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RESEARCH NOTE

Improved in vitro propagation, solasodine accumulation and assessment of clonal fidelity in regenerants of *Solanum trilobatum* L. by flow cytometry and SPAR methods

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Abstract An efficient protocol for the development of genetically uniform clones of a valuable medicinal plant *Solanum trilobatum* L. has been established. An optimal shoot regeneration response was observed in a modified Murashige and Skoog medium (M-MS) containing 25 mM ammonium nitrate, 2 mg l^{-1} 6-benzyl adenine and 0.1 mg l^{-1} indole-3-acetic acid using in vitro derived node and shoot tip explants. Consequently, the multiple shoot buds were elongated in MS medium supplemented with 0.5 mg l^{-1} Gibberellic acid. The in vitro regenerated shoots were rooted best in MS medium containing 1.5 mg l^{-1} indole-3-butyric acid and successfully acclimatized in the field. The single primer amplification reaction (SPAR) approach, including random amplified polymorphic DNA, inter simple sequence repeats and directed amplification of minisatellite DNA regions markers did not identify any genetic polymorphism among in vitro regenerants. Similarly flow cytometry analysis illustrated that the DNA content and genome size of micropropagated plants were equivalent to that of intact plants from field. In addition, the accumulation of solasodine in micropropagated plants was confirmed by thin layer chromatography and further quantified by high performance liquid chromatography analysis as 2.47 mg g^{-1} DW which is comparable to field grown plants. Thus the protocol can be effectively exploited for commercial propagation of this species to obtain solasodine and also in genetic transformation studies.

Keywords Acclimatization · Flow cytometry · Genetic stability · Micropropagation · *Solanum trilobatum* · Solasodine

Solanum trilobatum L. is a thorny medicinal shrub (Solanaceae) globally distributed in Indo-Malaysia and chiefly found in Southern India. The plant is extensively used in Indian traditional and folklore medicines to treat a wide array of ailments (Purushothaman et al. 1969), the therapeutic property of the plant being mainly attributed to glycoalkaloids such as β-solamarine and solasodine (Balakrishna et al. 1992). Solasodine ($C_{27}H_{43}O_2N$) is a nitrogen analogue and alternative of diosgenin, a prime raw material for the synthesis of steroid drugs in pharmaceutical industry (Mann 1978). The increasing demand of this multipurpose medicinal plant for its active principles has led to indiscriminate harvesting from wild affecting the status of wild plants (Dhavala et al. 2009). Moreover, propagation through seeds is inefficient due to poor germination of seeds (Alagumanian et al. 2004). Thus an effective strategy is required for rapid and large-scale proliferation of *S. trilobatum*.

Tissue culture is a potential tool for rapid mass multiplication of plants and holds great promise for controlled production of useful secondary metabolites, but the major problem encountered with tissue culture is somaclonal variation, which is often heritable (Breiman et al. 1987) and authentication of true-to-typeness remains crucial. Though several regeneration protocols are available for *S. trilobatum* (Arockiasamy et al. 2002; Jawahar et al. 2004; Alagumanian et al. 2004; Dhavala et al. 2009) to date no

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attempt has been made in appraising the genetic stability and measurement of active principles of micropropagated plants of *S. trilobatum*. Here, we have achieved successful plant regeneration in M-MS medium using node and shoot tip explants, evaluated the genetic integrity of in vitro regenerants by molecular markers (RAPD, ISSR and DAMD) and flow cytometry, and compared the levels of solasodine in micropropagated plants in relation to intact plants.

Explants were obtained from field grown plants of *S. trilobatum* established in the nursery of Department of Biotechnology, Alagappa University, Karaikudi and surface sterilized with 2 % Bavistin for 10–15 min followed by a brief rinse in 70 % alcohol (30 s) then treated with 0.1 % (w/v) mercuric chloride for 3–4 min and thoroughly washed in sterile distilled water thrice. The explants were incubated on MS medium (Murashige and Skoog 1962) and M-MS medium containing 25 mM ammonium nitrate supplemented with 3 % (w/v) sucrose, 0.8 % agar, and varying concentrations of plant growth regulators (PGRs). All cultures were maintained at 24 ± 2 °C under a light–dark cycle of 16:8, with a light intensity of 70 μ mol m^{-2} s^{-1}. The shoots were elongated in MS medium with GA_3 (0.1–1 mg l^{-1}). The elongated shoots (4–5 cm) were rooted in MS medium having different concentrations of IBA (0.5–3 mg l^{-1}). Plantlets with well developed roots were removed after 5 weeks and transferred to polypropylene Magenta™ vessels (Sigma, St. Louis, USA) containing equal proportions of autoclaved sand, red soil and vermiculite and maintained in culture room for 2 weeks. Thereafter they were transferred to earthen pots containing a mixture of 1:1 ratio of garden soil and vermiculite and kept in greenhouse for 2–3 weeks and finally transferred to the field for acclimatization. The experiments were carried out in a completely randomized design in which each treatment had 20 explants and all experiments were repeated thrice. The results were presented as a mean ± SE of three independent experiments. The data were analyzed using one-way analysis of variance (ANOVA) and the treatment means with significant differences were distinguished using Duncan's multiple range test (DMRT) at 5 % probability level. For analyzing the results of flow cytometry Student's *t* test was used at $P = 0.05$. All statistical analyses were carried out using SPSS version 15.0 statistical package.

Total Genomic DNA was isolated from donor mother plant and in vitro raised plants using HiPurA kit (Himedia, Mumbai, India) according to the manufacturer's instruction. The PCR reactions were carried out in a 25 μl reaction containing 1× Taq buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 μM primer (Sigma, St. Louis, USA), 1 U of Taq DNA polymerase (MBI, Fermentas, Lithuania) and 50 ng of template DNA. In a thermal cycler Eppendorf (Deutschland, Germany) the amplification was performed as follows: initial denaturation at 94 °C for 5 min, 42 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for RAPD, 55 °C for ISSR and DAMD for 1 min, extension at 72 °C for 2 min. The last cycle was followed by a final extension at 72 °C for 7 min. Amplified products were resolved in 1.5 % agarose gel in 1× TAE buffer, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc XR, Bio-Rad, Quarry Bay, Hong Kong).

The leaves of 2 years old in vitro shoots grown in M-MS medium containing 2 mg l^{-1} BA and 0.1 mg l^{-1} IAA were used for flow cytometry analysis. Nuclei were isolated as described by Dolezel et al. (2007). For each sample, at least 5,000–8,000 nuclei were analyzed in FACS Aria III (BD Biosciences, India) flow cytometer using the FACSDiva software package. *Solanum lycopersicum* L. 'Stupicke´ polnı´ rane´' (2C = 1.96 pg, Dolezel et al. 2007) was used as an internal standard.

Solasodine was extracted from field grown and in vitro plant materials based on a modified Bradley's method (Bradley et al. 1978). Dry plant powder (500 mg) was refluxed with 30 ml of 1 N HCl for 2 h at 100 °C and filtered. The pH of filtrate was adjusted to 10 using a dilute ammonia solution (10 % v/v) and the basic solution was extracted thrice with chloroform ($CHCl_3$) to ensure complete extraction. The $CHCl_3$ extracts were pooled and evaporated to dryness. For thin layer chromatography, the plant extracts and Solasodine standard (Sigma, St. Louis, USA) were dissolved in $CHCl_3$ at 10 mg ml^{-1} concentration and 10 μl was spotted on a precoated silica gel aluminium sheet 60F254 (Merck, Germany). The mobile phase used for separation of solasodine consisted of *n*-hexane:acetone (4:1 v/v). TLC plates were viewed under UV hood at 365 nm after spraying with anisaldehyde reagent and dried at 100 °C. For HPLC analysis, the above extracts were redissolved in 100 % HPLC grade methanol and filtered through 0.2 μm membrane filter. An aliquot of 20 μl was injected into Waters 2998 liquid chromatography (Waters, Milford, MA, USA) equipped with the photodiode array detector. Separation was achieved on Symmetry® C18 column (4.6 mm × 250 mm, 5 μm) using the mobile phase methanol: water (70:30), flow rate 1.0 ml min^{-1} at 30 °C and UV absorption at 205 nm. Data was analyzed using Empower 2 software.

Field collected explants showed best morphogenic response in MS medium containing 2 mg l^{-1} of BA and 0.1 mg l^{-1} of IAA, with higher shoot regeneration frequency (80, 76 %), number of shoots per explant (8.2 ± 0.40, 5.2 ± 0.73) and average shoot length (5.9 ± 0.32, 5.2 ± 0.73 cm) for node and shoot tip explants respectively (Online Resource 1). The efficiency of shoot regeneration was greatly influenced by addition of IAA at

Table 1 Effects of plant growth regulators on multiple shoot regeneration from in vitro derived node and shoot tip explants of *S. trilobatum* L. in M-MS medium

Plant growth regulators (mg l^{-1})			Node		Shoot tip	
BA	Kn	IAA	Response (%)	No. of shoots per explant	Response (%)	No. of shoots per explant
0.0	0.0	0.0	65	2.2 ± 0.64^{a}	56	1.4 ± 0.53^{a}
1	–	0.1	84	18.0 ± 0.55^{c}	78	11.6 ± 1.07^{d}
2	–	0.1	100	42.0 ± 0.54^{h}	98	25.0 ± 1.34^{g}
3	–	0.1	90	25.0 ± 0.75^{e}	88	15.2 ± 0.71^{e}
–	1	0.1	79	10.6 ± 0.70^{b}	78	6.4 ± 0.99^{b}
–	2	0.1	95	26.8 ± 1.03^{f}	91	12.6 ± 1.55^{d}
–	3	0.1	81	16.8 ± 0.93^{c}	80	8.4 ± 0.94^{c}
1	0.5	0.1	91	25.2 ± 1.40^{e}	89	13 ± 0.62^{d}
2	1	0.1	95	36.2 ± 0.83^{g}	92	18.6 ± 0.85^{f}
3	1.5	0.1	89	20.2 ± 0.30^{d}	87	9.2 ± 0.71^{c}

Mean values within a column having the same alphabet are not statistically significant ($P = 0.05$) according to Duncan's multiple range test

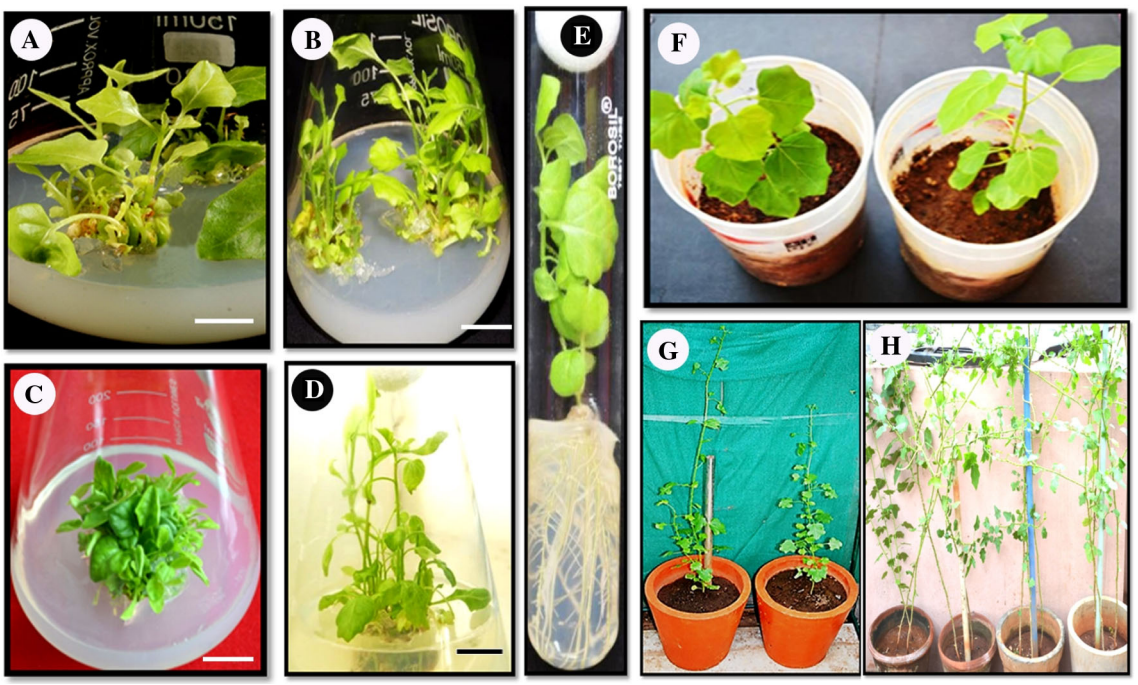

Fig. 1 In vitro plant regeneration from node and shoot tip explants of *S. trilobatum* L. **a** Multiple shoot induction from shoot tip explant in M-MS medium with 2 mg l^{-1} BA and 0.1 mg l^{-1} IAA after 4 weeks of culture (*bar* 0.8 cm). **b** Elongation of multiple shoot buds induced from shoot tip explants in MS medium with 0.5 mg l^{-1} GA_3 (*bar* 1 cm). **c** Multiple shoot induction from node explant in M-MS medium with 2 mg l^{-1} BA and 0.1 mg l^{-1} IAA after 4 weeks of culture (*bar* 1.2 cm). **d** Elongation of multiple shoot buds induced from nodal explant in MS medium with 0.5 mg l^{-1} GA_3 (*bar* 1 cm). **e** In vitro rooting of regenerated shoots in MS medium with 1.5 mg l^{-1} IBA. **f** Transfer of rooted plants to soil and maintenance under laboratory conditions. **g** Acclimatized plants maintained in greenhouse for 3 weeks. **h** Acclimatized plants in field

0.1 mg l^{-1} along with cytokinins, while further increases in concentration resulted in basal callus formation. Nodal explants were better than shoot tips. Interestingly, when in vitro derived explants were cultured in M-MS medium containing PGRs, multiple shoot buds were developed. A 100 % bud break appeared within 5 days, subsequently developing into a tuft of shoot buds at the bottom of explant after 4 weeks, regardless of PGR combination and concentration. A fivefold increase in mean shoot number per explant (42 ± 0.54 per node and 25 ± 1.34 per shoot tip) (Table 1; Fig. 1a, c) was achieved, when the same hormonal combination of 2 mg l^{-1} BA and 0.1 mg l^{-1} IAA was added in M-MS medium. The role of modified MS medium with different nitrogen levels for the improvement

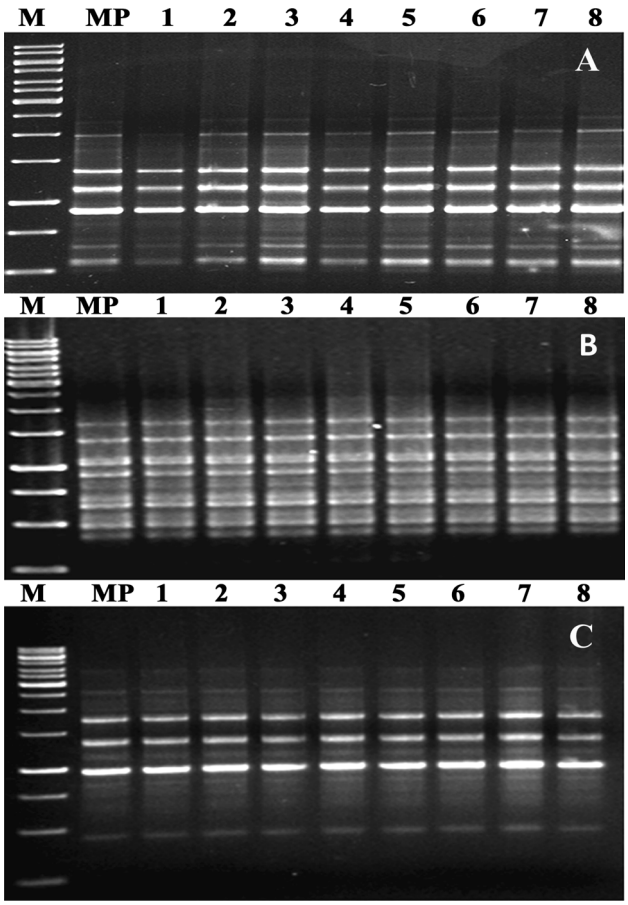

Fig. 2 Genetic similarity of plants regenerated from node and shoot tip explants of *S. trilobatum* revealed by **a** RAPD (OPH 04) **b** ISSR (A1) **c** DAMD (6.2H) markers. *Lane M* 1 Kb ruler, *lane MP* mother plant, *lanes 1–4* In vitro plants regenerated from node, *lanes 4–8* in vitro plants regenerated from shoot tip

of plant regeneration has often been reported previously (Poddar et al. 1997; Ivanova and Van staden 2009; Shirdel et al. 2011; Rahman et al. 2011). The in vitro regenerated shoots attained maximum elongation (10.8 ± 0.94 cm (node) and 9.2 ± 0.62 cm (shoot tip) using 0.5 mg $l^{-1}GA_3$ in MS medium (Online Resource 2) (Fig. 1b, d). The maximum frequency of root induction (77 %), mean number of roots (10.6 ± 0.35) and mean root length (4.6 ± 0.44 cm) was recorded in MS medium containing 1.5 mg l^{-1} IBA (Online Resource 3) (Fig. 1e). Rooted plantlets were initially maintained in laboratory conditions (Fig. 1f) then in greenhouse (Fig. 1g), in due course transferred to the field with 100 % survival rate (Fig. 1h).

For genetic fidelity analysis a total of 10 RAPD, 5 ISSR and 5 DAMD primers were used to identify genetic variation among 8 (4 from node and 4 from shoot tip derived shoots) randomly selected micropropagated plants established in the lab over a period of 2 years. The SPAR methods produced totally 155 PCR amplification products (71 RAPD, 38 ISSR and 46 DAMD) (Online Resource 4). The amplification products were monomorphic in nature across all micropropagated plants in comparison to the donor mother plant (Fig. 2). Among three marker systems used, DAMD primers resulted in higher number of amplification products with an average of 9.2 bands per primer than RAPDs (7.1 bands per primer) and ISSRs (7.6 bands per primer). The SPAR methods have been extensively used for genetic stability analysis of micropropagated plants like *Robinia ambigua* (Guo et al. 2006), *Aegle marmelos* (Pati et al. 2008) and *Kaempferia galanga* (Mohanty et al. 2011).

Flow cytometry is commonly employed to analyze the nuclear DNA content, genome size and ploidy stability of in vitro regenerated plants (Mallon et al. 2010; Ghimire et al. 2012; Vujovic' et al. 2012). In present study, flow cytometry analyses revealed that there was no significant difference ($P = 0.05$) among the 2C nuclear DNA content of in vitro cultured plants with that of donor mother plant (Fig. 3). To our knowledge, this is the first estimation of the genome size of *S. trilobatum* which, at 1C, was found to be in the range of 1,184–1,194 Mbp. The mean coefficient of variation (CV) values obtained was below 5 (Online Resource 5). Low CV value is a measure of accuracy of

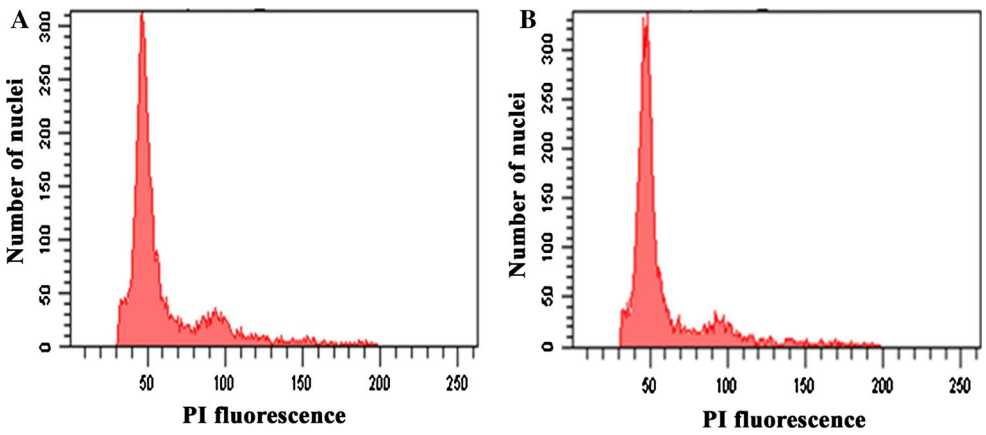

Fig. 3 Histograms show nuclear DNA content and ploidy stability of **a** Field grown plant, **b** In vitro cultured plant

analysis (Galbraith et al. 2002) and depicts the reliability and applicability of this protocol for other *Solanum* species. The results from flow cytometry analysis strongly suggest that the micropropagation protocol described here does not induce ploidy changes. Thin layer chromatography analysis indicated the presence of solasodine in both in vivo and in vitro plant extracts, as a single band with an Rf value of 0.8 at 365 nm (Online Resource 6). A similar range of Rf value (0.78) for solasodine was reported by Bhatnagar et al. (2004) in *Solanum laciniatum* Ait. Further HPLC analysis has revealed that, the micropropagated plants accumulated 2.47 ± 0.04 mg g^{-1} DW of solasodine, while the solasodine content of field grown plants was found to be 2.38 ± 0.02 mg g^{-1} DW. Anirudhan et al. (2009) has reported almost similar quantity of solasodine (2.32 mg g^{-1} DW) in field grown plants of *S. trilobatum*. The results of our study signify that, in vitro grown plants of *S. trilobatum* can serve as a good source of solasodine which can be exploited in pharmaceutical industries.

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