a 40 μm BD Falcon cell strainer into a 5 mL round bottom centrifuge tube. The filtrate was incubated at 4 °C for 10 min. The suspension was analyzed using BD FACSCalibur (BD Bioscience, San Jose, CA). A minimum of 10,000 nuclei were measured per sample. Three randomly selected in vitro-derived and seed-germinated plants with a total of three young leaves per plant were used in this study. The histograms of relative fluorescence intensity from in vitro-derived plants were compared with seed-germinated plants to determine the changes of DNA content based on the following formula: (Fluorescence intensity of sample/Fluorescence intensity of standard) × Genome size of soybean (2.5 pg) (Doležel and Bartos 2005). Fluorescence intensity of nuclei extracted from young leaves of soybean (*Glycine max* cv. Polanka) was used as the standard. Based on the calculated DNA content, genome size (bp) of *M. bracteata* was then estimated by using the following formula: $(0.978 \times 10^9) \times \text{DNA content (pg)}$ (Doležel et al. 2003).

Simple sequence repeats (SSR) analysis

SSR analysis was performed to evaluate the genetic fidelity of the in vitro-derived plants. A seed-germinated plant and ten randomly selected in vitro-derived plants were analyzed using ten SSR primers (Table 1). Genomic DNA was extracted from leaves of the selected plants using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Amplification was carried out in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 2.5 mM of MgCl₂, 1 μM of each forward and reverse primer, 1 mM of dNTP mix and 0.05 U of *i*-Taq DNA polymerase (iNtRON, Korea). The amplification reaction was carried out in a thermocycler (Bio-Rad Laboratories, Hercules, CA) with the following cyclic profiles: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at different temperatures based on the SSR primer (Table 1), 30 s extension at 72 °C for 30 s and a final extension cycle at 72 °C for 5 min. The PCR products were resolved on a 1% (w/v) agarose gel and visualized using a gel documentation system.

Data collection

The percentage of seed germination was recorded at days 4, 8 and 12 of culture. Seeds were defined as germinated when both plumule and radicle emerged from the seed coat. Each treatment consisted of 30 seeds with five seeds laid out per Petri dish and the entire experiment was repeated three times. Seed vigor index was calculated according to

Table 1 List of SSR primers used

Name	Primer sequence	Annealing temperature (°C)	Presence of bands
CBT 02	F: TCC TCC CTC CCT TCG CCC ACT G R: CGA TGT TCG CCA TGG CTG CTC C	60	No
CBT 03	F: ATC AGC AGC CAT GGC AGC GAC R: AGG GGA TCA TGT GCC GAA GGC	58	Yes
CBT 04	F: ACC CTC TCC GCC TCG CCT CCT C R: CTC CTC CTC CTG CGA CCG CTC C	58	Yes
CBT 07	F: CGA TCC ATT CCT GCT GCT CGC G R: CGC CCC CAT GCA TGA GAA GAG	53	Yes
RM 1	F: GCG AAA ACA CAA TGC AAA AA R: GCG TTG GTT GGA CCT GAC	46	Yes
RM 72	F: CCG GCG ATA AAA CAA TGA G R: GCA TCG GTC CTA ACT AAG GG	46	Yes
RM 135	F: CTC TGT CTC CTC CCC CGC GTC G R: TCA GCT TCT GGC CGG CCT CCT C	58	Yes
RM 154	F: ACC CTC TCC GCC TCG CCT CCT C R: CTC CTC CTC CTG CGA CCG CTC C	60	No
RM 205	F: CTG GTT CTG TAT GGG AGC AG R: CTG GCC CTT CAC GTT TCA GTG	53	No
RM 287	F: TTC CCT GTT AAG AGA GAA ATC R: GTG TAT TTG GTG AAA GCA AC	49	Yes

the following formula: Germination (%) × Seedling length on Day 12 (mm) (Abdul-Baki and Anderson 1973).

Statistical analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test at a significance level of $p < 0.05$. Experiments that involved more than one classification criterion were analyzed with two-way analysis of variance.

Results and discussion

Effect of sulfuric acid treatments on seed germination

Several scarification methods have been widely used to break seed dormancy, such as boiling the seeds in water, clipping the seed coat with a large nail clipper or submerging the seeds in concentrated sulphuric acid (Hermansen et al. 2000). We selected sulphuric acid to scarify the seeds since a large number of seeds can be scarified at one time (Fig. 1a, b). We determined the suitable exposure time by submerging the seeds in concentrated sulphuric acid (98%) for 10, 30, and 60 min. We found that the seeds submerged in the sulphuric acid for 30 min showed the highest percentage of germinated seeds (36.4% at Day 12) (Table 2). Similar results were also observed in *Corchorus olitorius* and *Hibiscus trionum* where sulphuric acid scarification for

30 min produced the highest percentage of seed germination (Velemplini et al. 2003; Chachalis et al. 2008). We also noted a lower percentage of germinated seeds (22.2% on Day 12) when scarified for 60 min. Low scarification efficiency has been reported in seeds exposed to sulphuric acid for a short period of time, while prolonging the exposure period might adversely affect the seed viability (Martín and Guerrero 2014). For instance, Hermansen et al. (2000) found that seed scarification for 90 min followed by 2 h water soaking increased the germination rate of *Dimorphandra mollis* but decreased the seed viability if incubated for > 90 min.

Effect of different disinfection protocols on seed germination

Surface sterilization is an important step in establishing in vitro plantlets. In this study, we surface sterilized *M. bracteata* seeds that have been scarified for 30 min in sulphuric acid using three surface sterilization treatments (95% ethanol, 50% Clorox or a combination of 95% ethanol and 50% Clorox). We found that the percentage of contaminated seeds treated in 95% ethanol and 50% Clorox alone was 6 and 7.3%, respectively, compared to the non-treated seeds (2.4%), indicating the unnecessary disinfectant treatment for *Mucuna* seeds (Table 3). Although the combination of ethanol and Clorox reduced the percentage of contaminated seeds to 0.7%, differences between the treatments were not significant.

Clorox is a mild sterilizing agents that has been widely used to disinfect plant material (Srivastava et al. 2010). It



Fig. 1 Seed germination and in vitro propagation process of *Mucuna bracteata*. **a** *M. bracteata* seeds enclosed with a testa layer (Bar=1 cm). **b** Scarified *M. bracteata* seeds without the testa layer (Bar=1 cm). **c** Leaf discs (L) and nodal segments (N) from in vitro seedling were used as explants used for shoot multiplication (Bar=1 cm). **d** The plumule (P) and radicle (R) were excised from 5-day-old in vitro germinating seed for cotyledonary node explant (Bar=1 cm). **e** Representative of leaf disc explants with callus forma-

tion cultured on semi-solid MS medium supplemented with different concentrations of BAP and kinetin (black arrows indicated region of callus formation) (Bar=1 mm). Shoot multiplication of cotyledonary node (**f**) and nodal segment (**g**) cultured on MS medium supplemented with 4.44 μM BAP (Bar=1 cm). **h** Root induction from plantlet cultured on MS media supplemented with NAA (Bar=1 cm). **i** Acclimatized plantlets of *Mucuna bracteata* in greenhouse condition after 4 weeks (Bar=10 cm)

kills microorganisms, including bacteria and some viruses, by oxidizing their biological molecules, such as proteins and nucleic acids (Bloomfield et al. 1991; Sawant and Tawar 2011). Surprisingly, our results showed that the seeds treated with 50% Clorox produced the highest percentage

of contaminated seeds. A similar finding was also reported by Barampuram et al. (2014), where the use of Clorox to surface disinfect the cotton seed was not successful. Moreover, the use of harsh and toxic chemicals might affect seed development (Barampuram et al. 2014). Since the percentages of

Table 2 The percentage of seed germination of *Mucuna bracteata* under different exposure times in sulfuric acid

Exposure period of sulfuric acid (min)	Percentage of seed germination (%)		
	Day 4	Day 8	Day 12
0	0.0±0.0 _a	0.0±0.0 _a	0.0±0.0 _a
10	6.9±2.82 _{ab}	10.3±4.10 _{ab}	11.1±4.80 _{ab}
30	11.4±4.31 _b	30.8±5.10 _c	36.4±6.30 _c
60	12.2±1.11 _b	21.1±4.84 _{bc}	22.2±4.01 _{bc}

*Mean±SE values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p < 0.05$

contaminated seeds were generally low in all treatments, we did not perform surface sterilization in our subsequent experiments. We speculate that the immersion of seeds in concentrated sulfuric acid during scarification process is sufficient to reduce contamination.

Effect of photoperiods and culture methods on seed germination

Besides scarification, we also investigated the effect of photoperiods and culture methods on the seed germination and development. We cultured the scarified seeds on semi-solid MS medium or wet cotton roll and incubated in the dark or under 16 h light regime. Our results showed that the percentage of germinated seeds in the dark condition (36.4–36.9% at Day 12) was higher compared to under light exposure (23.6–31.7% at Day 12), probably due to its shade-tolerant characteristic (Table 4). Earlier studies also reported that seeds with hard seed coat usually have light-independent germination (Huang et al. 2004; Chauhan and Johnson 2008). Culturing the seeds on either semi-solid MS medium or wet cotton roll did not significantly affect the seed germination. Taken together, we treated the *M. bracteata* seeds with 100% sulphuric acid for 30 min and incubated in dark condition on wet cotton roll in our subsequent experiments.

Table 4 The percentage of seed germination of *Mucuna bracteata* in different culture conditions

Light regime	Culture method	Percentage of seed germination (%)		
		Day 4	Day 8	Day 12
Dark	Solid MS	11.4±4.31	30.8±5.10	36.4±6.30
	Cotton roll	17.5±6.61	34.4±9.60	36.9±8.50
Light	Solid MS	3.3±2.20	11.7±8.20	23.6±5.20
	Cotton roll	6.7±4.41	24.2±5.80	31.7±7.90

Effect of hydropriming conditions on seed germination

Imbibition is important for seed germination as it signals the resumption of embryo growth and the activation of DNA repair mechanisms from dormancy state and metabolic quiescence (Carbonera et al. 2015). The success of seed priming is strongly depended on plant species/genotype and physiology, seed quality as well as the priming method used. There are several priming techniques, namely hydropriming, osmopriming, solid matrix priming, chemopriming, and biopriming. In this study, we selected hydropriming and osmopriming techniques after considering the ease of handling and relatively lower cost compared to other techniques. We imbibed *M. bracteata* seeds in sdH₂O for 0, 6, 24, and 48 h and cultured on double layers of wet cotton roll with different volumes of sdH₂O to promote the seed germination. We found that seeds imbibed for 6 h and cultured on wet cotton roll with 10 mL of sdH₂O produced the highest percentage of germinated seeds (43.0%) on the twelfth day (Table 5). Increased volumes of sdH₂O to the cotton roll and longer hydropriming period have negatively affected the seed germination. A similar finding has also been reported by Eskandari (2013). This might be attributed to oxygen deprivation where gas diffusion was heavily constrained due to excessive hydration. Seed germination was often characterized with marked increased in oxygen uptake (Weitbrecht et al. 2011). Thus, oxygen availability was crucial. The stress caused by low oxygen availability may induce rapid restriction of metabolism that could affect seed germination (Geigenberger 2003).

Despite having the same germination rate, we found that seed vigor index for seeds imbibed for 6 h and cultured on

Table 3 The percentage of seed germination and contamination of *Mucuna bracteata* using different sterilization methods

Treatment	Percentage of seed germination (%)			Contamination (%)
	Day 4	Day 8	Day 12	
No treatment	1.2±1.37	23.0±3.91	33.1±3.23	2.4±1.21
95% EtOH	1.3±0.99	19.2±3.09	32.7±3.69	6.0±6.00
50% Clorox	6.0±2.39	23.3±3.85	34.7±4.81	7.3±6.36
95% EtOH: 50% Clorox	2.4±1.54	13.3±3.39	28.3±4.70	0.7±0.67

Table 5 The percentage of seed germination of *Mucuna bracteata* under different hydropriming conditions

Imbibition period (h)	Volume of sdH ₂ O to moisten cotton layer (mL)	Percentage of seed germination (%)			Seed vigor index
		Day 4	Day 8	Day 12	
0	0	0.0 ± 0.0 _a	0.0 ± 0.0 _a	0.0 ± 0.0 _a	0
	10	0.0 ± 0.0 _a	33.0 ± 3.8 _{ef}	42.0 ± 4.0 _e	2463
	15	0.0 ± 0.0 _a	24.0 ± 6.2 _{cdef}	31.0 ± 4.4 _{de}	1814
	40	1.1 ± 1.1 _a	19.0 ± 7.0 _{bcd}	23.0 ± 6.2 _{cd}	1035
6	0	0.0 ± 0.0 _a	0.0 ± 0.0 _a	0.0 ± 0.0 _{ab}	0
	10	8.9 ± 4.4 _{abc}	37.0 ± 3.8 _f	43.0 ± 3.3 _e	2473
	15	11.0 ± 1.1 _{bcd}	33.0 ± 5.1 _{ef}	39.0 ± 8.0 _e	2067
	40	2.2 ± 1.1 _{ab}	14.0 ± 1.2 _{bd}	17.0 ± 1.9 _{bc}	1339
24	0	2.9 ± 1.6 _{ab}	8.3 ± 1.9 _{abc}	9.3 ± 2.1 _{ab}	465
	10	12.0 ± 6.2 _{bcd}	27.0 ± 2.2 _{def}	33.0 ± 2.8 _{de}	2310
	15	12.0 ± 7.4 _{bcd}	37.0 ± 6.0 _f	39.0 ± 5.0 _e	1950
	40	9.1 ± 3.1 _{abc}	12.0 ± 4.8 _{abc}	14.0 ± 6.6 _{bc}	700
48	0	9.4 ± 2.8 _{abc}	22.0 ± 3.5 _{bcd}	24.0 ± 2.9 _{cd}	120
	10	17.0 ± 5.5 _{cd}	27.0 ± 3.9 _{def}	32.0 ± 4.0 _{de}	2320
	15	21.0 ± 1.1 _d	33.0 ± 5.9 _{ef}	34.0 ± 5.3 _{de}	2720
	40	18.0 ± 3.4 _{cd}	34.0 ± 5.2 _{bcd}	21.0 ± 4.7 _{bcd}	1750

*Mean ± SE values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p < 0.05$

wet cotton roll with 10 mL of sdH₂O (2473) was higher than that of seeds without imbibition and cultured on wet cotton roll with similar volume of sdH₂O (2463) (Table 5). Seed vigor is one of the important aspects in seed development. Previous reports demonstrated a strong correlation between seed vigor with field emergence and performance (Egli and Rucker 2012; Singh et al. 2014; ur Rehman et al. 2014). Seed vigor has been shown to be affected by the ratio of sucrose/raffinose family oligosaccharides in leguminous *Medicago truncatula* (Vandecasteele et al. 2011) and exogenous application of folic acid and AA in *Pisum sativum* (Burguieres et al. 2007). Considering the importance of seed vigor during seedling development, imbibition period of 6 h followed by culturing on wet cotton roll with 10 mL of sdH₂O was regarded as optimal condition for germinating seeds of *M. bracteata*.

Effect of osmopriming and antioxidant treatments on seed germination

We also imbibed the *M. bracteata* seeds in an osmopriming agent to investigate their effect on seed germination. We found that 55.0% of seeds germinated in 10% of PEG solution, whereas only 33.0% seeds germinated in sdH₂O (Table 6). Numerous studies have reported that PEG is able to enhance seed germination in many plant species (Tobe et al. 2000; Bittencourt et al. 2005; Yasari et al. 2013). Osmopriming protects seeds from oxidative damage caused by reactive oxygen species (ROS) and accelerates seed germination process (Paparella et al. 2015). However,

Table 6 The percentage of seed germination of *Mucuna bracteata* under osmopriming and different antioxidant treatments

Treatment	Percentage of seed germination (%)			Seed vigor index
	Day 4	Day 8	Day 12	
sdH ₂ O	9.0 ± 4.5 _a	27.0 ± 4.9 _a	33.0 ± 5.0 _a	1898
PEG	17.0 ± 2.6 _{ab}	42.0 ± 2.9 _b	55.0 ± 3.9 _b	2946
AA	20.0 ± 1.7 _b	30.0 ± 4.9 _{ab}	39.0 ± 4.4 _a	2640
AC	20.0 ± 1.7 _b	40.0 ± 3.0 _{ab}	44.0 ± 2.7 _{ab}	3740

*Mean ± SE values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p < 0.05$

osmopriming using PEG is not economically viable as it is expensive and difficult to be removed from the mixture. Since phenolic compounds are a major antioxidant constituent in *Mucuna* species (Sridhar and Bhat 2007; Surveswaran et al. 2007), we applied hydropriming together with AA or AC to promote the seed germination. Our results revealed that the percentage of germinated seeds on AC and AA were 44.0 and 39.0%, respectively, albeit significantly ($p < 0.05$) lower than osmopriming treatment (55.0%) (Table 6). However, the seed vigor index was highest in seeds germinated on AC (3740) compared to osmopriming treatment (2946) or hydropriming treatment alone (1898). These results demonstrated that the benefits of osmopriming treatment could be offset in hydropriming treatment by culturing the seeds on wet cotton layer supplemented with either AC or AA. Since excessive accumulation of phenolic compounds could

adversely affect the seed development (Muscolo et al. 2001; Chon et al. 2002), some studies applied exogenous AA or AC to scavenge ROS or absorb phenolic compounds during seed hydration and germination phases (Thomas 2008; Mohammadi et al. 2014).

Effect of different types and concentrations of cytokinins on shoot induction and multiplication

In this study, we initiated shoots from leaf discs, nodal segments, and cotyledonary nodes on MS medium supplemented with different types of cytokinins at different concentrations (Fig. 1c, d). Our results demonstrated that cotyledonary nodes on MS medium containing 4.44 μM BAP alone and in combination with 4.65 μM kinetin were significantly different compared to other treatments, as they induced the highest number of shoots per explant (4.83–5.60) (Table 7). In comparison, MS medium without supplementation of plant growth regulators produced longer shoots than other treatments. No shoot was formed from the leaf discs in any treatment after 2 months of culture (Fig. 1e). The highest percentage of callus formation in leaf discs was 48.3% on MS medium supplemented with 17.8 μM BAP (Table 7).

In general, we found that cotyledonary nodes were more effective than nodal segments and leaf discs for shoot initiation (Fig. 1f, g). This was in agreement with the study carried out by Husain et al. (2008), where cotyledonary nodes of leguminous tree (*Pterocarpus marsupium* Roxb.) are suitable explants for shoot induction and multiplication. This might be due to the proliferating nature of pre-existing meristems at cotyledonary nodes compared to nodal segments (Distabanjong and Geneve 1997). We also observed that supplementation of cytokinin at low concentration helped to induce higher number of shoots as indicated by

previous studies (Al-Bahrany 2002; Chand and Singh 2004; Husain et al. 2008). BAP has been considered to be one of the most effective cytokinin (Tan et al. 2011). Several studies have shown that BAP was more effective than kinetin for shoot induction (Grzegorzczak-Karolak et al. 2015; Wei et al. 2015; Zaheer and Giri 2015). Kinetin is known to promote shoot elongation while BAP enhances bud proliferation and multiplication (Diallo et al. 2008). Since we aim to develop a suitable plant host system for genetic transformation in the future, cotyledonary nodes were selected as explants for shoot induction and multiplication on MS medium supplemented with 4.44 μM BAP.

Effect of different types and concentrations of auxins on root induction

Shoots of at least 5 cm in height started to root after 2 weeks of culture (Fig. 1h). Root initiation was influenced by the type of explants. We found that cotyledonary node-derived shoots produced the highest percentage rooting response (92.9%) with a mean of 1.38 roots per shoot and a mean length of 4.55 cm after 1 month of culture on MS medium supplemented with 10.7 μM NAA (Table 8). Only 43.3% nodal segment-derived shoots developed roots after 1 month of culture on MS medium containing 16.1 μM NAA. NAA is a synthetic auxin that has been widely used to induce roots for many plant species (Al-Bahrany 2002; Tan et al. 2011; Wei et al. 2015). It has been found to be more effective than IBA in inducing roots for plant species, such as *Morus alba* (Balakrishnan et al. 2009), *Arbutus andrachne* (Mostafa et al. 2010), and *Vanilla planifolia* (Tan et al. 2011).

A total of 100 rooted plantlets were transplanted in polybags containing garden soil and maintained in the

Table 7 Effect of different concentrations of BAP and kinetin on shoot multiplication of *Mucuna bracteata*

Cytokinin (μM)		Cotyledonary nodes		Nodal segments		Leaf
BAP	Kinetin	Number shoots per explants	Shoot length (cm)	Number shoots per explants	Shoot length (cm)	Callus formation (%)
–	–	1.43 \pm 0.03 _a	14.36 \pm 2.05 _d	1.04 \pm 0.04 _a	2.79 \pm 0.60 _{ab}	0.0 \pm 0.00 _a
4.44	–	5.60 \pm 0.20 _e	6.45 \pm 0.42 _c	1.42 \pm 0.14 _{bcd}	3.84 \pm 0.55 _b	23.9 \pm 5.10 _{cd}
8.88	–	3.63 \pm 0.41 _d	3.89 \pm 0.27 _{ab}	1.41 \pm 0.13 _{bcd}	2.70 \pm 0.46 _{ab}	35.0 \pm 5.27 _d
13.3	–	1.90 \pm 0.20 _{ab}	2.86 \pm 0.34 _a	1.50 \pm 0.19 _d	3.31 \pm 0.33 _{ab}	46.9 \pm 5.58 _e
17.8	–	2.91 \pm 0.07 _{cd}	2.95 \pm 0.22 _a	1.16 \pm 0.07 _{abc}	2.46 \pm 0.31 _{ab}	48.3 \pm 5.33 _e
4.44	4.65	4.83 \pm 0.64 _e	6.27 \pm 0.38 _c	1.46 \pm 0.14 _{cd}	3.28 \pm 0.31 _{ab}	21.3 \pm 4.76 _c
–	4.65	1.93 \pm 0.12 _{ab}	5.37 \pm 0.54 _{bc}	0.94 \pm 0.06 _a	1.84 \pm 0.53 _a	7.7 \pm 7.69 _a
–	9.29	1.80 \pm 0.20 _{ab}	7.24 \pm 0.79 _c	0.94 \pm 0.06 _a	3.03 \pm 0.60 _{ab}	23.9 \pm 6.36 _{bc}
–	13.9	1.52 \pm 0.07 _a	7.40 \pm 1.32 _c	1.11 \pm 0.06 _{ab}	3.18 \pm 0.54 _{ab}	9.8 \pm 4.21 _{ab}
–	18.6	2.57 \pm 0.46 _{bc}	5.48 \pm 0.57 _{bc}	1.04 \pm 0.04 _a	3.77 \pm 0.53 _b	20.0 \pm 5.21 _{bc}

*Mean \pm SE values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p < 0.05$

Table 8 Effect of different concentrations of NAA on root induction of *Mucuna bracteata*

NAA (μM)	Cotyledonary nodes			Nodal segments		
	Percentage of shoots forming roots (%)	Number roots per shoot	Root length (cm)	Percentage of shoots forming roots (%)	Number roots per shoot	Root length (cm)
0.0	36.7 \pm 3.33 _a	1.30 \pm 0.23 _a	7.06 \pm 1.46 _a	3.33 \pm 3.33 _a	1.00 \pm 0.00 _a	2.00 \pm 0.00 _a
2.69	78.9 \pm 6.76 _b	1.73 \pm 0.09 _a	5.59 \pm 0.55 _{ab}	10.0 \pm 0.00 _a	1.67 \pm 0.67 _a	8.94 \pm 2.40 _b
5.37	90.5 \pm 2.38 _b	1.71 \pm 0.06 _a	4.61 \pm 0.16 _{ab}	6.67 \pm 3.33 _a	1.00 \pm 0.00 _a	3.75 \pm 2.25 _{ab}
10.7	92.9 \pm 0.00 _b	1.38 \pm 0.09 _a	4.55 \pm 0.12 _{ab}	20.0 \pm 0.00 _b	1.33 \pm 0.17 _a	4.33 \pm 0.82 _{ab}
16.1	89.5 \pm 6.75 _b	1.45 \pm 0.10 _a	4.21 \pm 0.67 _b	43.3 \pm 3.33 _c	3.20 \pm 0.30 _b	6.50 \pm 0.59 _{ab}

*Mean \pm SE values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p < 0.05$

greenhouse. Survival of 89% of the plantlets with uniform growth was recorded after 4 weeks of transplanting (Fig. 1i).

Estimation of DNA content and genome size via flow cytometry analysis

Flow cytometry analysis revealed that in vitro-derived and seed-germinated plants had about similar fluorescence intensity, suggesting the ploidy stability among the in vitro-derived plants. The mean peak of fluorescence intensity for in vitro-derived and seed-germinated plants was 121.2 ± 0.83 and 123.1 ± 2.19 , respectively, with coefficients of variation (CV) within an acceptable range of 1.33–4.70% (Fig. 2). Flow cytometry analysis was often employed to assess the trueness-to-type of regenerants for a variety of species such as *Solanum trilobatum* (Shilpha et al. 2014), *Pinus elliotii* (Nunes et al. 2016) and *Vavilovia formosa* (Ochatt et al.

2016). We have also estimated the DNA content of *M. bracteata* to be 1.48 ± 0.01 pg based on the fluorescence intensity of the in vitro-derived plants and projected the genome size of *M. bracteata* is about 1448 ± 9 Mb. This information provides base line data for future cytogenetic study of *M. bracteata* and molecular characterization of *Mucuna* populations in general. To date, this is the first report on an estimation of the genome size and DNA content for *M. bracteata*.

SSR analysis

Genetic fidelity analysis of in vitro-derived and seed-germinated plants was further confirmed with SSR analysis. Seven out of ten primer pairs tested produced bands (Table 1). The bands appeared to be monomorphic across all the plants, indicating the clonal fidelity of in vitro-derived plants (Fig. 3).

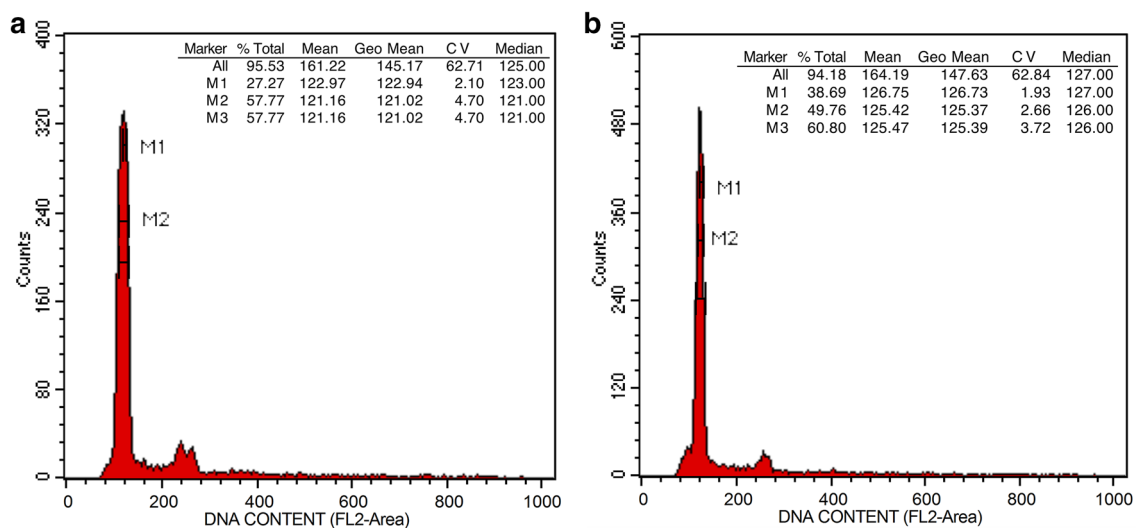


Fig. 2 Representative flow cytometry histogram of relative fluorescence intensity of propidium iodide-stained nuclei harvested from young leaves of *Mucuna bracteata*. **a** In vitro-derived plant; and **b** seed-germinated plant

Fig. 3 Representative gel image for SSR analysis using CBT 03 primer. The image demonstrated genetic similarity between in vitro-derived and seed-germinated *Mucuna bracteata*. Lane M: 1 kb ladder; Lane S: seed-germinated plant; Lanes 1–10: in vitro-derived plants and Lane –ve: negative control

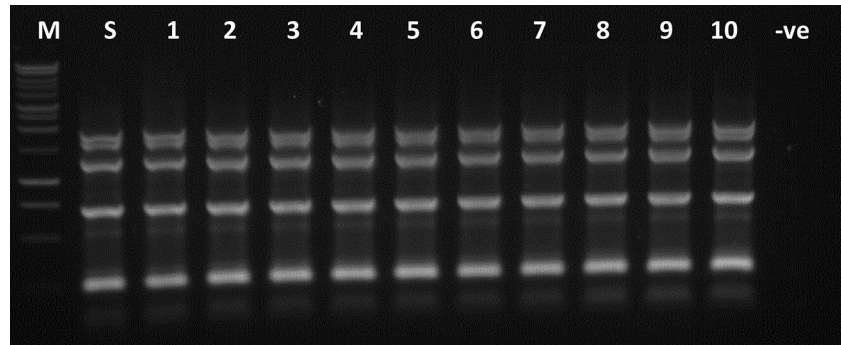
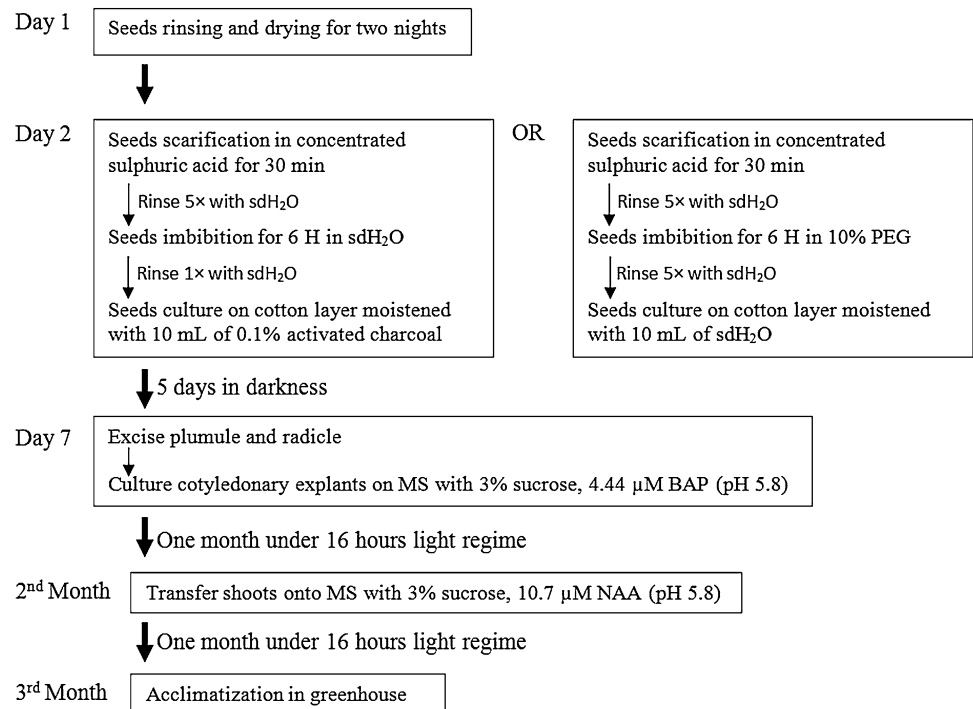


Fig. 4 General workflow for seed germination and in vitro propagation of *Mucuna bracteata*



Conclusion

We have successfully developed simple and efficient protocols for seed germination and micropropagation of *M. bracteata* in 3 months (Fig. 4). The generated plantlets showed genetic stability after confirmation with SSR analysis. Further analysis using flow cytometry has allowed the first estimations on the DNA content and genome size of *M. bracteata* that provides base line data for future molecular cytogenetic study of *M. bracteata*. The established protocols could be scalable to mass produce *M. bracteata* plantlets as an alternative source of supply for plantation companies. The protocols may also facilitate future improvement strategies especially for the application of –omic technologies in the study and utilization of this important tropical cover crop.

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Author contributions NAA conducted the experiments and analyzed data; NAA, BCT, RYO, NK conceived the idea, designed the experiments and wrote the paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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