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MINI-REVIEW

Indian sarsaparilla, *Hemidesmus indicus* (L.) R. Br. ex Schult: tissue culture studies

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Abstract

Hemidesmus indicus (L.) R. Br. ex Schult is commonly known as anantmul or Indian sarsaparilla. The roots of this plant, which display a wide range of medicinal, biological, and phytopharmaceutical properties, are used in the pharmaceutical and food industries. Conventionally, the plant is propagated by seed germination or vegetatively, but the efficacy of traditional methods has some limitations: plants derived from seed germination are prone to seed-borne diseases, or plantlet production using vegetative propagation is limited. In contrast, plant tissue culture allows for large-scale propagation and secondary metabolite production in vitro without sacrificing plants from their natural habitats. Many efforts have been made over 40 years of research to establish efficient micropropagation protocols to speed up cultivation of this plant, including callus-mediated in vitro propagation, somatic embryogenesis, and shoot multiplication using cotyledenory nodes, stem segments, shoot tips, and nodal explants. Among these explants, nodal explants are the most commonly used for *H. indicus* micropropagation. The application of adenine sulfate, citric acid, ascorbic acid, and arginine may be useful in preventing explant browning, premature leaf senescence, and shoot tip abscission during in vitro culture. This review provides insight into micropropagation, use of synthetic seeds for short-term germplasm preservation, and in vitro production of secondary metabolites such as 2-hydroxy-4-methoxybenzaldehyde, lupeol, vanillin, and rutin, from in vitro root and callus cultures. Furthermore, unexplored and possible innovative areas of research in *Hemidesmus* biotechnology are also discussed.

Key Points

- *Hemidesmus indicus has multiple therapeutic applications.*
- *H. indicus roots are used in confectionary and pharmacy.*
- *This review comprehensively assesses H. indicus tissue culture.*
- *Challenges and future research of H. indicus biotechnology are discussed.*

Keywords 2-hydroxy-4-methoxybenzaldehyde (MBALD) · Acclimatization · Explants · Micropropagation · Synthetic seeds

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Introduction

Hemidesmus indicus (L.) R. Br. ex Schult (syn. *Periploca indica* L.), a monophyletic plant (The Plant List 2020), is represented by 264 vernacular names in eight languages (FRLGHT 2020), but is most commonly known in English as Indian sarsaparilla. It is taxonomically distinct from "true" sarsaparilla *Smilax febrifuga*, in the Smilacaceae (Nandy et al. 2020). To date, *H. indicus* has been assigned to different families, including the Periplocaceae (Benerjee and Ganguly 2014), Asclepiadaceae (Efloras 2020), and Apocynaceae (The Plant List 2020), but has now been placed in the Apocynaceae following a phylogenetic reclassification (The Plant List 2020; Nandy et al. 2020). The growth form of the plant is a twining shrub with a woody rootstock, as a vine, and has opposite leaves and subsessile flowers in lateral cymes (Benerjee and Ganguly 2014; Efloras 2020). The plant has five partite calyces that have a glandular base, a greenish-purple corolla with five fleshy lobes that form below the sinus, free filaments, and granular pollen (Benerjee and Ganguly 2014; Efloras 2020).

The ethnobotanical, phytochemical, and pharmacological value of *H. indicus* has been extensively discussed by Nandy et al. (2020). This plant shows anticancer activity against colorectal cancer (Turrini et al. 2018), breast cancer (Suryavanshi et al. 2019), and leukemia (Turrini et al. 2019). An aromatic aldehyde (phenolic), 2-hydroxy-4-methoxybenzaldehyde (MBALD), accumulates in *H. indicus* roots, which are used as an ingredient in sherbet or flavored sweet drinks and bakery products (Patnaik and Debata 1996; Chakraborty et al. 2008; Fiori et al. 2014). MBALD, which is also the main constituent (97.9%) of the essential oil of *H. indicus* (Sreelekha et al. 2007), is produced by the shikimate pathway (Kundu et al. 2012). Due to the multipurpose nature of *H. indicus*, the National Medicinal Plant Board (NMPB) of India identified it as a "highly traded (500-1000 MT/year) medicinal plant" (NMPB 2020).

Indian sarsaparilla can be propagated by vegetative cuttings or seed germination, but the success of these methods is low, 60% and 46%, respectively, and seedlings produced using seed germination are prone to damping-off disease (Raghuramulu et al. 2005). Moreover, plants produced via vegetative propagation are prone to the transfer of diseases from stock plants to seedlings and are unsuitable for large-scale propagation. Hence, alternative methods to propagate plants would be useful for large-scale production not only of infection-free material and thus standard (cloned) biomass but also of source material for the production of secondary metabolites and essential oil. Since active ingredients are present in roots, field harvesting is essential. However, field harvesting is very difficult and laborious, so in vitro root culture offers an opportunity for the production of standardized quality root material for the pharmaceutical industry. In this review, we focus on the micropropagation, use of synthetic seeds, and in vitro production of secondary metabolites of Indian sarsaparilla.

Tissue culture and in vitro propagation

In vitro plant cell, tissue, and organ cultures are not only used for large-scale production of quality planting material, allowing for continuous supply to meet demand (Mukherjee et al. 2019; Wen et al. 2019; Teixeira da Silva et al. 2019; Mitra et al. 2020), but also useful for cryopreservation (Kulus and Zalewska 2014; Teixeira da Silva and Kulus 2014; Bi et al. 2017; Kulus 2019), secondary metabolite production (Isah et al. 2018; Teixeira da Silva et al. 2019), and genetic improvement (Chang et al. 2018; Sood et al. 2019). In vitro cultures derived from plant cells or tissues are a vital strategy for the large-scale production of pharmaceutically important plant secondary metabolites, which can reduce overexploitation of natural populations (Isah et al. 2018; Mukherjee et al. 2019; Mitra et al. 2020).

A complete tissue culture protocol for Indian sarsaparilla is provided in Fig. 1, while details of the choice of stock plant, explant collection, surface disinfection, and aseptic culture, as well as shoot induction and multiplication, rooting, and acclimatization, have been detailed in Tables 1 and 2.

Explant selection for in vitro culture establishment

The physiological condition of the stock plant, the choice of explant, its size, position, or orientation may affect the outcome of in vitro propagation. Actively growing shoots from field-grown plants, which are likely to be the most responsive explant for in vitro conditions, were extensively used in several protocols for the tissue culture of Indian sarsaparilla (Table 2). However, only few reports are available on explants from seedlings raised in vitro (Raghuramulu et al. 2003; Saryam et al. 2012a, b; Purohit et al. 2014).

For Indian sarsaparilla, the easiest method is to use an explant with a predetermined meristem such as a shoot tip or node (Table 1). Sreekumar (1997) conducted a comparative study to select the optimal explant among roots, shoot tips, nodes, internodes, and leaves from 1-year-old field-grown plants and tested shoot regeneration on full-, half-, or quarter-strength Murashige and Skoog (1962) (MS) medium, B5 medium (Gamborg et al. 1968), Schenk and Hildebrandt (1972) (SH), or woody plant medium (WPM) (Lloyd and

Fig. 1 Micropropagation of *Hemidesmus indicus* (Indian sarsaparilla) from nodal explants and subsequent acclimatization of plantlets. **a** Culture initiation from nodal explant on Murashige and Skoog (MS) medium with 8.88 μM BA, 8.81 μM AA, 135.5 μM AdS, 4.78 μM CA, and 4.36 μM Arg for 30 days. **b**, **c** Shoot multiplication during subculture on MS medium with 4.44 μM BA, 2.33 μM KIN, 0.57 μM IAA, 50 mg/l AA, 135.5 μM AdS, 4.78 μM CA, and 4.36 μM Arg for 30 days. **d** In vitro shoots rooted on ¼-strength MS medium with 14.66 μM IBA for 30 days. **e** Rooted plants in nursery poly-bags containing Soilrite®, manure, garden soil, and vermicompost (1:1:1:1, v/v) and maintained in a greenhouse for 6–7 weeks. **f** Hardened plantlets were transferred to pots containing soil for 3–4 weeks. **g** Acclimatized plantlets were transferred to the field (96% survival). Culture conditions were based on Shekhawat and Manokari (2016). Unpublished photographs. AA, ascorbic acid; AdS, adenine sulfate; Arg, arginine; BA, 6-benzyladenine; CA, citric acid; IAA, indole-3-acetic acid; KIN, kinetin

McCown 1980) supplemented with combinations of 2.22 μM 6-benzyladenine (BA) and 1.07 μM 1-naphthaleneacetic acid (NAA). Cultures were kept in the dark for 4 days, and then exposed to a 12-h photoperiod (50–60 μmol m^{-2} s^{-1}). Sreekumar (1997) found that nodes were more effective for axillary shoot multiplication (92% of explants receptive, 9.37 shoots per node, approximately 2.8 cm long shoots) than other explant types when cultured on full-strength MS medium. In contrast, Sreekumar et al. (2000) reported best results for shoot multiplication (95% of explants receptive, 9.3 shoots per explant, 7.2 cm long shoot) on ½MS medium supplemented with 2.22 μM BA and 1.07 μM NAA. Misra et al. (2005), on the other hand, reported that nodes formed callus more effectively than leaves or roots from field-grown plants.

Even though nodes and shoot tips were the most frequently used explants for Indian sarsaparilla tissue culture (Table 1), stem segments (either nodes or internodes) (Heble and Chadha 1978; Sarasan and Nair 1991; Sarsan et al. 1994; Sreekumar 1997; Sreekumar et al. 2000), leaves (Sarasan and Nair 1991; Sarsan et al. 1994; Sreekumar 1997; Sreekumar et al. 2000; Misra et al. 2005; Ghatge and Dixit 2006; Ghatge 2007; Shanmugapriya and Sivakumar 2011; Pathak and Joshi 2017), roots from mature stock plants (Heble and Chadha 1978; Sreekumar 1997; Sreekumar et al. 2000; Misra et al. 2005), cotyledonary nodes from in vitro germinated seeds (Purohit et al. 2014), or root segments from seedlings raised in vitro (Raghuramulu et al. 2003) have also been employed. Sreekumar et al. (2000) noted that nodes collected from actively growing shoots on the second and third node (average of 9 shoots per node), counting from the shoot tip, were more responsive to axillary shoot multiplication than from nodes 4 to 8 on MS medium with 2.22 μM BA and 1.07 μM NAA. In contrast, Nagahatenna and Peiris (2007) found that the first and second nodes could not induce axillary shoots, the third node only produced callus at the base of the explant, while the fourth and fifth nodes induced the most axillary shoots (average of 2.5 shoots per node) on MS medium with 8.88 μM BA and 0.5 μM NAA.

Table 1 Explant type size, culture vessel, and explant disinfection used to establish *Hemidesmus indicus in vitro* cultures (chronological listing of papers for which information and full texts were available)

Explant type and size	Disinfection procedure	Culture vessel, medium, and explant density	Reference
Shoot tips, stems, roots (2 mm)	NR	NR	Heble and Chadha (1978)
Shoot tips, stem segments, leaf discs (size NR)	RTW (3 h) → 0.1% $HgCl_2$ (5 min) → post-$HgCl_2$ treatment NR	NR	Sarasan and Nair (1991)
Actively growing shoots (12–15 cm) → defoliated; nodes (0.5 cm long with one axillary dormant bud)	90% EtOH (15 s) → 0.1% $HgCl_2$ (5–6 min) → 3–4× SDW	150 mm × 25 mm (length × diameter), 20 ml, 1 explant/tube, plugged with non-absorbent cotton	Patnaik and Debata (1996)
Shoots (5–6 leaves) → defoliated → twig (0.5–1.5 cm, with or without nodes)	RTW (10 min) → 1% Labolene (6–8 min) → RTW (time NR) → 1–5 cm cuttings → 5% NaOCl (5–10 min) → 70% EtOH (30–40 s) → 0.1% $HgCl_2$ (5–10 min) → *n*× SDW	150 mm × 25 mm, 20 ml, 1 explant/tube, plugged with non-absorbent cotton → 100 or 250 ml Erlenmeyer flasks. For *Agrobacterium* infection studies: 250 ml culture bottles with polycarbonate cap	Sreekumar (1997)
Sreekumar (1997)	RTW (10 min) → 1% Labolene (6–8 min) → RTW (time NR) → 1–5 cm cuttings → 5% NaOCl (5–10 min) → 70% EtOH (30–40 s) → 0.1% $HgCl_2$ (5–10 min) → *n*× SDW	NR in the manuscript but details available in Sreekumar (1997) thesis	Sreekumar (1997); Sreekumar et al. (1998)
Terminal shoot cuttings with 5–6 leaves → shoot tips, nodes, internodes (~ 0.5 cm long), leaves (0.5 cm^2), roots (~ 1.0 cm)	RTW (10–15 min) → 1% Labolene (4–5 min) → 2× DW. Defoliated stem cuttings (2–5 cm) → 0.1% $HgCl_2$ (6–8 min) → 5–6× SDW. Roots 4–5 cm, 2–3 m-old plants → *n*× RTW → 5% (v/v) Steriliq (5 min) → 0.1% $HgCl_2$ (5–7 min) → 4–5× SDW	Not mentioned in Sreekumar et al. (2000)	Sreekumar (1997); Sreekumar et al. (2000)
Mature fruits → seeds → disinfection → ½MS + 2% sucrose + 2.22 μM BA + 1 μM NAA or PGR-free ½MS (SGM) → root segments (1–1.5 cm) from 60-day-old plants	15% H_2O_2 3 min → 4–5× SDW	150 mm × 25 mm, 15 ml, 1 explant/tube, plugged with cotton	Raghuramulu et al. (2003)
Nodal segments (1 cm)	RTW 30 min → 5% liquid detergent (5 min) → RTW → 70% EtOH (5 s) → 0.1% $HgCl_2$ (5 min) → 5× SDW	NR	Misra et al. (2003)[1]
Defoliated actively growing shoots → nodes (5 mm)	2 drops/100 ml DW Tween-20 (10–12 min) → *n*× SDW → 0.1% $HgCl_2$ (10 min) → 3–4× SDW	NR	Saha et al. (2003)
Shoots → defoliated → nodes (1–2 cm)	RTW 10 min → few drops of Tween-80 → 1% Savlon (10 min) → 0.1% $HgCl_2$ 7 min → *n*× SDW	Culture tubes (150 mm × 25 mm, 15 ml), 1 explant/tube	Siddique et al. (2003); Siddique and Bari (2010, 2006)[2]
Leaves, roots, nodes (size NR)	RTW → 5% mild detergent (5 min) → 2× DW → 70% EtOH 5 s → 0.1% $HgCl_2$ (3 min, nodes; 5 min, roots; 2 min, leaves) → 5× SDW	NR	Misra et al. (2005); Misra and Mehrotra (2006)
Leaves, nodes, internodes (size NR)	RTW (30 min) → Laboline (time NR) → *n*× DW → 0.2% Bavistine (2 h) → *n*× SDW → 0.1% $HgCl_2$ (5 min, leaves; 6 min,	Culture tubes (150 mm × 25 mm, 20 ml), 1 explant/tube; conical flask plugged with non-absorbent cotton wrapped with cheese	Ghatge and Dixit (2006); Ghatge (2007); Ghatge et al. (2008)

Table 1 (continued)

Explant type and size	Disinfection procedure	Culture vessel, medium, and explant density	Reference
	stems) → 4× SDDW → 70% EtOH (30 s) → 3× SDDW	cloth	
Nodes (1–2 cm)	0.1% Bavistin™ (2 d) → nodes → 3× Teepol™ → 0.1% Bavistin (30 min) → vacuumed 10% Clorox + 2 drops Tween-20 (5 min) → shake in Clorox (5 min) → 4× SDW	Culture tubes (details NR)	Nagahatenna and Peiris (2008, 2007)
Nodes (1–2 cm)	RTW (30 min) → twigs with 2–3 nodes → 0.1% $HgCl_2$ + 2 drops/100 ml Tween-20 (2–5 min) → 0.1% $HgCl_2$ (2–4 min) → *n*× SDW	Borosilicate culture tubes (150 mm × 25 mm, 15 ml), 1 explant/tube, plugged with non-absorbent cotton wrapped with cheese cloth for multiplication Borosilicate glass bottles (dimension: NR), 30 ml	George (2009)
Nodes, first two expanded leaves (0.5–1 cm) from top (leaf disc and nodal cutting size NR)	RTW (time NR) → DDW + few drops of surfactant (10–20 min) → 0.5% NaOCl (3–5 min) → *n*× SDW	NR	Shanmugapriya and Sivakumar (2011)
Seeds → explant type and size NR disinfected → ½MS (SGM)	RTW (30 min) → Bavistin conc. NR (2 h) → detergent (10 min) → RTW → 70% EtOH (30 s) → 0.1% $HgCl_2$ (6–10 min) → 4–6× SDW	NR	Saryam et al. (2012a, 2012b)[2]
Nodes (2 cm)	Teepol + NaOCl conc. NR (5 min) → 1% Bavistin (5 min) → 3× DW → 20% bleach [5.25% NaOCl] (3 min) → 3× DW	Culture tubes (details NR)	Cheruvathur et al. (2013)
Shoot tips (size NR)	10% Teepol → RTW → 70% EtOH (45 min) → SDW → 0.1% $HgCl_2$ (2 min) → 2× SDW	Culture tubes (150 mm × 20 mm, 15 ml)	Sudarmani and Hasina (2013)
Young shoots with 3–4 nodes → 1–1.5 cm nodes	*n*× RTW → shoot tip or 1st, 2nd, and 3rd nodes → RTW → 5% Teepol (time NR) → RTW (5 min) → *n*× DW → 0.1% $HgCl_2$ (2 min) → 2–3× SDW	Culture tubes (150 mm × 20 mm, 15 ml); Erlenmeyer flasks (250 ml capacity; 50 ml); culture bottles (dimensions NR): 40 ml. Non-absorbent cotton plugs wrapped in surgical cloth for flasks and culture tubes	Gopi (2014); Sindura (2014)
Nodes (2–3 cm)	RTW + Tween-20 (30 min) → antifungal powder conc. NR (30 min) → 3X SDW → $HgCl_2$ conc. NR (5–6 min) → 70% EtOH (15–20 s) → 5× SDW	Petri dishes and test tubes (details NR)	Khan (2014)
Seeds → disinfection → ½MS (SGM) → cotyledenory nodes from germinated seeds	0.1% $HgCl_2$ (5 min) → 4–6× SDW	Culture bottles (details NR)	Purohit et al. (2014)
Nodes from one year old plant from glasshouse (size NR)	RTW (20 min) → Tween-20 (time NR) → 4–5× DW → 0.1% $HgCl_2$ (5–8 min) → *n*× SDW	Culture tube (20 ml)	Devi et al. (2014)
Node (size NR)	RTW → Tween-20 (5–10 min) → 0.5% $HgCl_2$ (5 min) → *n*× SDW	Culture tubes and flasks (details NR)	Singh and Shalini (2015)
Node (1–2 cm)	RTW → 0.1% Bavastin (2 min) → 0.05% $HgCl_2$ (2 min) → 70% EtOH (40s) → 4–6× SDW	Culture tubes (125 mm × 25 mm)	Reddy et al. (2016)

Table 1 (continued)

Explant type and size	Disinfection procedure	Culture vessel, medium, and explant density	Reference
Stem segments with 1–2 nodes (3–5 cm)	0.1 % Bavistin (5–7 min) → *n*× SDW → 0.1% $HgCl_2$ (4–5 min) → 7–8× SDW	Culture tubes/culture bottles and flasks (details NR)	Shekhawat and Manokari 2016
Shoot tips (0.5–1 cm)	*n*× RTW → Teepol (5 min) → *n*× DW → 0.1% $HgCl_2$ (2–3 min) → *n*× SDW	Culture tubes (details NR)	Verma and Vashistha (2016)
Leaves → lamina with midrib (1 cm^2)	RTW (1 h) → Laboline (time NR) → 0.01% Bavistin (3 min) → 0.1% $HgCl_2$ (3 min) → *n*× NR	Test tubes and flasks (details NR)	Pathak and Joshi (2017)
Nodes (2 cm)	RTW + Laboline (1 h) → 0.01% Bavistin (4 min) → 2× SDW → 0.1% $HgCl_2$ (4 min) → 2× SDW	Test tubes (details NR)	Pathak et al. (2017)
Leaves, nodes, and internodes (size NR)	RTW (10–15 min) → 2 drops Tween 20 in 100 ml DW (1 min) → DW → 0.1% $HgCl_2$ (6–8 min) → DW → 5% $NaOHCl_2$ (15–20 min) → 4–5× DW	Borosil glass tubes (25 × 150 mm) 20 ml	Prashanti et al. (2017)
Stem segments, leaf base and cotyledons (size NR)	Teepol (10 min) → 0.1% $HgCl_2$ (5 min) → 3–4× SDW	Test tubes (details NR), 18 ml	Patidar (2017)
Tender twig with 2–3 nodes → nodes (1–1.5 cm)	Tween 20 (10 min) → RTW (30 min) → several × DW → 0.1% $HgCl_2$ (5 min) → no further washes with SDW	NR	Maity (2018a)
Shoot buds (size NR)	3–4× SDW → 0.1% $HgCl_2$ (7 min) → 4–5× SDW	NR	Maity (2018b)
Nodes (1–1.5 cm)	1% (w/v) Bavistin (15 min) → 5% Teepol (15 min) → *n*× SDW → 0.1% $HgCl_2$ (3 min) → 5–6× SDW → For the encapsulation the nodal explants (0.4–0.6 cm) were trimmed from both the end sides having axillary buds	100 ml capacity wide mouth flask (45–50 cm^3 medium)	Yadav et al. (2019)

BA, N^6-benzyladenine (BA is used throughout even though BAP (6-benzylaminopurine) may have been used in the original (Teixeira da Silva 2012a); *Bavistin*™, carbendazim; *d*, day(s); *DW*, distilled water; *EtOH*, ethanol; *$HgCl_2$*, mercuric chloride; *MS*, Murashige and Skoog (1962) medium; *NAA*, α-naphthaleneacetic acid; *NaOCl*, sodium hypochloride; *NR*, not reported in the study; *n*×, multiple times; *PGR*, plant growth regulator; *RTW*, running tap water; *s*, second(s); *SDW*, sterilized distilled water; *SGM*, seed germination medium

[1] Full text not available

[2] Duplicate publication(s)

Table 2 Micropropagation and tissue culture of *Hemidesmus indicus*

Morphogenetic responses	Culture medium, PGRs, and additives1	Culture conditions2 (temperature, light source and intensity, photoperiod)	Remarks, experimental outcome and maximum productivity, acclimatization, and variation	Reference3
Callus culture and secondary metabolites	Lin and Staba + 9.04 μM 2,4-D + 0.93 μM KIN (CIM). Lin and Staba + 11.43 μM IAA (SIM). Carbon source: NR. Solidification agent: NR.	25 ± 2 °C. FT. Light intensity NR. Continuous light.	Stem callus formed shoot buds in 4 w. Shoot buds grew into plantlets with well-developed root system. Roots and shoot tips formed callus. All cultures formed phytosterols but 16-hydropregnenolone detected only in stem-derived callus. Acclimatization NR.	Heble and Chadha (1978)
Root culture for secondary metabolites	Nodes → MS + 9.78 μM IBA (RIM). Roots → B5 + 9.78 μM IBA + 4% sucrose. pH 5.6 (liquid culture).	Darkness. 70 rpm (continuous shaking).	550 mg/flask root dry weight with 0.18% MBALD every 5th d. Consistent biomass production over 21 m of culture.	Sreekumar (1997); Sreekumar et al. (1998)
Shoot regeneration from callus	MS + 9.3 μM KIN + 5.37 μM NAA (CIM). MS + 11.63 μM KIN + 5.37 μM NAA (SIM). MS + 19.54 μM IBA + 4.65 μM KIN (RIM). pH 5.8. Carbon source: NR. 0.8% agar.	25 ± 2 °C. CWFT. 2000 lux. 14-h PP.	Callus induction 85%, light green friable callus in 4 w. Shoot regeneration 86–90%, 4.3 shoots/callus, 3 cm long shoots in 8 w. Root regeneration 80%, 2.2 roots/shoot in 35 d. Rooted plants → pots with sand + soil (1:1) covered with beakers → details of survival of micropropagated plants NR.	Siddique et al. (2003); Siddique and Bari (2010, 2006)
Shoot regeneration from callus	MS + 5.3 or 10.7 μM NAA + 0.9 or 2.3 or 4.6 μM KIN (CIM; leaves). MS + 45.3 μM 2,4-D + BA 8.8, 22.2, 44.4 μM (CIM; roots). MS + 2.2, 4.4, 8.8 μM BA + 1.0 or 2.6 μM NAA (SIM). pH 5.8. 3% sucrose (CIM), 4% sucrose (SIM). 0.8% agar.	26 ± 1 °C. CWFT. 70 μmol m^{-2} s^{-1}. 16-h PP.	Regeneration from callus NR. Shoot cultures, callus from roots and leaf explants produced lupeol, vanillin, and rutin (validated by HPTLC).	Misra et al. (2005)
Shoot regeneration from callus	MS + 4.52 μM 2,4-D (CIM). MS + 8.88 μM BA + 28.59 μM IAA (SIM). ½MS + 9.78 μM IBA (RIM). NN basal (SEIM/SEGM). pH 5.8. 3% sucrose. 0.8% agar.	24 ± 2 °C. CWFT. 2000 lux. 16-h PP.	70% aseptic cultures. Shoot regeneration 95%, 8.9 shoots/node. Root regeneration 91%, 5.1 roots/shoot. 85% SE, 14 SEs/100 mg callus. Rooted plants → plastic cups with autoclaved soil + sand + compost (various ratios) → shade 2 w in culture room → field. 92% survival. During acclimatization, single spray of 0.2% bavistin.	Ghatge and Dixit (2006); Ghatge (2007); Ghatge et al. (2008)
Shoot regeneration from callus	MS + 20 μM BA + 1 μM IAA (CIM/SIM). ¼ liquid MS + 20 μM IBA (RIM). pH 5.8. 3% sucrose (CIM/SIM); 1% (RIM). 0.8% agar (CIM/SIM).	26 ± 2 °C. Light source and intensity NR. 16-h PP.	Callus → shoot regeneration > 90%, 15–20 shoots/node. Root regeneration 100%, 8.8 roots/shoot. Rooted plants → cups with cocopit + sand (1:1) → covered with polythene, irrigated with sterile distilled water at 2-d intervals, 26 ± 2 °C, 16-h PP (time NR). 100% survival.	Pathak and Joshi (2017)
Shoot regeneration from callus	MS + 2.22 μM BA + 1.07 μM NAA (CIM). MS + 0.88 μM (SIM/RIM). pH 5.8 ± 0.1. 3% sucrose. 0.5% agar.	22–24 °C. Light source and light intensity NR. 16-h PP.	Yellowish nodular callus → 11.4 shoots and 8.2 roots (source from callus or from individual shoot not specified). Rooted plants → several rinses in sterilized distilled water → soil (22–24 °C, in shade for 2 w). 90% survival.	Maity (2018a)
Shoot regeneration from callus	MS + 4.28 μM NAA + 11.63 μM KIN (CIM). MS 5.37 μM NAA + 2.79 μM KIN (SIM). ½MS + 5.37 μM NAA pH 5.8. 3% sucrose for CIM and SIM, 4% for RIM. 0.8% agar.	25 ± 2 °C. Fluorescent light 1200 lux. 16-h PP.	14% callus formation from stem segment. 10.3 shoots/callus cluster. 8–14 roots (average = 12.9 cm long). Acclimatization NR.	Patidar (2017)

Table 2 (continued)

Morphogenetic responses	Culture medium, PGRs, and additives1	Culture conditions2 (temperature, light source and intensity, photoperiod)	Remarks, experimental outcome and maximum productivity, acclimatization, and variation	Reference3
Somatic embryo regeneration from callus and synthetic seed	MS + 14.66 μM IBA (CIM). ½MS + 9.78 μM IBA (SEM). ½MS + 9.78 μM IBA (SEGM). MS + 17.76 μM BA + 4.34 μM GA_3 (SEMM). Synseeds of torpedo stage SE complexed in MS + 4% sodium alginate in 100 μM $CaCl_2$ + 3% sucrose (pH 5.8) → orbital shaker at 60–80 rpm (20 min) → 2× SDW → stored at 4 °C for 120 d. MS + 9.3 μM KIN + 2.46 μM IBA (synseed germination medium). All media: pH 5.8. 3% sucrose (7.5% in SEGM). 0.8% agar.	25 ± 2 °C. CWFT. 60 μmol m^{-2} s^{-1}. 16-h PP.	Callus 83%. SE induction 92% cultures with 32 SEs/callus. > 90% SE germination with > 40 SEs/callus. Out of 35 torpedo stage SEs, 33 plants converted into plants on SEMM. Plantlet conversion from synseeds: 100%, 2.4 cm long plants in 30 d. Rooted plants → plastic cups with garden soil + sand (1:1) → covered with polythene 4 w (25 ± 2 °C, 16-h PP) → greenhouse 2 m → field. 94% survival.	Cheruvathur et al. (2013)
Shoot regeneration from root segments	MS + 13.32 μM BA + 2.69 μM NAA (SIM). RIM: PGRs NR. pH 5.8. Carbon source NR. 0.8% agar.	25 ± 2 °C. CWFT. 2000 lux. PP NR.	Shoot regeneration 65%, 5 shoots/root segment, shoot length NR. Rooted plants → pots with vermiculite + sand (3:1) → mist chamber (time NR) → field. 85% survival.	Raghuramulu et al. (2003)
Shoot regeneration from root segments	MS + KIN (conc. NR) (SIM). ½MS + 9.78 μM (RIM). pH 5.8. 3% sucrose. 0.8% agar.	NR	Shoot/root regeneration: data difficult to interpret. Acclimatization details and survival NR.	Khan (2014)
Shoot multiplication from shoot tips	Shoot tips → MS + 2% or 3% sucrose + 2.69 μM NAA + 2.22 μM BA + 1.45 μM GA_3 (SIM). MS + 34.85 μM NAA + 2.22 μM BA + 1.45 GA_3 (SMM). ½MS + 0.97 μM IBA (RIM). Stems → MS + 4.52 μM 2,4-D + 2.22 μM BA (CIM/SEIM).	25 ± 2 °C. FT. 30–45 μmol m^{-2} s^{-1}. 12-h PP.	Multiple shoots from 83% nodal explants. Experimental protocol and results unclearly specified / quantified. Acclimatization NR.	Sarasan and Nair (1991)
Shoot multiplication from shoot tips	MS + 0.72 μM BA + 0.22 μM KIN (SIM). ½MS + 0.51 μM IBA + 0.22 μM KIN (RIM). pH 5.8. Gelling agent NR. Carbon source NR.	25 ± 2 °C. 16-h PP.	Shoot regeneration 87.1%, 3.2 shoots/shoot tip. Root regeneration 95.7%, 4.5 roots/shoot. 77% survival.	Sudarmani and Hasina (2013)
Shoot multiplication from shoot tips	MS + 8.88 μM BA (SIM). ½MS + 0.49 μM IBA (RIM). pH 5.8. 3% sucrose. 0.6% agar.	25 ± 2 °C. CWFT. 30–35 μmol m^{-2} s^{-1}. 12-h PP. Root suspension: gyratory shakers at 80 rpm.	93% aseptic cultures. Shoot regeneration: shoot tips, 1st, 2nd, 3rd nodes: 90%, 70%, 65%, 62%. 0.65–0.83 cm long shoots in 4 w. Root regeneration: 3 roots/shoot. Rooted plants → plastic cups with soil + sand (1:2) → mist chamber for 3 w irrigated daily → field. 80% survival.	Gopi (2014); Sindura (2014)
Shoot multiplication from nodes and shoot tips	Exp 1: MS + 2.22 μM BA (SIM). Exp 2: ½MS + 2.22 μM BA +1.07 μM NAA (SIM). ¼MS + 9.8 μM IBA (RIM). pH 5.8. 3% sucrose. 0.8% agar.	25 ± 2 °C. CWFT. 50–60 μmol m^{-2} s^{-1}. 12-h PP.	Exp 1: shoot regeneration: 84%, 6.6 shoots/node, 6.7 cm long shoots in 5 w. Exp 2: shoot regeneration: [shoot tips: 70%, 6.6 shoots/shoot tip, 7.3 cm long shoots]; [nodes 95%, 9.3 shoots/node, 7.2 cm long shoots]; [internodes 62%, 4.1 shoots/internode, 6 cm long shoots]; [leaves 70%, 2.7 shoots/leaf, 4 cm long shoots]; [roots 20%, 1.7 shoots/root, 1.6 cm long shoots] in 5 w. Root regeneration 98%, 12 roots/shoot, 3.4 cm long roots in 3 w. Rooted plants with 10–12 roots, 0.5–1.5 cm long roots → 3 cm diameter pots with river sand + farmyard manure (2:1) →	Sreekumar (1997); Sreekumar et al. (2000)

Table 2 (continued)

Morphogenetic responses	Culture medium, PGRs, and additives1	Culture conditions2 (temperature, light source and intensity, photoperiod)	Remarks, experimental outcome and maximum productivity, acclimatization, and variation	Reference3
			mist chamber, 60–80% RH, 50% sunlight for 3 w → field (15 cm^3 pits, 90 cm spacing, in river sand + farmyard manure + top soil (1:2:1). 97% survival. 0.12%/g dry weight MBALD in 1-y-old micropropagated plants.	
Shoot multiplication from nodes	MS + 1.15 μM KIN + 0.054 μM NAA (SIM). MS + 1.15 μM KIN + 0.054 μM NAA (SMM). MS + 7.35 μM IBA (RIM). pH 5.8. 2% sucrose. 0.8% agar.	25 ± 2 °C. CWFT. 3000 lux. 14-h PP.	Shoot regeneration 95%, 8 shoots/node, 7.3 cm long shoots in 5 w. Root regeneration 86%, 4.3 roots/shoot, 4.4 cm long roots in 4 w. Rooted plants → earthen pots with garden soil + sand + compost (2:1:1) → shade 2 w → field. 70% survival.	Patnaik and Debata (1996)
Shoot multiplication from nodes	MS + 4.44 μM BA + 2.69 μM NAA + 80.41 μM AdS (SIM). ½MS + 9.78 μM IBA + 5.37 μM NAA + 1.2 μM AC (RIM). pH 5.8. 0.75% sucrose. 0.8% agar.	25 ± 2 °C. CWFT. 70 μmol m^{-2} s^{-1}. 16-h PP.	Shoot regeneration 5.13 shoots/node, 4.27 cm long shoots in 10 d. Root regeneration 5.1 roots/shoot, 4.8 cm long roots in 30 d. Rooted plants → pots with sand + manure (1:1) → mist chamber with 80–90% RH for 30 d → field. 70% survival.	Misra et al. (2003)
Shoot multiplication from nodes	MS + 8.88 μM BA + 0.53 μM NAA + 17.61 μM AA + 14.61 μM glutamine (SIM). ½MS + 7.37 μM IBA + 14.61 μM glutamine (RIM). pH 5.7. 2% sucrose. 0.8% agar.	24 ± 1 °C. CWFT. 48 μmol m^{-2} s^{-1}. 16-h PP.	Shoot regeneration 7.4 shoots/node, 3.8 cm long shoots in 5 w. Root regeneration 3.5 roots/shoot, 3.7 cm long roots in 45 d. Rooted plants → pots with vermiculite and light intensity = 3000 lux → pots with garden soil + sand (1:3) in greenhouse. 85% survival.	Saha et al. (2003)
Shoot multiplication from nodes	MS + 8.88 μM BA (SIM). ½MS + 7.34 μM IBA (RIM). 81.3 μM AdS. 0.1% streptomycin. 0.1 g/l AA. pH 5.8. 3% sucrose. 0.8% agar.	25 ± 2 °C. Warm fluorescent light. 900–1500 lux. 16-h PP.	76% aseptic cultures. 2 shoots/node 2.8–3.2 cm long shoots in 3 w. Root regeneration 38%, 3.7 roots/shoot, 3.7 cm long roots in 12 w. Rooted plants → pots with sterilized soil + sand (1:3). 100% survival.	Nagahatenna and Peiris (2008, 2007)
Shoot multiplication from nodes	MS + 4.65 μM KIN + 0.49 μM IBA + 81.3 μM AdS (SIM). MS + 1.22 μM IBA (RIM). pH 5.8. 3% sucrose. 0.7% agar.	22 ± 2 °C. CWFT. 35–40 μmol m^{-2} s^{-1}. 12-h PP.	Shoot regeneration 80%, 5.8 shoots/node, 6.5 cm long shoots. Root regeneration 100%, 7.6 roots/shoot, 8.2 cm long roots. Rooted plants → cups with soil + sand + cow dung (1:1:1) → humid chambers 3–4 w → nursery 1 m → field. Survival NR.	George (2009)
Shoot multiplication from nodes	MS + 22.2 μM BA (SIM). pH 5.8. 3% sucrose. 0.65% agar.	25 ± 2 °C. CWFT. 2000–3000 lux. PP NR.	Shoot regeneration 75%. Rooted plants → trays with soil + vermiculite (1:1) → irrigated with ¼MS without vitamins and sucrose for 1 w. Rooting details and survival NR.	Shanmugapriya and Sivakumar (2011)
Shoot multiplication from nodes	MS + 4.44 μM BA (SIM). MS + 5.37 μM NAA + 2.40 μM AC (RIM). pH 5.8. 3% sucrose. 0.8% agar.	25 ± 4 °C. CWFT. 2000 lux. 16-h PP.	Shoot regeneration 75–80%, 10–12 shoots/node, 2–3 cm long shoots. Root regeneration 85–90%, 3–5 cm long roots. Rooted plants → pots with vermiculite → covered with polythene 2 w → uncovered plants in diffused light 1 m → field. Survival NR.	Saryam et al. (2012a, 2012b)[4]

Table 2 (continued)

Morphogenetic responses	Culture medium, PGRs, and additives1	Culture conditions2 (temperature, light source and intensity, photoperiod)	Remarks, experimental outcome and maximum productivity, acclimatization, and variation	Reference3
Shoot multiplication from nodes	MS + 9.3 μM KIN + 4.44 μM BA (SIM). MS + 9.78 μM IBA (RIM). pH 5.8. 3% sucrose. 0.8% agar.	22 ± 2 °C. CWFT. 3000 lux. 14–16-h or 8–10-h PP.	Shoot regeneration 80%, 8–10 shoots/node. Root regeneration 80%, 6 roots/shoot. Rooted plants → glasshouse. Survival NR.	Devi et al. (2014)
Shoot multiplication from cotyledonary nodes	½MS (SGM). MS + 8.88 μM BA + 2.69 μM NAA (SIM). MS + 4.44 μM BA + 2.33 μM KIN (SMM). MS + 2.85 μM IAA (RIM). pH 5.8. 3% sucrose. 0.8% agar.	24–25 °C. CWFT. 70 μmol m^{-2} s^{-1}. 16-h PP. Seed germination: darkness 2 d → 16-h PP.	Seed germination 94%. 80% shoot induction (6.8 shoots/cotyledenory node). Rooting in 16–18 d. Acclimatization in sterilized sand + soil (1:3). 95% survival.	Purohit et al. (2014)
Shoot multiplication from nodes	MS + 13.52 μM /l BA (SIM). MS + 11.43 μM IAA (RIM). pH 5.8. 3% sucrose. 0.8% agar.	24 ± 4 °C. 2000 lux. 16-h PP.	Shoot regeneration 90%, 2–4 shoots/node, 1 cm long shoots. Root regeneration 77%, 2–5 roots/shoot, 1–3.5 cm long roots. Acclimatization NR.	Singh and Shalini (2015)
Shoot multiplication from nodes	MS + 4.44 μM BA (SIM). MS + 2.23 μM BA + 2.23 μM KIN (SMM). ½MS + 0.41 μM IBA (RIM). pH 5.8. 3% sucrose. 0.8% agar.	25 ± 2 °C. CWFT. 50 μmol m^{-2} s^{-1}. 16-h PP.	Shoot regeneration 75%, 5.6 shoots/node. Shoot multiplication 10.2 shoots/node. Root regeneration 10 roots/shoot. Rooted plants → sterilized soil + manure (1:1) moistened with tap water and was covered with polythene bag for 1 w to maintain humidity. 80% survival.	Reddy et al. (2016)
Shoot multiplication from nodes	MS + 8.88 μM BA (SIM). MS + 4.44 μM BA + 2.33 μM KIN + 0.57 μM IAA (SMM). ¼MS + 14.66 μM IBA (RIM). pH 5.8. 3% sucrose. 0.8% agar. Additives for SIM + SMM: 8.81 μM AA + 135.5 μM AdS + 4.78 μM CA + 4.36 μM arginine.	25 ± 2 °C. CWFT. 40-50 μmol m^{-2} s^{-1}. 12-h PP.	Shoot regeneration 100%, 9 shoots/node, 5.2 cm long shoots. Shoot multiplication 272 shoots/node, 12.6 cm long shoots. Root regeneration 97%, 62 roots/shoot, 7.6 cm long roots. In vitro rooted plants → glass bottles with autoclaved Soilrite® + ¼MS. 91% survival. Ex vitro rooting 5–6 cm long shoots → 1954 μM IBA (5 min): 96% rooting, 45 roots/shoot, 6.9 cm long roots → cups with 55 g Soilrite + ¼MS → covered with polythene cups → after rooting, covering removed at night and kept during day (10 d) → nursery poly-bags with Soilrite + manure + garden soil + vermicompost (1:1:1:1). Greenhouse 80–90% RH, 25 ± 2 °C. 98% survival.	Shekhawat and Manokari (2016)
Shoot multiplication from nodes	MS + 8.88 μM BA + 5.71 μM IAA (SIM). ½MS + 2.46 μM IBA (RIM). pH 5.8. 3% sucrose. 0.8 % agar.	25 ± 2 °C. CWFT. 3500 lux. 16-h PP.	Shoot regeneration 87.4%, 7.6 shoots/node, 4.8 cm long shoots. Root regeneration 80%, 5 roots/shoot, 4.5 cm long roots. Rooted plants → plastic cups with sterilized soil + sand + vermiculite (2:1:1) → covered with perforated polythene bags → 25 ± 2 °C, CWFT, 3500 lux, 16-h PP (2 w) → irrigated with ¼MS on alternate days for 1 m → pots with garden soil + sand + vermiculite (2:1:1) → glasshouse 4 w → soil. Survival NR.	Verma and Vashistha (2016)

Table 2 (continued)

Morphogenetic responses	Culture medium, PGRs, and additives1	Culture conditions2 (temperature, light source and intensity, photoperiod)	Remarks, experimental outcome and maximum productivity, acclimatization, and variation	Reference3
Shoot multiplication from nodes	MS + 10 μM BA + 5 μM KIN (SIM). RIM: same as Pathak and Joshi (2017). pH 5.8. 3% sucrose. 0.8 % agar.	26 ± 2 °C. CWFT. 40 μmol m^{-2} s^{-1}. 12-h PP.	Shoot regeneration 100%, 11 shoots/node. Root regeneration not quantified. Rooted plants → acclimatization same as Pathak and Joshi (2017). Survival NR.	Pathak et al. (2017)
Shoot multiplication from nodes	MS + 8.88 μM BA + 21.68 μM AdS (SIM). MS + 22.87 μM IAA (RIM). pH 5.8. 2% sucrose. 0.8 % agar.	25 ± 2 °C. Light source and light intensity NR. 16-h PP.	Shoot regeneration 87%, 8.51 shoots/node, 7.78 cm long shoots. Root regeneration 80%, 7.91 roots/shoot, 11.6 cm long roots. Rooted plants → cups with sterilized sand and soil (2:1)→ acclimatization in greenhouse (acclimatization conditions NR). 78% survival.	Prashanti et al. (2017)
Shoot multiplication from nodes	MS + 8.88 μM BA (SIM). PGR-free MS (RIM). pH 5.8 ± 0.1. 3% sucrose. 0.7% agar.	22–24 °C. Fluorescent tube 12000 lux. 16-h PP.	Shoot regeneration 10 shoots/node. Root regeneration 9.3 roots/shoot. Acclimatization NR.	Maity (2018b)
Shoot multiplication from nodes and synthetic seed production	Nodal segment encapsulated in 3% sodium alginate in 100 mM $CaCl_2$ with 3 min. of exposure → MS + 5 μM BA + 0.5 μM IBA (SIM). ½ liquid MS + 1.0 μM IBA (RIM)	25 ± 2 °C. CWFT. 50 μmol m^{-2} s^{-1}. 16-h PP.	Shoot regeneration 84%, 5.53 shoots/synseed. Root regeneration 84.3%, 17 roots/shoot. Rooted plantlets → Soilrite™ (75% Peat moss and 25% horticulture mark expanded perlite) + ¼MS inorganic nutrients → 89.3% survival	Yadav et al. (2019)

AA, ascorbic acid; *AC*, activated charcoal; *AdS*, adenine sulfate; B_5 medium, or Gamborg medium (Gamborg et al. 1968); *BA*, N^6 -benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original (Teixeira da Silva 2012a); *CA*, citric acid; *CI*, culture initiation; *CIM*, callus induction medium; *CWFT*, cold white fluorescent tubes; *d*, day(s); *FT*, fluorescent tubes; GA_3, gibberellic acid; *IAA*, indole-3-acetic acid; *IBA*, indole-3-butyric acid; *KIN*, kinetin (6-furfuryl aminopurine); Lin and Staba medium (Lin and Staba 1961); *m*, month(s); *MBALD*, 2-hydroxy-4-methoxybenzaldehyde; *MS medium*, Murashige and Skoog (1962); *NAA*, α-naphthaleneacetic acid; *NN medium*, Nitsch and Nitsch (1969); *NR*, not reported in the study; *PP*, photoperiod; *RH*, relative humidity; *RIM*, root induction medium; rpm, revolutions per minute; *SE*, somatic embryo; *SEIM*, somatic embryo induction medium; *SEGM*, somatic embryo germination medium; *SGM*, seed germination medium; *SIM*, shoot induction medium; *SMM*, shoot multiplication medium; *TDZ*, thidiazuron (*N*-phenyl-*N*'- 1,2,3-thiadiazol-5-ylurea); *w*, week(s); *y*, year(s)

[1] Even though calli was used in the original, the term callus has been used here based on recommendation of (Teixeira da Silva 2012b)

[1a] Hormone concentration was standardized for all studies by converting mg/l to μM to better understand and compare the effects of hormones on in vitro morphogenesis (Beyl 2011)

[2] The original light intensity reported in each study has been represented

[3] Since limited information was available, either because only the abstract or only incomplete portions of the paper were available, despite requests to authors, the following studies have not been summarized in this table (Malathy and Pai 1998; Sarsan et al. 1994)

[4] Duplicate publication(s)

Explant disinfection

Explant disinfection is one of the most essential steps of the tissue culture protocol because infection limits the success of all ensuing steps and practical applications (Teixeira da Silva et al. 2016). A summary of the disinfection protocols used for *H. indicus* in vitro regeneration is provided in Table 1. Ghatge (2007) tested mercuric chloride ($HgCl_2$) for 1–7 min on leaf and nodal explants, finding that 0.1 mg l^{-1} $HgCl_2$ for 5 min was effective, resulting in more than 80% aseptic cultures without any sign of browning of explants. A 10-fold higher concentration of $HgCl_2$ and exposure for 2 min was sufficient to disinfect nodal segments (Sharma and Yelne 1995). When 0.1% $HgCl_2$ for 1–5 min was used for shoot tips or 2 min for nodes, there was only 10–12% microbial contamination (Gopi 2014; Sindura 2014). However, none of these studies conducted statistical analyses, weakening the strength of their observed conclusions.

Light intensity and photoperiod

The source, spectrum, and intensity of light, as well as the photoperiod, are useful parameters to regulate plant growth in vitro (Batista et al. 2018). Despite this, no comparative studies on light conditions on tissue culture or secondary metabolite production are available for *H. indicus*, while many protocols provide poor or no details about the light source, photoperiod, or light intensity (Table 2). Future studies on *H. indicus* in vitro culture should optimize conditions such as the use of wide-spectrum light-emitting diodes (Miler et al. 2019) while also assessing the impact of culture vessel in photoautotrophic micropropagation (Xiao et al. 2011). Sreekumar (1997) was able to rapidly induce shoots from node, leaf, shoot tip, and root segments when inoculated in the dark for 4 days and then exposed to a 16-h photoperiod. Heble and Chadha (1978) were able to induce callus and shoot buds under continuous light, but plant production was poor.

Nutrient medium, carbon source, media additives, and plant growth regulators

The choice of nutrient medium, carbon source, media additives, and plant growth regulators (PGRs) all impact the success of in vitro growth and morphogenesis. Sreekumar (1997) and Ghatge (2007) tested various nutrient media with *H. indicus*, noting that the choice of nutrient medium affected the outcome of in vitro growth. Sreekumar (1997) found that MS medium was more responsive to shoot regeneration than other media, including B5 (Gamborg et al. 1968), Nitsch (Nitsch and Nitsch 1969), White (White 1934), SH (Schenk and Hildebrandt 1972), and WPM (Lloyd and McCown 1980). Similarly, Ghatge (2007) also found MS medium to be more responsive to shoot regeneration than B5 and Nitsch media (details in Table 2).

The carbon source, which provides additional carbohydrate and energy for in vitro explants that are not autotrophic, is a vital component of plant tissue culture media (Yaseen et al. 2013). The most frequently used carbon source for *H. indicus in vitro* culture (mainly shoot regeneration) is 3% sucrose (Table 1), but other concentrations of sucrose have also successfully been used, such as 2% (Patnaik and Debata 1996; Sreekumar 1997; Saha et al. 2003), 4% (Sreekumar 1997; Sreekumar et al. 1998), and 7.5% (Misra et al. 2003). Misra et al. (2005) used 4% sucrose for shoot induction from nodes and 3% sucrose for callus induction from leaves and roots. The development of photoautotrophic micropropagation will be useful for cost-effective and commercial-scale propagation of this plant (Xiao et al. 2011).

Similar to the vast majority of plants cultured in vitro, in *H. indicus*, the choice of PGR affects the outcome of regeneration. On MS medium supplemented with 13.32 μM BA, internode length and thickness of axillary shoots could be increased when the concentration of ammonium nitrate was reduced (Malathy and Pai 1998). Table 2 provides a detailed summary of how the choice of PGR affects organogenesis in *H. indicus*. Shekhawat and Manokari (2016) reported that in the most effective protocol for axillary shoot multiplication, about 272 shoots were obtained from a single node with a 98% survival of micropropagated plants (Table 2).

Misra et al. (2003) used 81.3 μM adenine sulfate (AdS) in shoot induction medium (SIM). Application of AdS prevented leaf abscission, callus formation, and accelerated shoot regeneration (Misra et al. 2003). Similarly, Nagahatenna and Peiris (2007) reported shoot tip abscision and premature leaf fall. Nagahatenna and Peiris (2007) used 81.3 μM AdS n in SIM, which was effective in the control of shoot tip abscission and premature leaf fall. In contrast, Patnaik and Debata (1996) also reported leaf abscission, but since there was no effect on plant growth, they did not use AdS or any other supplements. Saha et al. (2003) used 17.61 μM ascorbic acid (AA) and 14.61 μM glutamine in SIM while Shekhawat and Manokari (2016) applied 135.5 μM AdS, 8.81 μM AA, 4.78 μM citric acid, and 4.36 μM arginine in SIM, solving the problem of explant browning as well as shoot tip abscission and premature leaf fall. In all these cases, the objective was to reduce explant browning and oxidation.

In vitro shoot multiplication from a predetermined meristem (shoot tip or node)

In vitro shoot multiplication using a predetermined shoot meristem (apical or axillary bud) is the most popular and

convenient method for micropropagation (Tables 1 and 2). The application of a predetermined meristem minimizes micropropagation steps and provides genetically uniform plants (Sandhu et al. 2018). There are only a few reports in which apical buds (shoot tips) were used for *H. indicus* micropropagation (Sarasan and Nair 1991; Sreekumar 1997; Sreekumar et al. 2000; Sudarmani and Hasina 2013; Gopi 2014; Sindura 2014). In most cases, axillary buds (nodal explants) were used for micropropagation (Patnaik and Debata 1996; Sreekumar 1997; Sreekumar et al. 2000; Misra et al. 2003; Saha et al. 2003; Nagahatenna and Peiris 2007, 2008; George 2009; Shanmugapriya and Sivakumar 2011; Saryam et al. 2012a, 2012b; Devi et al. 2014; Singh and Shalini 2015; Reddy et al. 2016; Shekhawat and Manokari 2016; Verma and Vashistha 2016; Pathak et al. 2017; Prashanti et al. 2017; Maity 2018b; Yadav et al. 2019).

Callus-mediated (indirect) regeneration and somatic embryogenesis

Callus-mediated regeneration has several advantages over direct regeneration from a predetermined meristem: callus can be utilized for investigating developmental biology (Ikeuchi et al. 2016) or somaclonal variation (Krishna et al. 2016), or utilized for cell suspension culture to produce secondary metabolites (Espinosa-Leal et al. 2018). There are few reports on callus-mediated regeneration for *H. indicus* (Table 2). Heble and Chadha (1978) made the first attempt to regenerate callus from shoot tips, leaves, and stems on Lin and Staba (1961) medium supplemented with 9 μM 2-4,dichlorophenoxy acetic acid (2,4-D) and 0.93 μM kinetin (KIN). When callus was transferred to Lin and Staba medium supplemented with 9.78 μM IBA, only stem-derived callus was able to regenerate adventitious shoots. Pathak and Joshi (2017) reported callus-mediated shoot regeneration from leaf explants when 20 μM BA and 1 μM IAA were added to MS medium. Yellowish nodular callus, which formed from nodes on MS medium containing 2.22 μM BA and 1.07 μM NAA (Maity 2018a), was able to produce shoots after transfer to MS medium with 0.88 μM BA.

Somatic embryos can be utilized for germplasm storage, micropropagation, or synthetic seed production. Only limited studies are available on somatic embryogenesis of *H. indicus* (Sarsan et al. 1994; Ghatge and Dixit 2006; Ghatge 2007; Nagahatenna and Peiris 2008; Cheruvathur et al. 2013), but several of these studies lacked sufficient histological or molecular evidence to support the unequivocal claim of somatic embryogenesis, while in most studies, acclimatization and survival of plantlets produced via somatic embryos was not quantified, except in the Cheruvathur et al. (2013) study. In the Cheruvathur et al. (2013) study, when callus was transferred to ½MS with 9.78 μM IBA, 92% of cultures produced about 32 somatic embryos per gram of callus, and upon transfer of these embryos to MS medium with 17.76 μM BA and 4.34 μM GA_3, more than 90% of somatic embryos converted into plantlets.

Synthetic seeds

Production of synthetic seeds using alginate encapsulation is an efficient method of propagation and short- to mid-term storage (Sharma et al. 2013; Faisal and Alatar 2019; Qahtan et al. 2019). For *H. indicus*, there are only two reports on synthetic seed production (Cheruvathur et al. 2013; Yadav et al. 2019). Cheruvathur et al. (2013) used 4% sodium alginate and 100 μM $CaCl_2$ to encapsulate somatic embryos, which were stored at 4 °C for 120 days, and the stored synthetic seeds show 100% plantlet conversion on MS medium supplemented with 9.3 μM KIN and 2.46 μM IBA. Yadav et al. (2019) used nodal cuttings with a single axillary bud encapsulated in 3% sodium alginate and 100 mM $CaCl_2$ and stored at 4 °C for 60 days. Maximum regeneration frequency (84%) was observed on MS medium supplemented with 5.0 μM BA and 0.5 μM IBA up to the first week, but regeneration frequency decreased thereafter (Yadav et al. 2019).

Rooting and acclimatization

Rooting of in vitro raised shoots of *H. indicus* commonly takes place on full, ½, or ¼ MS nutrient medium, generally in the presence of IBA (Table 2). The induction of a profuse root system in vitro fortifies the chances of successful acclimatization and survival of plantlets when transferred ex vitro (Shekhawat and Manokari 2016). Despite the importance of the acclimatization step (Pospíšilová et al. 1999), parameters such as relative humidity, substrate choice, environmental conditions, treatment with antimicrobial agents, irrigation, light intensity, or relative humidity, which are some of the important factors that can determine the fate of acclimatized micropropagated plants, have not been reported for *H. indicus*. Misra et al. (2003) used ½MS with 9.78 μM IBA, 5.37 μM NAA, and 1.2 μM activated charcoal for in vitro rooting, yielding five roots. Saha et al. (2003) tested 14.61 μM glutamine along with 7.37 μ IBA in MS medium produced average 3.5 roots per shoot. In contrast, Shekhawat and Manokari (2016) reported 62 roots (in vitro rooting) from one shoot on ¼MS medium supplemented with 14.66 μM IBA, and about 45 roots per shoot (ex vitro rooting) when in vitro raised shoots were treated with 1954 μM IBA for 5 min. Although many reports describe the acclimatization step in a greenhouse, limited information is available about the survival and genetic fidelity of regenerants (Table 2). These crucial aspects need to be focused on in future research since they

can affect the chemical composition of plants and thus influence the constituents of medicinally, pharmaceutically important compounds, or secondary metabolites (Shekhawat and Manokari 2016). Only a single study by Khan (2014) compared the anatomy of mother plants versus micropropagated plants, the latter forming more trichomes than the former. However, results were not quantified for cellular observations between stock plants and micropropagated plants. Hence, future histological studies on micropropagated plants can be useful, especially cell wall lignification and cuticle formation, for understanding the cellular basis of acclimatization. Saha et al. (2003) found a stable number of chromosomes ($2n = 22$) in micropropagated plants after acclimatization.

Phytochemical stability and in vitro production of secondary metabolites

Pathak et al. (2017) compared the chemical profile of in vitro–derived plants versus stock plants by high-performance thin-layer chromatography and quantified lupeol content. They found similar banding in plants derived from cytokinin-containing medium but variation in chemical constituents in shoots derived from auxin-supplemented medium. Highest lupeol content (0.187 mg g^{-1} dry weight (DW) was observed in in vitro shoots grown on MS medium supplemented with 10 μM BA and 5 μM KIN, equivalent to levels in stock plants (0.185 mg g^{-1} DW). Devi et al. (2014) conducted a phytochemical analysis of alkaloids, flavonoids, saponins, phenols, and tannins from stock plants followed by micropropagation, but a phytochemical analysis of micropropagated plants was not performed. Using gas chromatography, George (2009) found no phytochemical differences between micropropagated and stock *H. indicus* plants.

Heble and Chadha (1978) conducted pioneer studies in *H. indicus* on the production of secondary metabolites (cholesterol, campesterol, and sitosterol) from shoot tips, leaves, and stem-derived callus. Lupeol, vanillin, and rutin were reported from micropropagated plants (Misra et al. 2003), as well as from in vitro shoots and callus cultures (Misra et al. 2005). Secondary metabolite production, especially of MBALD, was reported by several researchers (Sreekumar 1997; Sreekumar et al. 1998, 2000; Gopi 2014; Sindura 2014). A higher concentration of MBALD (0.12% DW basis; ~ 2–3-fold more) was found in micropropagated plants than in stock plants (Sreekumar et al. 2000). Table 2 provides details of the culture conditions and medium composition of these studies. Ghatge (2007) reported a quantitative analysis of total phenolics, flavonoids, and alkaloids of callus from leaf and stock plant parts. The total phenolic, flavonoid, and alkaloid contents of leaves of stock plants (phenolics, 42.56 mg g^{-1} fresh weight (FW); flavonoids, 13.51 mg g^{-1} FW; alkaloids, 20.65 mg g^{-1} FW) were higher than callus culture (phenolics, 2.85 mg g^{-1} FW; flavonoids, 3.09 mg g^{-1} FW; alkaloids, 3.09 mg g^{-1} FW). Thus, optimization of the protocol for high-quality secondary metabolites from tissue culture is essential.

Conclusions and future perspectives

Most of the reports available for *H. indicus* are on in vitro shoot multiplication, except for a few studies on callus-mediated organogenesis, somatic embryogenesis, synthetic seed production, and MABLD production. Anatomical studies to confirm somatic embryogenesis would be useful for understanding the developmental biology of this medicinal plant. Physiological studies during in vitro culture and acclimatization are totally unexplored areas of research. The application of molecular markers, genetic engineering, cryoconservation for long-term preservation, temporary immersion systems, photoautotrophic systems, low cost systems of micropropagation, elicitation, and genetic engineering of in vitro cultures for the production of secondary metabolites are some areas of research that deserve a special focus to advance the biotechnology of this medicinal plant species.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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