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MICROPROPAGATION

Micropropagation of *Hibiscus moscheutos* L. 'Luna White': effect of growth regulators and explants on nuclear DNA content and ploidy stability of regenerants

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Abstract

Hibiscus moscheutos L., also known as hardy hibiscus, is valued for its medicinal and ornamental attributes. It is usually propagated via seeds or cuttings. The purpose of this investigation was to develop a dependable micropropagation for *H. moscheutos* 'Luna White'. To that end, the effect of four explant types (leaf, root, node, shoot tip) and two growth regulators 6-benzylaminopurine (BA) and *meta*-Topolin (*mT*) (6-(3-hydroxybenzylamino) purine) on *in vitro* growth of *H. moscheutos* was investigated. Genetic stability of the *in vitro* grown plants was assessed using flow cytometry, and chromosome count was investigated. No shoots were obtained from leaf or root explants. An efficient protocol for micropropagation of *H. moscheutos* using two explant types, 2-node and shoot tip explants, and two cytokinins (BA and *m*T) capable of producing true-to-type regenerants was established. Both BA and *mT* can be used at 2 μM or 4 μM using either 2-node or shoot tip explants. No significant difference was found between the nuclear DNA contents of seed-derived and in vitro grown plants ($P < 0.05$). The mean 2C DNA and monoploid 1Cx-values of seed-derived plants were 3.25 ± 0.08 pg and 1.62 ± 0.04 pg, respectively, compared with 3.26 ± 0.06 pg and 1.63 ± 0.02 pg, respectively, for *in vitro* grown plants. The chromosome number of both seed-derived plants and regenerants was determined to be $2n = 2x = 38$. The mature regenerants obtained were fertile and phenotypically similar to seed-derived plants.

Keywords Cytokinin · Hardy hibiscus · *meta*-Topolin · Rose mallow · Somaclonal variation

Introduction

Over 300 annual and perennial species belonging to the family Malvaceae are included in the genus *Hibiscus* (Pfeil *et al.* 2002; Akpan 2007). *Hibiscus* species are multipurpose plants used as sources of food, medicines, and/or ornamentals (West and Preece 2004). Among these species is *H. moscheutos* L. also known as mallow, common rose mallow, giant rose mallow, swamp mallow, and hardy hibiscus. *Hibiscus moscheutos* L. is a perennial shrub native to wetland areas of North America and desired for its showy white to pink flowers (Barrios and Ruter 2019).

Traditional methods have been used to develop new stable cultivars that can be then mass propagated by sowing F_1 seeds or rooting shoot tip cuttings (West and Preece 2004). However, seed-propagated seedlings take a long time to reach maturity (West and Preece 2004). Limitations to this type of propagation include a lack of readily available shoot tips and/or sufficient space. Micropropagation, an effective tool for research and commercial production, can be used to alleviate these limitations.

Micropropagation is often achieved with the aid of growth regulators such as thidiazuron (TDZ; N-phenyl-1, 2, 3-thidiazole-5ylurea), zeatin (6-(4-hydroxy-3-methylbut-2-enylamino) purine), 6-benzylaminopurine (BA), and *meta*-Topolin (*mT*) or 6-(3-hydroxybenzylamino) purine, an

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aromatic cytokinin (Strnad *et al.* 1997). TDZ, a substituted phenyl urea (Mok *et al.* 1982; Ricci *et al.* 2001), was first discovered as a cotton defoliant (Arndt *et al.* 1976), but it has since been widely used as a growth regulator in a large number of species (Jaiswal and Sawhney 2006). TDZ has been used in micropropagation of *H. moscheutos*, but higher TDZ concentrations resulted in stunted and chlorotic shoots, leading to shoot tip dieback (West and Preece 2004), as well as the development of structural anomalies such as hyperhydricity, stunting, fasciation, and distortion in *Rhododendron mucrunulatum* Turcz and cotton (Novikova *et al.* 2020; Personal observations). Naturally occurring zeatin ($86.9 mg L^{-1}) is far more expensive than BA ($0.19 mg L^{-1}) (Sigma-Aldrich 2020). There is probably no growth regulator without any level of negative effects on tissue cultured plants, but the negative effects depend on the growth regulator concentration, length of time the explants are in culture, and plant species (Trolinder and Goodin 1983; Bairu *et al.* 2007). BA is the most widely used in micropropagation because it is one of the most effective and affordable cytokinins available (Werbrouck *et al.* 1996). Successful micropropagation also may depend on the explant source. Improved *in vitro* shoot multiplication with rooting and reduced or total elimination of hyperhydricity have been reported in micropropagated plants using *mT* (Werbrouck *et al.* 1996; Bairu *et al.* 2007; Novikova *et al.* 2020). Micropropagation from preformed structures such as shoot tips or axillary buds is generally assumed to be less prone to genetic instability, but somaclonal variation has been reported in tissue culture systems using this method (Ahuja 1998; Rani and Raina 2000; Bairu *et al.* 2008). *In vitro* regenerated plants are not always free from genetic changes due to somaclonal variation (Pramanik and Datta 1986; Trolinder and Gooding 1987; Stelly *et al.* 1988), so assessing genetic fidelity of regenerants is an important factor in the micropropagation of genetically uniform plants. Methods of evaluating genetic stability include flow cytometry analysis and chromosome counts (Stelly *et al.* 1989; Currais *et al.* 2013; Sakhanokho *et al.* 2020).

The objective of this investigation was to optimize the micropropagation protocol for *H. moscheutos*; therefore, we evaluated various concentrations of the cytokinins BA and *mT* as well as four explant sources: leaf, root, nodal segments, and shoot tip. Genetic stability of the regenerants was assessed using flow cytometry and chromosome count analysis.

Materials and Methods

Experiment 1 Plant Material F_1 seeds obtained from a controlled self-pollination of a *H. moscheutos* L. 'Luna White' plant were used. For seed germination, an *in vitro* germination medium (MS0) was used. The MS0 consisted of Murashige and Skoog (1962) salts supplemented with 30 g L^{-1} sucrose. For all experiments, the pH of the solution was adjusted to 5.8 before addition of 8 g L^{-1} agar and autoclaving for 20 min at 121 °C and 15 psi. Then, the medium was transferred to 100 × 25 mm Petri dishes. Seeds were scarified for 10 min in 98% sulfuric acid and subsequently thoroughly rinsed at least three times with tap water under a fume hood. Afterwards, the seeds were surface-sterilized by stirring in 70% ethanol for 1 min and 10% (v/v) bleach and 1 drop of Tween-20 (Sigma-Aldrich, St. Louis, MO) for 5 min on a rotary shaker at 130 rpm. The seeds were then rinsed at least 3 times with sterilized deionized water. The surface-sterilized seeds were transferred to 100 × 25 mm Petri dishes containing the seed germination medium and placed in an incubator where the temperature was kept at 25 °C for 16 h light, 22 °C for 8 h dark and light intensity at 50 μmol m^{-2} s^{-1}. Relative humidity was maintained at 55%. About 90% of seeds germinated after 5 d in the germination medium. Six days after seed germination, seedlings were transferred to double Magenta GA-7 (Caisson Labs, Smithfield, UT) vessels containing the same MS0 medium for further growth for at least 28 d.

Effect of Growth Regulators and Explant Types Four types of explants, root, leaf, nodal segment, and shoot tip, were excised from the seedlings growing in double Magenta jars after 28 d. The nodal explants consisted of 2-node segments. The root and leaf explants were transferred into Petri dishes containing MS0 amended with 6-benzylaminopurine (BA) (0, 2, 4, or 6 μM) or *meta*-Topolin (*mT*) (0, 2, 4, or 6 μM). All the growth regulators were purchased from Sigma-Aldrich (St. Louis, MO) except *m*T which was obtained from PhytoTech Labs (Lenexa, KS). The nodal and shoot tip explants were transferred into double Magenta jars also containing media consisting of MS0 amended with BA (0, 2, 4, or 6 μM) or *mT* (0, 2, 4, or 6 μM). After 90 d, the effect of growth regulators and explant types on plant growth was assessed by counting the number of roots and shoots, measuring the shoot length (cm), and evaluating vigor based on visual rating. Visual vigor rating was based on a 1 to 3 scale, with 1 being poor and 3 being the most vigorous. Overall plant appearance and health was used for rating; a plant rated 1 had no shoots with very little life; an explant rated 1 was dead or mostly brown with very little sign of life; a plantlet with a 2-vigor rating had some green shoots and leaves but its overall health (e.g., dead leaves) was less than desirable; and a rating of 3 indicated a plant that was green with several shoots and looked healthy. The presence or absence of roots was not considered for the visual rating of plants. Each plant was independently rated by two evaluators.

Experiment 2 In experiment 1, explants subjected to higher concentrations (4 or 6 μM) of either cytokinin failed to form roots after 90 d in culture; therefore, we decided to set up a

separate second experiment. In experiment 2, explants were treated with the various concentrations of BA (0, 2, 4, or 6 μM) and *mT* (0, 2, 4, or 6 μM) for 56 d and transferred afterwards to a medium without any growth regulator and allowed to grow for 49 d. Plants were first grown in double Magenta jars containing the same MS0 cytokinin-amended media as in experiment 1. Double Magenta jars were used to reduce any space or physical restriction and thus allow the plants to grow taller and facilitate multiple shoot production. After 56 d, explants consisting of 2-node segments and shoot tips were excised from these plants and transferred to 25 mm × 150 mm test tubes containing MS0, a medium including no growth regulators. After 49 d of growth in MS0, the number of shoots, number of roots, vigor, and shoot length (cm) of the plantlets were determined.

Experimental design and statistical analysis To determine the effect of the cytokinins BA and *mT* on *in vitro* micropropagation of *H. moscheutos* 'Luna White', 4 concentrations (0, 2, 4, 6 μM) of each cytokinin were used. For both experiment 1 and experiment 2, the experimental design was a completely randomized design (CRD). For the leaf and root explants, the experimental unit was a 100 × 25 mm Petri dish containing 2 to 3 leaf or root explants. Five Petri dishes were used for each explant (leaf or root). For the nodal segment and shoot tip explants, the experimental unit was the double Magenta vessel. Each treatment was replicated 10 times, and the whole experiment repeated once. Duncan's multiple range test was used for mean separation at the 5% level, except for the nuclear DNA content for which Student's t-test was used to determine whether the nuclear DNA content of seed-derived plants was different from that of *in vitro* grown plants at the 5% probability level. SAS software (SAS 9.4, Cary, NC) was used for statistical analysis.

Rooting and acclimatization Fifteen plantlets per treatment were randomly selected and transferred to small (approximately 1 L) pots containing finely ground, aged pine bark in the greenhouse under a mist system (approximately 26 °C, ~70% humidity). Each pot was covered with a 1-gallon (3.8 L) Ziplock plastic bag, and 4 to 5 small holes were punctured in each plastic bag by the third day for gradual acclimatization of plantlets. The plastic bags were removed after 7 d. All rooted and non-rooted plantlets developed roots and were hardened in pots in the greenhouse.

Additionally, ten *in vitro* grown plants were randomly chosen from among those hardened in the greenhouse and allowed to flower in the greenhouse. All *in vitro* grown plants flowered, and five plants were randomly chosen and self-fertilized via controlled pollination in the greenhouse. Mature, brown seed pods showing signs of splitting were harvested and their seeds collected. Fifty seeds from each plant were sown for a germination test of harvested seeds.

Nuclear DNA content determination Preliminary results on the genome size of BA- or *mT*-treated *in vitro* grown *H. moscheutos* 'Luna White' plants showed no difference: therefore, 10 plants from among the BA- and *mT*-treated plants were randomly selected to compare their nuclear DNA content with that of seed-derived plants using flow cytometry analysis following a procedure described elsewhere (Islam-Faridi *et al.* 2020a; Sakhanokho *et al.* 2020) with minor modifications. Briefly, two fresh leaves of each of the seed-derived or *in vitro H. moscheutos* plants were placed in a Petri dish and co-chopped with the internal standard, *Sorghum bicolor* 'Tx623' (2C 1.67 pg) (Price *et al.* 2005) to an equal size (approximately 0.5 cm^2), and resuspended in 500 μL nuclei extraction buffer. The extraction buffer mixture was pipetted through a filter to remove large debris, then a nuclei staining solution (propidium iodide, RNAse, and 5% polyvinylpyrrolidone-40,000) was added. The mixture was covered to protect against light and incubated in a refrigerator at 4 °C for 15 min, and nuclear DNA content was determined using a BD Accuri™ C6 flow cytometer and a BD Accuri C6 software version 1.0.264.21 (BD BioSciences, Ann Arbor, MI). At least 5000 events (nuclei) were gated for each run. Fluorescence ratios were calculated and converted to nuclear DNA content and expressed in picograms (pg) as follows: sample 2C-value (picograms) = reference 2C-value × [(sample 2C mean peak)/(reference 2C mean peak)]; and genome sizes were converted to megabases (Mbp) using the formula 1 pg = 978 Mbp (Doležel *et al.* (2003). Sample monoploid 1Cx-value (pg) was calculated by dividing the 2C-value by the ploidy level (x = 2) of *H. moscheutos* 'Luna White' (Greilhuber *et al.* 2005).

Chromosome Count Chromosome spreads were prepared for 5 seed-derived and 5 *in vitro* randomly selected from both shoot tip– and node-derived plants using procedures previously described (Jewell and Islam-Faridi 1994; Sakhanokho *et al.* 2020) with some minor modifications. Actively growing root tips about 1.0 cm long were harvested and immediately pretreated with 2 mM 8-hydroxyquinoline for 4.0 h in the dark at room temperature (RT, 22–24 °C), rinsed with double-distilled H_2O and then fixed in 4 EtOH:1 GAA (95% ethanol:glacial acetic acid) and stored at RT overnight before processing for enzyme digestion for chromosome spread. The root tips were processed for enzyme treatment within 7 d after harvest, and fixed root tips were rinsed with deionized water, mildly hydrolyzed (0.2N HCl) at 60 °C for 15 min, rinsed with deionized water, then rinsed in cold 0.01M citrate buffer (20 min standing at RT) before enzyme digestion. The enzyme mixture consisted of 2% cellulase RS (w/v), 1% macerozyme R10 (w/v) (Yakult Pharmaceutical Industry Company, Tokyo, Japan), 2% pectolyase Y23 (w/v) (Kyowa Chemical Products, Tokyo, Japan), 30% cellulase [(v/v), C2730, 30% pectinase (v/v), P2611, Sigma-Aldrich], and

40% 0.01 M citrate buffer (pH 4.5). The enzyme digestion time varied from 24 to 35 min based on the thickness of root tips. The chromosome spreads were stained with 1% Azure-B (Sigma, St. Louis, MO) and dried overnight in a 37 °C incubator and made permanent with a drop of Permount (Fisher Scientific, Fair Lawn, NJ). Chromosome spreads were viewed under a 63X plan apo-chromatic objective, and digital images were recorded under a green filter using bright field microscopy (AxioImager M2, Carl Zeiss, Göttingen, Germany). The chromosome spread images were processed with Adobe Photoshop (Adobe Systems Inc., NY, NY).

Results and Discussion

Effects of Growth Regulators and Explant Source In experiment 1, the effects of BA (0, 2, 4, 6 μM) and *mT* (0, 2, 4, 6 μM) and four explant types (leaf, root, nodal section, and shoot tip) on morphogenic response of *H. muscheutos* 'Luna White' were evaluated based on observation of the dependable variables which included shoot length, number of shoots, number of roots, and vigor based on visual rating (Table 1). Among the explant sources evaluated, leaf and root explants failed to form any shoot at all in any of the BA or *mT* treatments, including the controls. The majority of leaf explants turned brown and died; however, abundant root formation occurred sporadically in some leaf explants after 28 to 42 d in culture (Fig. 1*d*), but no shoot formation was observed for the leaf explant. This phenomenon has been reported in chrysanthemum (Trigiano *et al.* 1994) as well as in other members of the family Malvaceae including cotton (Sakhanokho *et al.* 2004) and kenaf (*Hibiscus cannabinus* L.) (Susanto and Mat Hussin 2019). Although not the intent of the current study, this phenomenon of rhizogenesis in leaf tissue culture, if optimized, could be useful for studies focused on better understanding, for example, root formation and development under various environmental conditions. As for the root explants, no changes in appearance, including color change and growth, occurred for the entire duration of the experiment despite multiple transfers to fresh media every 28 d.

For all the growth factors evaluated, the analysis of variance showed that the effects of BA and *mT* were highly significantly different ($P > 0.001$), but the effect of explant and explant*growth regulator interactions were not significantly different ($P \leq 0.05$) except for plantlet vigor for which both explant and BA had a significant effect ($P > 0.05$) (Table 2). For shoot length, the measurements ranged from 0.88 ± 0.44 cm when shoot tips were treated with 6 μM to 12.50 ± 6.08 cm when nodal segments were exposed to MS0 medium (the control medium with no growth regulators) (Table 1). Regardless of the source of explant or growth regulator, plantlet length was significantly ($P > 0.05$) reduced with increasing

Table 1. Effect of plant growth regulators (6-benzylaminopurine (BA), meta-Topolin (*mT*)) and explant source on micropropagation of *Hibiscus moscheutos* L. 'Luna White'

Treatment no.	Explant source	BA (μM)	*mT* (μM)	Mean ± SD							
				Shoot length (cm)α		Number of shoots		Number of roots		Vigor	
1	NSß	0	0	12.50 ± 6.08[a]	c	1.00 ± 0.00	g	25.40 ± 16.17	b	2.70 ± 0.67	a
2	NS	0	2	5.15 ± 2.29	b	2.10 ± 1.20	bcde	0.10 ± 0.03	c	2.70 ± 0.48	a
3	NS	0	4	2.40 ± 0.46	bc	2.10 ±0.88b	cde	0.00 ± 0.00	c	2.80 ± 0.42	a
4	NS	0	6	2.28 ± 0.67	bc	2.78 ± 0.97	abcd	0.00 ± 0.00	c	2.44 ± 0.53	ab
5	NS	2	0	2.90 ± 1.90	bc	2.40 ±1.17	abcde	0.40 ± 0.09	c	2.60 ± 0.84	a
6	NS	4	0	2.11 ± 0.74	bc	1.89 ± 1.90	defg	0.00 ± 0.00	c	2.44 ± 0.73	ab
7	NS	6	0	1.85 ± 0.81	c	1.90 ± 0.57	defg	0.00 ± 0.00	c	2.30 ± 0.82	ab
8	ST	0	0	11.10 ± 9.18	a	1.10 ± 0.32	fg	38.60 ± 4.11	a	2.40 ± 0.97	ab
9	ST	0	2	2.60 ± 1.02	bc	2.90 ± 1.10	abc	2.30 ± 0.48	c	2.20 ± 0.42	ab
10	ST	0	4	1.65 ± 0.58	c	3.00 ± 0.82	ab	0.10 ± 0.03	c	2.30 ± 0.48	ab
11	ST	0	6	1.40 ± 0.66	c	3.10 ± 1.37	a	1.40 ± 0.23	c	2.30 ± 0.67	ab
12	ST	2	0	1.50 ± 0.71	c	2.10 ± 0.63	bcde	0.20 ± 0.06	c	2.80 ± 0.42	a
13	ST	4	0	1.17 ± 0.61	c	1.56 ± 0.53	efg	0.11 ± 0.03	c	1.89 ± 0.60	bc
14	ST	6	0	0.88 ± 0.44	c	2.00 ± 0.53	cdef	1.63 ± 0.23	c	1.38 ± 0.52	c

α Data are expressed as means ± SD (standard deviation) of at least 20 observations per treatment

Data were collected 90 d after transfer to double Magenta jars

ß NS = nodal segment; ST = shoot tip

Means within the same *column* and with the same *letters* are not significantly different according to Duncan's multiple range test ($P < 0.05$)

Figure 1. Micropropagation of *Hibiscus moscheutos* L. 'Luna White'. (***a***) Mature tissue cultured *H. moscheutos* 'Luna White' growing in a greenhouse. This plant was regenerated in a medium containing 2 μM *meta*-Topolin (*mT*). (***b***) Flower from the same *in vitro* grown plant. (***c***) Seedlings from seeds obtained from a controlled self-pollination of *in vitro* grown plants. (***d***) Leaf explants sporadically produced roots but not shoots.

concentrations of growth regulator. On the other hand, compared with the control plants, the number of shoots grown in the presence of BA or *mT* rose with increased concentration of growth regulator in general regardless of the type of explant used. Control plants derived from both shoot tips and nodal sections developed significantly ($P > 0.05$) more roots than BA- and *mT*- treated plants (Table 1). Nodal segments treated with *mT* (4, 6 μM) and BA (4, 6 μM) failed to form roots although the plants looked healthy and vigorous (Table 1).

***In Vitro* Rooting** Explants subjected to higher concentrations of both BA and *mT* (4 or 6 μM) failed to form roots after 90 d in culture. This was probably due to the effect of these growth regulators similar to that of Bairu *et al.* (2008) reported of rooting. We decided to set up a second and separate experiment, experiment 2, in which explants were treated with the various concentrations of BA (0, 2, 4, or 6 μM) or *mT* (0, 2, 4, or 6 μM) for 56 d before transferring directly to a rooting medium without any growth regulator and allowed to grow for 49 d. For the *in vitro* rooting stage, we chose a medium without growth regulators (e.g., auxins) as plants subjected to multiple subcultures and growth regulators for a long period of time tend to undergo somaclonal variation or genetic changes (Nehra *et al.* 1992; Bairu *et al.* 2006). BA- and *mT*-treated explants continued to produce multiple shoots even after transfer to a medium without growth regulators, and their root formation improved dramatically (Tables 3 and 4). For example, 100% and 25% of explants grown in the presence of 4 μM *mT* and 6 μM *mT*, respectively, formed roots (Table 4). Similarly, 100 and 75% of explants previously grown in media containing 4 μM BA and 6 μM BA formed roots. Interestingly, in addition to root and multiple shoot formation, the other growth factors (shoot length and vigor) of both BA- and *mT*-treated explants were similar to those of control plants (Table 3).

Table 2. Analysis of variance for vigor based on visual rating of *in vitro* grown *Hibiscus moscheutos* L. 'Luna White' plants

Source	df	SS[a]	MS[b]	F-value	Significance
Explant	1	1.61811741	1.61811741	3.97	0.0485
BA[c]	3	8.49816716	2.83272239	6.95	0.0002
mT	3	0.43377778	0.14459259	0.35	0.7857
Explant*BA	3	3.17239329	1.05746443	2.59	0.0556
Explant**mT*	3	0.43377778	0.14459259	0.35	0.7857

[a] SS = sum of squares

[b] MS = mean square

[c] BA= 6-benzyladenine; *mT* = *meta*-Topolin

Nuclear DNA Content Determination Genetic stability of the microshoots grown in the presence of BA (0, 2, 4, or 6 μM) or *mT* (0, 2, 4, or 6 μM) was evaluated using flow cytometry. Flow cytometry analysis showed a single peak for both seed-derived leaves and leaves from *in vitro* grown plants, suggesting there was no mixoploids or change in ploidy level in *in vitro* grown plants. Analysis of the relative DNA contents of *H. moscheutos* 'Luna White' with the internal standard *S. bicolor* 'Tx623' resulted in two peaks representing the G1 nuclei of *H. moscheutos* and *S. bicolor* 'Tx623', respectively (Fig. 2 *a–b*). Only the flow cytometry runs with coefficients of variation within the acceptable range, i.e., lower than 5% (Doležel and Bartoš 2005), were included in the nuclear DNA analysis. The mean 2C DNA and monoploid 1Cx-values of seed-derived plants were 3.25 ± 0.08 pg and 1.62

Table 3. Effect of plant growth regulators (6-benzylaminopurine (BA)), meta-Topolin (mT) on *in vitro* growth of *Hibiscus moscheutos* L. 'Luna White' after transfer to MS0, a medium without growth regulators

Treatment no.	Explant source	BA (μM)	*mT* (μM)	Mean ± SD							
				Shoot length (cm)		Number of shoots		Number of roots		Vigor	
1	NS[b]	0	0	1.5 ± 0.58[c]	b	3.00 ± 2.00	ab	10.50 ± 4.71	ab	3.00 ± 0.00	a
2	NS	0	2	2.25 ± 1.26	ab	4.50 ±1.00	ab	10.38 ± 5.66	ab	3.00 ± 0.00	a
3	NS	0	4	2.25 ± 0.50	ab	3.75 ± 1.26	ab	13.38 ± 3.54	ab	3.00 ± 0.00	a
4	NS	0	6	0.75 ± 0.50	b	1.25 ± 0.25	b	5.63 ± 4.25	b	1.75 ± 0.96	b
5	NS	2	0	2.25 ± 0.96	ab	3.00 ± 2.30	ab	8.38 ± 3.64	ab	2.75 ± 0.50	a
6	NS	4	0	1.75 ± 0.50	ab	4.50 ± 0.58	ab	16.75 ± 2.6	a	3.00 ± 0.00	a
7	NS	6	0	2.13 ± 0.83	ab	2.13 ± 0.22	ab	9.44 ± 6.41	ab	2.50 ± 0.76	ab
8	ST	0	0	1.50 ± 1.00	b	3.25 ± 1.30	ab	9.50 ±7.05	ab	2.50 ± 0.58	ab
9	ST	0	2	3.25 ± 1.70	a	5.50 ± 1.29	ab	16.63 ± 2.50	a	3.00 ± 0.00	a
10	ST	0	4	1.75 ± 1.50	ab	4.50 ± 3.70	ab	11.38 ± 7.34	ab	2.50 ± 1.00	ab
11	ST	0	6	2.50 ± 1.00	ab	7.00 ± 1.00	a	11.50 ± 7.69	ab	2.75 ± 0.50	a
12	ST	2	0	1.25 ± 0.50	b	2.50 ± 0.26	b	10.13 ± 9.69	ab	2.25 ± 0.96	ab
13	ST	4	0	1.75 ± 0.96	ab	4.00 ± 0.31	ab	12.75 ± 4.99	ab	3.00 ± 0.00	a
14	ST	6	0	2.50 ± 2.12	ab	7.50 ± 2.12	a	17.50 ± 0.71	a	3.00 ± 0.00	a

Data are expressed as means ± SD (standard deviation) of at least 20 observations per treatment

NS = nodal segment; ST = shoot tip

Means within the same *column* and with the same *letters* are not significantly different according to Duncan's multiple range test ($P < 0.05$)

± 0.04 pg, respectively, compared with 3.26 ± 0.06 pg and 1.63 ± 0.02 pg, respectively, for *in vitro* grown plants (Table 5). There was no statistical difference in nuclear DNA content between seed-derived and *in vitro* grown plants ($P \leq 0.05$). To our knowledge, this is the first report of genome size in *H. moscheutos*.

Chromosome Count Among genetic changes leading to somaclonal variation are various types of chromosomal

Table 4. Percentage of root formation in *Hibiscus moscheutos* L. 'Luna White' explants (nodal segments and shoot tips) from experiment 1 and experiment 2. In experiment 1, media containing growth regulators BA (0, 2, 4, or 6 μM) and *mT* (0, 2, 4, or 6 μM) were used. Explant cuttings from experiment 1 were transferred to MS0 medium containing no growth regulators. Data represent the number of explants that formed out of the total number of explants in a treatment

Treatment no.	Explant source[a]	BA[b] (μM)	*m*T[b] (μM)	Percentage of root formation (%)	
				Experiment 1	Experiment 2
1	NS	0	0	90	75
2	NS	0	2	10	100
3	NS	0	4	0	100
4	NS	0	6	0	25
5	NS	2	0	80	100
6	NS	4	0	0	100
7	NS	6	0	0	75
8	ST	0	0	70	75
9	ST	0	2	40	100
10	ST	0	4	10	75
11	ST	0	6	30	75
12	ST	2	0	10	75
13	ST	4	0	11	75
14	ST	6	0	40	100

[a] NS = nodal segment; ST = shoot tip

[b] BA= 6-benzyladenine; *mT* = *meta*-Topolin

Table 5. Average (Mean ± SD*) nuclear DNA content of *Hibiscus moscheutos* L. 'Luna White' obtained from seeds and *in vitro* culture

Source	Ploidy (*x*)	2C DNA (pg)		1Cx-DNA (pg)		1Cx-Mb (Mbp)	
Seed-derived plants	2	3.25 ± 0.08[b]	a	1.62 ± 0.04	a	1587.70 ± 37.2	a
In vitro regenerated plants	2	3.26 ± 0.06	a	1.63 ± 0.02	a	1593.40 ± 35.6	a

Ten seed-derived plants and 10 *in vitro* grown plants were used for the flow cytometry analysis. Two different leaves were collected from each plant and separately co-chopped with the internal standard *Sorghum bicolor* 'Tx623' (Peak 1, 2C 1.67 pg)

* SD = standard deviation

Means within the same *column* belonging with common *letters* are not significantly different according to Student's t-test ($P < 0.05$)

changes including disturbed ploidy and chromosome number as well as changes in chromosome architecture such as duplications, translocation, deletion, and inversions of chromosome segments (Bednarek and Orłowska 2020). Flow cytometry may be used to detect aneuploidy (addition or deletion of one or more chromosomes) (Pfosser *et al.* 1995; Roux *et al.* 2003) but not to count chromosomes, so we used conventional chromosome count to determine the exact number of chromosomes for both seed-derived and in *vitro* grown plants. The chromosome number $2n = 2x = 38$ is generally reported for *H. moscheutos* (Skovsted 1935; Small 2004; Wise and Menzel 1971; Barrios and Ruter 2019). The first chromosome count in *H. moscheutos* was conducted by Skovsted (1935) who reported $x = 19–20$ as basic chromosome numbers for *H. moscheutos* and *H. palustris*, which are considered synonyms by some taxonomists. We counted $2n = 2x = 38$ for both seed-derived and *in vitro* grown plants (Fig. 3*a–b*). In general, the plant genome contains a very high amount of heterochromatic DNA [mainly AT-rich (Schweizer 1976)] concentrated in the pericentromeric region as revealed by the dark Azure-B stain (braces in Fig. 3*a*). Late prophase chromosomes of a seed-derived plant with their euchromatin structures indicated by arrows and braces are shown in Fig. 3*a*, which also shows two satellites indicated by arrowheads. A satellite chromosome is a chromosome with a segment separated from the main body of the chromosome by a secondary constriction, which is known as nucleolus organizing region (NOR). Sometimes, the satellites might be detached during chromosome preparation from the mother chromosomes and counted as individual chromosomes, which would result in additional chromosome counts (Islam-Faridi *et al.* 2020b). *H. moscheutos* contained one pair of satellite chromosomes that are clearly separated by lightly stained secondary constrictions (arrows, Fig. 3*b*). Our chromosome spreads clearly show that the basic chromosome number for *H. moscheutos* is $x = 19$. The earlier report (Skovsted 1935) of $x = 20$ for *H. moscheutos* was probably due to erroneous counting of the two satellites as complete chromosomes. Metaphase chromosome spread of an *in vitro* grown *H. moscheutos* plant with the same number $2n = 2x = 38$ is shown in Fig. 3*b*, confirming the genetic fidelity of the regenerants at the chromosome level.

The results of both flow cytometry analysis and chromosome spread strongly suggested that the regenerants obtained in this study were genetically stable. However, subtle genomic DNA mutations/rearrangements are not readily easy to detect

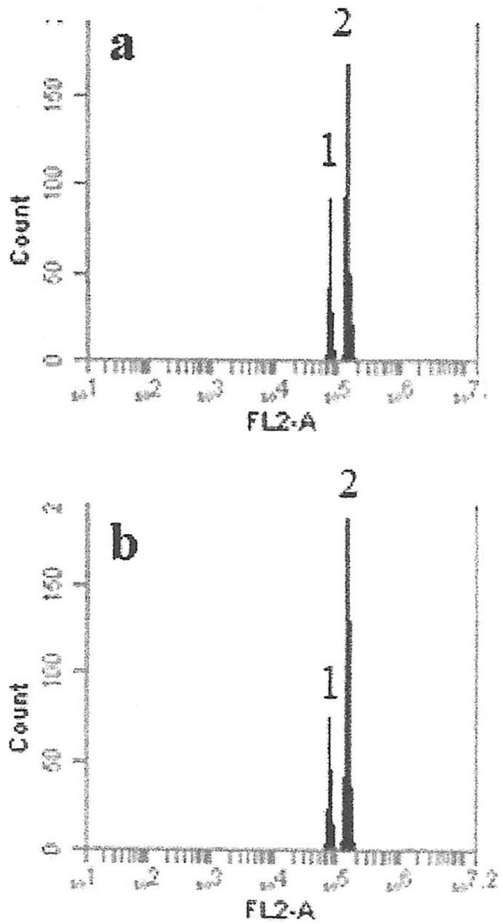

Figure 2. Representative histogram of nuclear DNA content estimation of *Hibiscus moscheutos* L. 'Luna White' using flow cytometry. Simultaneous analysis of nuclei isolated from the internal standard *Sorghum bicolor* 'Tx623' (Peak 1, 2C 1.67 pg) and *H. moscheutos* 'Luna White'. (***a***) Internal standard *S. bicolor* 'Tx623' (Peak 1, 2C 1.67 pg) and seed-derived *H. moscheutos* L. 'Luna White' plants (Peak 2, 2C 3.25 ± 0.08 pg). (***b***) Internal standard *Sorghum bicolor* 'Tx623' (Peak 1, 2C 1.67 pg) and *in vitro* grown *H. moscheutos* 'Luna White' plants (Peak 2, 2C 3.26 ± 0.06 pg). The two peaks represent populations of nuclei in G1 phase of cell cycle.

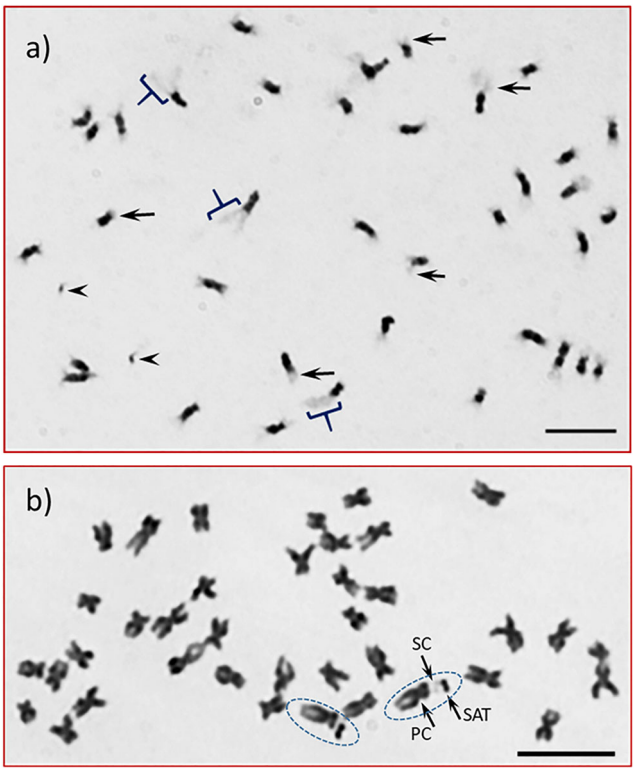

Figure 3. Root tip somatic chromosome spread from (***a***) seed-derived *Hibicus moscheutos* 'Luna White' and (***b***) *in vitro* grown *H. moscheutos* 'Luna White'. In ***a***, late prophase chromosomes ($2n = 2x = 38$) of a seed-derived plant with their euchromatin structures indicated by arrows and braces. Satellites (*arrowheads*) are detached or separated from their respective mother chromosomes. In ***b***, metaphase chromosomes ($2n = 2x = 38$) of an *in vitro* grown *H. moscheutos* 'Luna White', which contained a prominent pair of satellite chromosomes (marked by *dotted oval-shaped circles* and the satellites are attached with their respective mother chromosomes), SC = secondary constriction, PC = primary constriction, SAT = satellite. *Bar* = 5 μm.

(Smýkml *et al.* 2007; Sun *et al.* 2013). Reduced or loss of fertility attributed to epigenetic factors such as changes in DNA methylation levels has been reported in *in vitro* regenerated plants (Sun *et al.* 2013). Therefore, even though the *in vitro* regenerated plants grown *ex vitro* in the greenhouse were phenotypically similar to seed-derived plants in terms of both vegetative growth and flowering patterns (Fig. 1*a–b*), a controlled self-pollination of *in vitro* grown plants was conducted. All regenerants produced flowers similar to those of seed-derived plants (Fig. 1*b*). Also, a seed germination test conducted on seeds derived from controlled self-pollination yielded a 90% germination rate and normal-looking seedlings (Fig. 1*c*).

Conclusions

In this study, we provided an efficient protocol for micropropagation of *H. moscheutos* using two explant types, 2-node and shoot tip explants, and two cytokinins (BA and *mT*) capable of producing true-to-type regenerants. Both BA and *mT* can be used at 2 μM or 4 μM using either 2-node or shoot tip explants for 56 d. The protocol developed in this investigation can be used for plant transformation, *in vitro* selection, and industrial mass micropropagation of improved *H. moscheutos* selections or cultivars. To our knowledge, this study is the first report on genome size in *H. moscheutos*. Finally, the chromosome number ($2n = 2x = 38$) of *H. moscheutos* was unequivocally confirmed.

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Declarations

Conflict of Interest The authors declare no competing interests.

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