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In vitro Micropropagation in *Boehmeria nivea* to Generate Safe Planting Materials for Large-scale Cultivation

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Abstract

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An efficient *in vitro* micropropagation protocol has been developed using nodal explants of ramie (*Boehmeria nivea*), with maximum shoots (42) per explant in 5 passages (passage duration: 21 days) on Murashige and Skoog medium supplemented with 2.0 mg/l 6-benzyladenine and 2.0 mg/l $AgNO_3$. ½ Murashige and Skoog medium containing 40% sucrose was found to be most effective for the rooting of *in vitro* developed shoots. Those plantlets were acclimatized and transferred to pots for hardening under glasshouse conditions. About 91% of mericlones survived and showed no ectopic expression in respect of any morphological character in comparison with the parental stock. Furthermore, clonal fidelity of the mericlones was confirmed by using DNA markers (random amplified polymorphic DNA and inter simple sequence repeats) and by polypeptide profiling through SDS-PAGE at a genomic and protein level, respectively, which showed the true-to-type nature of the *in vitro* micropropagated plants. Thus the protocol developed can be used to generate safe planting material for large-scale cultivation of ramie.

Keywords: clonal fidelity; molecular markers; ramie; tissue culture; vegetative propagation

Ramie (*Boehmeria nivea* /L./ Gaud.), commonly known as Chinese grass, is the strongest and finest natural vegetable fibre crop in the world obtained from the bark of the plant. It is native to China, where it has been domesticated and which is the major producer, followed by Brazil, Indonesia, Philippines, Korea, Vietnam, Japan, Taiwan, Columbia, Malaysia and France. Ramie has been used as blending material with other fibres in the textile and other ancillary industries over centuries owing to its excellent fibre quality (Batra & Bell 1975). It is endowed with excellent fibre properties like high tensile strength (Goda *et al.* 2006), which slightly increases when wet (Fontanelli 1998). Excellent thermal conductivity, appreciable coolness, enhanced ventilation function, moisture absorption capacity and antibacterial properties are the major merits of ramie fibres, which make it highly compatible for textile industries. Ramie seeds are extremely small in size with very low germination percentage, hence seeds are not appropriate to propagate ramie plants for large-scale cultivation. Conventionally, it is propagated through rhizomes, stemlets, stem cuttings, which are not only bulky in nature but also endowed with short shelf-life (~10 days) and low field establishment rate. Furthermore, those are not safe planting material from a phytosanitary standpoint since collected from the standing crop growing *in natura*, they are harbouring many pathogens. *In vitro* culture techniques offer an option for efficient and rapid multiplication of planting materials when high uniformity of the progenies is required to solve the scarcity of planting materials for area expansion under ramie cultivation.

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The aim of the present research work was to establish a reliable and reproducible plantlet regeneration protocol using nodal explants by capitalizing the meristematic potential of the axillary meristem to develop *in vitro* micropropagated ramie plants *en masse* and confirmation of their true-to-type nature through a clonal fidelity test.

MATERIAL AND METHODS

Source of explants. Young nodal explants (8–10 cm) of ramie var. Kanai (R67-34) were collected from 2–3 months-old field grown material maintained at ICAR – Central Research Institute for Jute and Allied Fibres (CRIJAF), Nilganj, Barrackpore, West Bengal (latitude 22°45'N, longitude 88°26'E).

Explant preparation and sterilization. Nodal segments of 8–10 cm length were excised and kept in an ice-cold antioxidant solution containing 0.1% (w/v) ascorbic acid and 0.15% citric acid for 20 min. Those explants were washed thoroughly under running tap water for 5 min. Under aseptic conditions, they were snap dipped for 10 s in 70% ethyl alcohol followed by immersion in 1 (N) H_2SO_4 for 1 min and quick dipping in 0.5 (M) Na_2CO_3 solution. Then they were washed with sterile ddH_2O 4–5 times and dipped in 2.0% NaClO with 0.1% Tween20 for 30 min and subsequently washed with sterile ddH_2O water 7–8 times. Finally the explants were air dried in horizontal Laminar Airflow Cabinet Klenzaids (Model 1590-R-48-24-30, Klenzaids, India) and were cut into 4–5 cm long pieces prior to culture.

Culture media and culture conditions. The nodal explants were placed vertically on 15 ml Murashige and Skoog basal medium (Murashige & Skoog 1962) with 3% (w/v) sucrose, 2.8 g/l Gelrite (w/v) used as a solidifying agent, pH adjusted to 5.7 ± 0.02 before autoclaving at 121°C under 1.05 kg/cm^2 pressure for 20 min in culture tubes (25 × 150 mm; No. 9820U08, Borosil®, India). All the cultures were kept at a temperature of 28 ± 2°C with 16/8 h photoperiod with 130 µE/m^2/s light provided by white fluorescent light and 70–80% relative humidity.

Shoot induction, multiplication and elongation. To study the effect of different cytokinins on bud breaking, nodal explants were cultured on Murashige and Skoog medium containing different concentrations of either 6-benzyladenine (BA) or kinetin (concentrations used: 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) and also with different concentrations (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) of silver nitrate ($AgNO_3$) in a separate experiment. For shoot multiplication, the mother culture (explants) was separated from newly formed shoot buds and the explants were transferred onto shoot multiplication media (Murashige and Skoog medium supplemented with 2 mg/l BA) and maintained up to five passages to study the effect of successive transfer on shoot multiplication. Subculturing was done at an interval of every 21 days for 5 passages. Individual shoots from the bunch of shoots were separated and transferred onto Murashige & Skoog medium with a reduced dose of BA (1 mg/l) for further elongation.

***In vitro* rooting**. For root induction under *in vitro* conditions, individual shoots with 3–4 fully expanded leaves (2–5 cm in length) from the elongation medium were transferred to half-strength of Murashige and Skoog medium fortified with different concentrations of sucrose (10, 20, 30 , 40 and 50 g/l).

Acclimatization of regenerated plantlets. Well-developed rooted individual *in vitro* micropropagated ramie plantlets of 6–8 cm height were taken out carefully, washed in ddH_2O to remove the adhered medium and placed in 10–12 ml Hoagland solution (Yoshida *et al*. 1976) pH 5.0 under 16/8 h photoperiod in culture room for 4–5 days in rimless culture tubes closed with cotton caps wrapped in cotton cloth.

Hardening of acclimatized plantlets. Survivor plantlets were transplanted into small plastic pots containing vermiculite, garden soil and sand (1:1:1) covered with polyethylene bags in the glasshouse and watered to maintain a high relative humidity. After 45–50 days, plantlets were transferred outside the glasshouse in larger cement pots filled with soil.

Experiment design and statistical data analysis. All the experiments were conducted following a completely randomized design (CRD) and were replicated thrice with 10 explants per replication in each treatment. Observations were recorded at 21 days interval. Data were statistically processed using the one-way analysis of variance (ANOVA), and significant differences between means were assessed by Duncan's multiple range tests (DMRT) at $P = 0.05$.

Clonal fidelity. Clonal fidelity using random amplified polymorphic DNA (RAPD) markers and ISSR (inter simple sequence repeats) and protein profiling using SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) was carried to confirm the true-to-type nature of the mericlones developed.

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Genomic DNA isolation. Fresh young leaves (~200 mg) from 3-months-old plants grown from each *in vitro* micropropagated plant were crushed in 1.0 ml cetyltrimethylammonium bromide (CTAB) extraction buffer (100 mM Tris HCl, 10 mM EDTA, pH 8.0; 1.4 M NaCl and 2% CTAB) for genomic DNA isolation as outlined by Murray and Thompson (1980), with minor modifications.

Polymerase chain reaction (PCR) analysis. PCR amplification was performed in a Veriti Thermal Cycler (Applied Biosystems, USA) using a set of five random RAPD and three ISSR primers for assessing the variation at a genomic level between the mother plant and different mericlones developed through *in vitro* micropropagation. Amplification reaction was carried out in 25 µl reaction volume containing 50–100 ng of genomic DNA, 5 µl of 1X reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 unit of Taq DNA polymerase (PCR components used from Promega) and 5 pmol of primer (GCC Biotech, Pvt. Ltd, India). Thermal cycling for RAPD was performed following the programme: 5 min at 94°C; followed by 44 cycles of 1 min at 94°C, 1 min at annealing temperature based on the primer Tm and 1 min at 72°C, followed by one cycle at 72°C for 8 min. For ISSR analysis the PCR programme as followed was: 4 min at 95°C; followed by 43 cycles of 30 s at 95°C, 1 min for annealing based on the primer Tm and 2 min at 72°C followed by 1 cycle of final extension at 72°C for 10 min. Amplified products were separated through electrophoresis in 1.6% agarose gel containing ethidium bromide in 0.5 X TBE buffer at 80 volts for 1.5 h. Gel was photographed under UV light using the gel documentation system. The size of the amplified product was estimated using 1kb DNA ladder.

Polypeptide profiling. To confirm clonal fidelity at a protein level, about ~200 mg of fresh leaf samples were collected from 3-months-old micropropagated plants and ground in liquid N_2 (–196°C) into fine powder and the protein was extracted by trichloro acetic acid (TCA) acetone method under cold conditions following the protocol of Deng *et al.* (2013) with minor modifications. Isolated protein was quantified following the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as standard. Polypeptides were electrophoresed on 12% SDS-PAGE according to Laemmli (1970). About 20 µg of crude extract along with gel loading dye was loaded in each well. The gel was stained using Coomassie brilliant blue and photographed with a Sony digital camera.

RESULTS AND DISCUSSION

To assess the bud breaking from nodal explants of ramie, two different cytokinins (BA and kinetin) were used. Bud breaking was observed on Murashige and Skoog medium supplemented with different concentrations of either BA (Figure 1A) or kinetin alone after 7–9 days of culture. However, nodal explants cultured on Murashige and Skoog medium containing different plant growth regulators (PGR) at different concentrations supplemented with $AgNO_3$ showed bud breaking within 2–5 days depending upon explant vigour (data not shown). However, differential growth rate of the sprouted buds from the axils of nodal explants on Murashige and Skoog medium with 2 mg/l $AgNO_3$ in addition to (*i*) 1 mg/l BA, (*ii*) 2 mg/l BA and (*iii*) 2 mg/l kinetin was distinctly discernible (Figure 1B). The medium without any PGR (control) showed no shoot induction even after 10 days.

Different concentrations of BA displayed an altered response in respect of shoot length and rate of multiplication. Concentrations of BA ranging from 0.5 to 2.0 mg/l were found to be more effective in comparison with higher levels (2.5, 3.0 mg/l) of BA for shoot induction. It was found that beyond 2.0 mg/l BA, bud breaking and shoot induction declined and further elongation of shoots was also reduced. Among different concentrations of BA tested, the most responsive dose was 2 mg/l BA (Table 1). Similarly, explants cultured on the medium supplemented with different concentrations of kinetin showed that as the concentration of kinetin increased from 0.5 to 2.0 mg/l, the rate of shoot induction and proliferation was also enhanced in respect of the number of shoots and shoot length and thereafter declined. Profuse and repetitive multiplication of shoots was observed (Figure 1C, D) on the medium supplemented with 2 mg/l BA fortified with 2 mg/l $AgNO_3$.

In the present study explants cultured on Murashige and Skoog medium supplemented with either BA or kinetin showed a similar kind of response. As the concentration of those hormones was increased, the *in vitro* culture response of explants was found to increase up to certain levels and then it decreased even with an increase in the concentration. However, among the two hormones, BA was found to be more responsive in bud breaking and shoot multiplication in comparison with kinetin. BA has been reported to be superior to kinetin in bud breaking and multiple shoot induction in many plants (Malek *et al.* 2010; Saha *et al.* 2012). A combination of α-naphthaleneacetic acid

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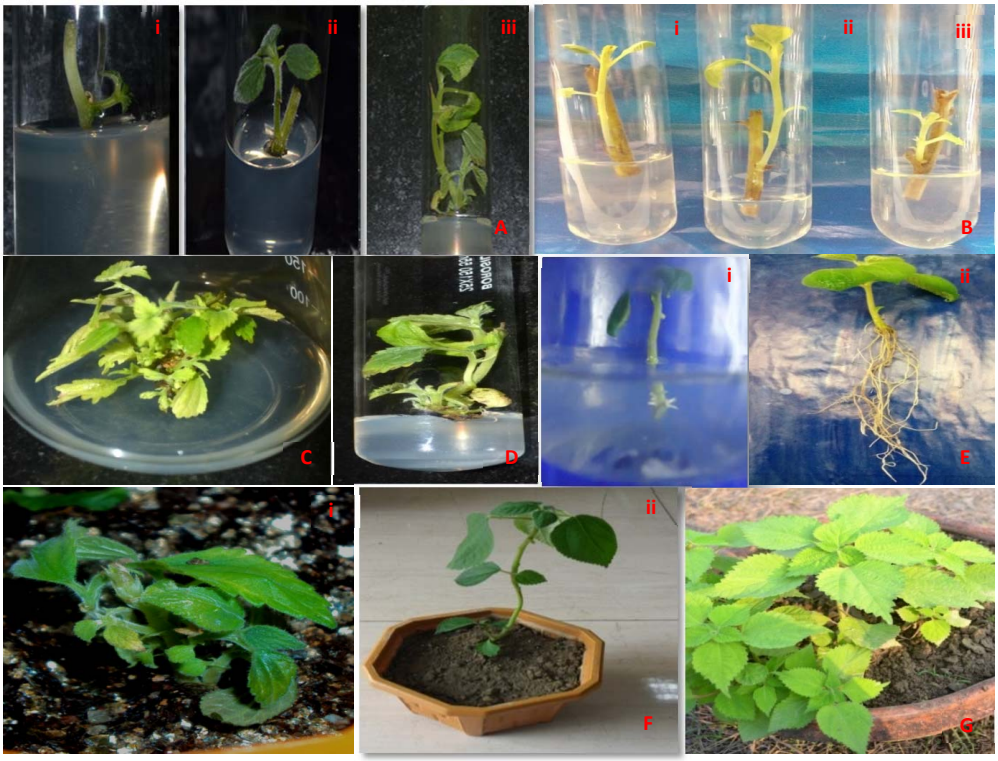

Figure1. *In vitro* clonal propagation of ramie: (A) bud breaking on Murashige and Skoog medium containing 2 mg/l BA, growth visible at 4 (*i*), 10 (*ii*) and 20 days (*iii*) of culture; (B) differential growth rate on Murashige and Skoog medium with 2 mg/l $AgNO_3$ in addition to 1 mg/l BA (*i*), 2 mg/l BA (*ii*) and 2 mg/l kinetin (*iii*); (C) mass multiplication under *in vitro* culture conditions; (D) separated shoots showing repetitive multiplication; (E) individual shoots on rooting medium: initiation of roots on 0.5 Murashige and Skoog with 40 g/l sucrose (*i*) and well developed root system and complete plant (*ii*); (F) hardened plantlet after 3 days of establishment (*i*) and plantlets transferred to pot growing vigorously (*ii*); (G) *in vitro* raised plants after 3 months of transplantation into pots in field

(NAA) and kinetin showed multiple shoots in ramie with shoot-tip cultures (Del Rosario & Corcolon 1991) whereas Sut *et al.* (2004) reported multiple shoot induction from shoot-tip and nodal segments with 1.5 mg/l BAP (6-benzylaminopurine).

Hyperhydricity, the most common phenomenon during *in vitro* micropropagation, was observed in the present study in plantlets cultured on Murashige and Skoog medium supplemented with kinetin (disproportionate root to shoot growth). However, the

Table 1. Effect of different concentrations of 6-benzyladenine (BA) or kinetin on multiple shoot induction from nodal explants of ramie

BA/kinetin (mg/l)	BA			Kinetin		
	shoot multiplication (%)	No. of shoots/explant	shoot length (cm)$^+$	shoot multiplication (%)	No. of shoots/explant	shoot length (cm)
0.0	0.0	0.0^{i}	0.00^{g}	0.0	0.0^{i}	0.00^{g}
0.5	62.77	1.33 ± 0.06^{ij}	1.28 ± 0.08^{def}	59.45	1.15 ± 0.12^{jk}	1.12 ± 0.02^{ef}
1.0	77.77	2.77 ± 0.09^{de}	2.13 ± 0.14^{bc}	68.88	2.009 ± 0.11^{gh}	1.58 ± 0.08^{cdef}
1.5	81.66	3.42 ± 0.13^{b}	2.21 ± 0.14^{de}	78.33	2.71 ± 0.06^{def}	2.08 ± 0.10^{bc}
2.0	93.88	6.09 ± 0.09^{de}	2.13 ± 0.04^{bc}	84.44	3.28 ± 0.06^{bc}	2.40 ± 0.13^{b}
2.5	71.66	2.94 ± 0.08^{cd}	1.96 ± 0.12^{bcd}	71.66	2.55 ± 0.12^{defg}	1.78 ± 0.04^{bcde}

$^+$Observations of 21-days-old shoots; values denote means ± SE of 30 cultured explants; for each column and treatment, means followed by the same letter are not significantly different according to Duncan's multiple range tests ($P < 0.05$)

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Table 2. Effect of different concentrations of $AgNO_3$ supplemented Murashige and Skoog medium containing 2 mg/l 6-benzyladenine (BA) in influencing shoot initiation

$AgNO_3$ (mg/l)	Leaf No.	Shoot length (cm)
0.0	8.60 ± 0.44^{a}	12.27 ± 0.74^{a}
1.0	8.07 ± 0.22^{ab}	6.07 ± 0.41^{c}
2.0	8.37 ± 0.29^{a}	5.12 ± 0.39^{c}
3.0	7.23 ± 0.29^{b}	3.36 ± 0.22^{b}

Values denote means ± SE of 30 cultured explants; for each column and treatment, means followed by the same letter are not significantly different according to Duncan's multiple range tests ($P < 0.05$)

Table 3. Effect of different concentrations of sucrose in 0.5 Murashige and Skoog medium on root induction from shoots of ramie

Sucrose (g/l)	Root induction (%)	No. of root/shoot	Root length (cm)
0.0	0.0	0.0^{g}	0.00^{f}
10	15.55	0.41 ± 0.12^{ef}	0.23 ± 0.01^{def}
20	18.88	0.49 ± 0.05^{e}	0.31 ± 0.01^{de}
30	34.44	1.02 ± 0.07^{c}	0.62 ± 0.16^{bc}
40	81.11	3.18 ± 0.06^{a}	1.24 ± 0.09^{a}
50	56.11	1.80 ± 0.11^{b}	0.84 ± 0.07^{b}

Values denote means ± SE of 30 cultured explants; for each column and treatment, means followed by the same letter are not significantly different according to Duncan's multiple range tests ($P < 0.05$)

same was not observed in plants cultured on the medium fortified with BA. To overcome hyperhydricity different concentrations of the widely used chemical $AgNO_3$ were used and the best results were observed on Murashige and Skoog medium containing 2.0 mg/l BA and 2.0 mg/l $AgNO_3$ with maximum bud breaking, shoot induction, shoot length and leaf area in ramie. The medium fortified with a high dose of $AgNO_3$ showed a decrease in the leaf number and shoot length (Table 2). BA in combination with $AgNO_3$ showed a reduced hyperhydricity effect and bud breaking occurred within 2–3 days depending upon the explant health (data not shown). Micropropagation on BA in combination with $AgNO_3$ was reported as the best combination in many plants like *Punica granatum* L. (Naik & Chand 2003), *Brassica* sp. (Chi & Pua 1989; Chi *et al.* 1991; Palmer 1992), *Helianthus annuus* (Chraibi *et al.* 1991) and *Nicotiana plumbaginifolia* (Purnhauser *et al.*1987).

For rooting *in vitro* developed shoots were cultured on 0.5 Murashige and Skoog medium supplemented with different concentrations of sucrose. However, no rooting was observed at a lower dose of sucrose, similar results were reported by Wainwright and Scrace (1989). Profuse rooting (Figure 1E) was observed on 0.5 Murashige and Skoog basal media containing 40 g/l sucrose (Table 3), near the cut surface of the shoot explants (basal end) (Figure 1F), similar results with 40 g/l sucrose were reported by Singh and Shymal (2001), which corroborated our observations. In ramie the root development with both kinetin and indole-3-butyric acid (IBA) was reported by Dumanois *et al.* (1986), BA and IBA by Sut *et al.* (2004) and on 0.5 Murashige and Skoog medium supplemented with NAA by Wang *et al.* (2007). The use of high concentrations of sucrose induced more rooting (Hazarika *et al.* 2000; Singh & Shymal 2001) and increased root length and root quality (George & Sherrington 1984). About

Table 4. Primers, their sequences and the number of bands of amplified fragments generated by RAPD and ISSR in both micropropagated and mother plants of ramie

Primers sequence (5'–3')	No. of bands		
	total	monomorphic	polymorphic
RAPD analysis			
1. OPA1 (5'CAGGCCCTTC3')	5	5	0
2. OPA2 (5'TGCCGAGCTG3')	4	4	0
3. OPA3 (5'AGTCAGCCAC3')	2	2	0
4. OPA5 (5'AGGGTCTTG3')	5	5	0
5. OPA6 (5'GGTCCCTGAC3')	2	2	0
ISSR analysis			
1. U885 (5'BHBGAGAGAGAGAGAGA3')	6	6	0
2. U840 (5'GAGAGAGAGAGAGAGAYT3')	3	3	0
3. U836 (5'AGAGAGAGAGAGAGAGYA3')	5	5	0

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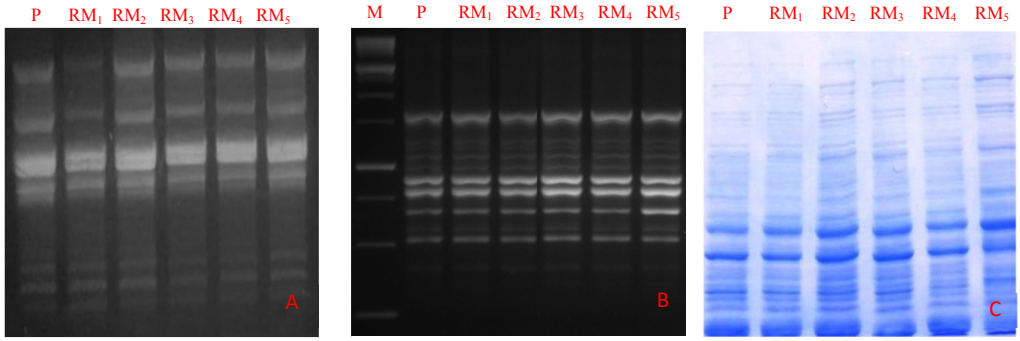

Figure 2. PCR amplification products obtained from the parental stock (P) and micropropagated plantlets (RM1-RM5) with a RAPD primer (OPA-5) (A); ISSR primer (ISSR-U836) (B); polypeptide profile (SDS-PAGE) of the parent and the mericlones in the same order in ramie (C)

91% of ramie plants were successfully hardened and established in the soil (Figure 1F, G).

Genetic fidelity of the clones developed through *in vitro* micropropagation is essential as somaclonal variation often appears under *in vitro* tissue culture conditions, which warrants the fidelity test to confirm their clonal nature. In the presents study, no ectopic expression of characters at the morphological level was observed, which has been confirmed by molecular assays involving PCR (RAPD and ISSR) and SDS-PAGE for protein. Five RAPD and three ISSR primers (Table 4) produced clear and distinct bands, which generated a total number of 108 and 102 bands among the mother and clones with an average of 18 and 12 bands per primer, respectively. None of the primers showed any deviating *in vitro* raised clones (Figure 2a, b) in comparison with the mother stock. The size of the band ranged from 260 to 2750 bp for RAPD and from 510 to 1600 bp for ISSR.

For clonal fidelity at a protein level the crude protein sample was loaded on 12% SDS-PAGE. From the polypeptide profile it was observed that all the samples loaded showed the same banding pattern without any deviation from the parental stock (Figure 2c). In nutshell no variation was detected in respect of both RAPD and ISSR primers and a uniform polypeptide profile confirms the true-to-parent nature of the mericlones.

CONCLUSION

Though several reports on the *in vitro* tissue culture of ramie have been published, none of them have been supported by the clonal fidelity test of regenerated plants. The protocol we have developed is reproducible and was found to display a high rate of shoot multiplication, which could be confidently used for large-scale commercial production, genetic transformation, *in vitro* germplasm conservation for long-term storage and maintenance of ramie crops in suboptimal gene bank for mid-term storage and to generate safe planting materials for large-scale ramie crop husbandry with ease and confidence.

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