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Organogenesis and evaluation of genetic homogeneity through SCoT and ISSR markers in *Helicteres isora* L., a medicinally important tree

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ABSTRACT

The present study develops the highly reliable and reproducible protocol for indirect organogenesis of *Helicteres isora* L., a medicinally important multipurpose plant of Sterculiaceae family. Murashige and Skoog (MS) medium supplemented with different plant growth regulators induced callus with different type and texture. The combination of 10.74 μM naphthalene acetic acid (NAA) and 5.71 μM indole-3-acetic acid (IAA) fortified with MS medium resulted best response (100%) in callus induction with fresh weight from leaf (3.75 ± 0.11 g) and internodal (3.30 ± 0.14 g) explants. Green compact nodular callus (GCNC) was transferred to shoot regeneration medium for shoot regeneration. The multiplication rate of adventitious shoots was influenced by various factors like explant type, media composition, plant growth regulator combinations and concentrations. MS medium supplemented with 2.69 μM NAA, 0.57 μM IAA and 4.92 μM N6-(2-isopentenyl) adenine (2ip) combination resulted in 80.95 ± 4.76 percentage of response with 8.43 ± 0.43 number of shoots per piece of callus derived from leaf explant. This combination was expressed 76.19 ± 4.76 percentage of response with 7.28 ± 0.28 number of shoots per internode-derived callus respectively. The addition of 50 mg l^{-1} glutamine in 2.69 μM NAA, 0.57 μM IAA and 4.92 μM 2iP combination enhanced the shooting frequency with 12.14 ± 0.83 and 9.14 ± 0.51 shoots from leaf and internodal explant-derived callus. Elongated shoots [4.92 μM 2iP and 1.44 μM of gibberellic acid (GA_3)] were achieved rooting in half-strength MS medium fortified with 4.90 μM indole-3-butyric acid (IBA) with 582.84 μM activated charcoal. The genetic fidelity between mother plant and *in vitro* plants was assessed through SCoT and ISSR marker systems. Both these analysis revealed that all the samples were found monomorphic in nature. This suggests that the current study standardised the true-to-type culturing protocol and authenticated the *in vitro* raised plantlets are remain free from somaclonal variations.

1. Introduction

Helicteres isora L. (Sterculiaceae) is commonly known as East Indian screw tree and widely distributed throughout India in dry deciduous forests up to 1500 m on hill slopes. It is a medicinally important multipurpose subdeciduous shrub or a small tree. Various parts of *H. isora* possess pharmacological activities, including antidiabetic (hypoglycaemic and hyperglycaemic), anticancer, antimicrobial, antispasmodic and to treat snake bite, dog bite, diarrhoea and blood purification from ancient days till nowadays (Pramod et al., 2012; Kumar and Singh, 2014). Also the plant has various bioactive compounds, such as cucurbitacin- B, isocucurbitacin- B, diosgenin, daucosterol, hibifolin, trifolin, rosmarinic acids and many from different parts of plant (Pramod et al., 2012; Kumar and Singh, 2014).

In vitro propagation studies most often widely used for *ex situ* conservation, mass propagation and also the bioactive compound production (Sajc et al., 2000; Nasim et al., 2010). *H. isora* has problem with seed setting as well as seed dormancy causes regeneration difficulties *in vivo* (Badave and Jadhav, 1998; Atluri et al., 2000). *H. isora* has been harvested indiscriminately for its extensive medicinal uses have made serious survival threat also the further possibility of field extinction. Indirect organogenesis is a better way of getting callus biomass as well as adventitious shoot regeneration protocol. Earlier investigation on indirect organogenesis of *H. isora* reported low frequency of response (<70%) with limited number of shoots using nodal explants (Shriram et al., 2008). Various factors such as age, season and explant type causes low responsiveness for woody plant species at *in vitro* conditions (Purohit and Kukda, 2004). However, in adventitious shoot culture system, the selection of explant is important criteria; hence, we have chosen leaf and internodal explants. Early reports of *in vitro* raised seedling-based explants were found successful for indirect organogenesis in many woody plant species (Lavanya et al., 2014; Piatczac et al., 2015). Somaclonal variation during the *in vitro* regeneration causes serious limitations over this techniques (Bhojwani and Dantu, 2013). Explant nature, genotype, PGR type then concentration, methodology and

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repeated subculture play a vital role in genetic variations (Bairu et al., 2011). Likely disorganised growth phase and long-term maintenance and repeated subculture can cause variation among propagules (Rani and Raina, 2000; Kuznetsova et al., 2006). Lakshmanan et al. (2007) recommended variety of DNA-based marker system for the analysis of genetic homogeneity as it will have different target regions in that genome. Start codon targeted polymorphism (SCoT) and inter-simple sequence repeats (ISSR) are the two highly reproducible marker systems widely utilised to analyse the genetic homogeneity between mother plants and tissue cultured plants (Bekheet et al., 2015; Bhattacharyya et al., 2016). There was no report on genetic variation studies for indirect organogenesis of *H. isora*; hence, it makes necessitate to check genetic fidelity. The present study investigates the highly reproducible protocol for *H. isora* through indirect organogenesis and validates the genetic homogeneity between mother plants and *in vitro* raised plantlets.

2. Materials and methods

2.1. Plant collection and explant selection

H. isora L. fruits were collected from Kolli hills on Western Ghats of Tamil Nadu, India. Plants were identified by Botanical survey of India, Coimbatore (Specimen No.177312). Matured dry fruits of *H. isora* were untwisted to dehisce the seeds. Seeds were acid treated (H_2SO_4, 98%) for 1 min, then the seeds were washed with running tap water for 10 min to remove debris followed by detergent wash (Teepol™, Sigma- Aldrich, India) for 1 min and thoroughly washed through tap water and then rinsed with ethanol for 30 s, then 3% NaOHCl for 1 min and 0.1% *w/v* $HgCl_2$ for 5 min. Finally, the seeds were five times washed with sterile distilled water. Treated seeds were further inoculated into sterile wet cotton bed for seed germination. Leaf and internode of 4- to 6-week-old seedlings were used as explants.

2.2. Media selection and culture conditions

Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) was used as basal medium with sucrose (20.0 g l^{-1}) and agar (0.7%, Agar Agar type II) for callus initiation and further proliferation, whereas shoot elongation and rooting was performed with 0.8% agar. The cultures were incubated in culture room maintained at 25 ± 2 °C, under 16 h photoperiod with a light intensity of 35 μΣ $m^{-2}S^{-1}$ from Philips cool white fluorescent tubes with 55–60% relative humidity.

2.3. Callus initiation and proliferation

Explants including internode (IN) (1 cm in length) and leaf (L) (0.5 sq. cm) were excised from 35-day-old *in vitro* germinated seedlings and placed horizontally on MS medium. The effects of both auxins and cytokinins were studied individually also in combination for callus initiation and proliferation. Auxins like 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) and picloram (PIC) then different cytokinins, 6-benzyl adenine (BA), 6-furfuryl amino purine (KN) and thidiazuron (TDZ) were fortified at various concentrations either alone or in combination. The frequencies of response, fresh weight and nature of callus were recorded after 40 days.

2.4. Shoot regeneration from callus

Green compact organogenic callus (~0.5 g fresh weight) were selected and further maintained in PGRs free MS basal medium for 1 week then subjected to shoot regeneration (SR) medium, MS medium (20 g l^{-1} sucrose) supplemented with different cytokinins [BA, N6-(2-isopentenyl) adenine (2ip) and TDZ] with the best suited auxins at a concentration of 2.69 μM NAA and 0.57 μM IAA for shoot initiation and multiplication. The effect of organic additives like amino acids (glutamine and proline), polyamines (putrescine and spermidine), sodium citrate and adenine sulphate were tested at various concentrations in best responded shoot regeneration medium.

2.5. Shoot elongation, rooting and acclimatisation

Microshoots (≤1 cm) were excised from 40-day-old cultures and transferred to microshoot recovery medium, half-strength MS medium with 20 g l^{-1} sucrose and devoid of any PGRs. Recovered shoots with 3–4 leaves were further introduced into shoot elongation medium, half-strength MS medium (20 g l^{-1} sucrose) fortified with 4.92 μM 2iP and various concentrations of GA_3. Developed shoots above 5–6 cm were introduced into root induction medium, half-strength MS medium containing various concentrations of auxins (IAA, IBA and NAA) and activated charcoal. Plantlets with developed roots were transferred to sterile paper cups (6 × 8 cm) consists of sterile red soil: sand: compost (120 g/cup) (1:1:1 v/v) and then transferred to pots containing red soil, sand and manure (2:1:1, v/v).

2.6. Isolation of genomic DNA

Seven acclimatised plants (60 days old), derived from *in vitro* cultures, were randomly selected for genetic homogeneity studies. Young leaves (0.1 g) grounded with liquid nitrogen in a pestle and mortar, then further processed to acquire genomic DNA using HiPurA plant genomic DNA miniprep purification kit (MB507-Himedia). Quantity and quality of isolated DNA's were recorded with Biophotometer (Eppendrof BioPhotometer Plus, Germany). The final quantity of isolated DNA was adjusted to 50 ng/μl.

2.7. SCoT analysis

Seventeen SCoT primers (Collard and Mackill, 2009) were initially scrutinised for reproducible multiple band formation, and those responded positively were trailed to analyse genetic homogeneity between mother plants and acclimatised plants. Final volume of 20 μl reaction mixture consists of 4 μl template DNA, 3 μl of SCoT primer (GeNei™, Bangalore, India), 3 μl of sterile distilled water and 10 μl of 2× PCR master mix [Tris–HCl pH 8.5, $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 U/μl Taq DNA polymerase, inert red dye and stabiliser] (Ampliqon, Denmark), used for amplification in thermal cycler (Eppendrof AG, Germany). The PCR programmed with initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C (60 s), annealing for 60 s at Ta, extension at 72 °C (120 s) and final extension for 5 min at 72 °C. The products of PCR were resolved at 50 V for 3 h in 1.2% Agarose gel with 1× TAE buffer and stained with ethidium bromide. Gels were photographed using gel documentation system (Alpha Imager EP).

2.8. ISSR analysis

Ten ISSR primers [Department of Biotecnology laboratory, University of British Colombia (UBC Set 09] were trailed initially to perform ISSR analysis. Final volume of 20 μl reaction mixture consists of 4 μl template DNA, 3 μl of SCoT primer (GeNei™, Bangalore, India), 3 μl of sterile distilled water and 10 μl of 2× PCR master mix (Ampliqon, Denmark) were used for amplification in thermal cycler (Eppendrof AG, Germany). The PCR programmed with initial denaturation at 94 °C for 5 min followed by 45 cycles of denaturation at 94 °C (45 s), annealing for 45 s at Ta, extension at 72 °C (90 s) and final extension for 7 min at 72 °C. The products of PCR were resolved at 50 V for 3 h in 1.2% agarose gel with 1× TAE buffer and stained with ethidium bromide. Gels were photographed using gel documentation system (Alpha Imager EP).