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Biotechnological advances in *Vitex* species, and future perspectives

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Abstract *Vitex* is a large genus consisting of 230 species of trees and shrubs with multiple (ornamental, ethnobotanic and pharmacological) uses. Despite this, micropropagation has only been used to effectively propagate and preserve germplasm a limited number (six) of *Vitex* species (*V. agnus-castus*, *V. doniana*, *V. glabrata*, *V. negundo*, *V. rotundifolia*, *V. trifolia*). This review on *Vitex* provides details of published micropropagation protocols and perspectives on their application to germplasm preservation and *in vitro* conservation. Such details serve as a practically useful user manual for *Vitex* researchers. The importance of micropropagation and its application to synthetic seed production, *in vitro* flowering, production of secondary metabolites, and the use of molecular markers to detect somaclonal variation *in vitro*, are also highlighted.

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1. Introduction

The *Vitex* L. genus (Lamiaceae) [51] contains mainly trees and shrubs and several species have well-established uses in ethnobotany, medicine, pharmacology and landscaping as ornamental plants [27,30,71,109,56,76]. The socio-economic importance of many *Vitex* species is thus irrefutable. However, the over-reliance on natural populations to derive such primary resources can strain the ecological balance of the environment in which they grow naturally, making collection from natural populations, in some cases, unsustainable. Fifteen *Vitex* species (*V. acunae* Borh. & Muniz, *V. ajugaeflora* Dop., *V. amaniensis* W. Piep, *V. cooperi* Standl., *V. evoluta* Däniker, *V. gaumeri* Greenm., *V. heptaphylla* A. Juss., *V. keniensis* Turrill, *V. kuylenii* Standl., *V. lehmbachii* Gürke, *V. longisepala* King & Gamble, *V. parviflora* Juss., *V. urceolata* C.B. Clarke, *V. yaundensis* Gürke, and *V. zanzibarensis* Vatke) are included in the IUCN Red Data list [46] for various reasons, but all related to unsustainable harvesting.

Depulped seeds of *V. doniana* Sweet germinate well after a hot water treatment [1] or after 21 days of hydration-dehydration cycles [31]. The seed germination of other *Vitex* species has not yet been studied. Thus, urgent attention is needed to conserve *Vitex* species. One biotechnological tool, *in vitro* propagation, provides a viable solution for the large-scale propagation of medicinally important plants [53,92,65], including cryoconservation [94] and genetic transformation [96]. The micropropagation of three *Vitex* species (*V. negundo*, *V. agnus-castus* and *V. trifolia*) from 29 published reports of *Vitex* sp. has recently been reviewed [9]. However, that review lacks vital details about disinfection methods, temperature, light source and intensity, photoperiod, basal medium, plant growth regulator type and concentrations, medium pH, carbon source type and concentrations for culture initiation, multiplication and rooting, all of which are essential factors that influence the outcome of the *in vitro* protocol for *Vitex* species. Consequently, our review explores these fine-scale details of the different steps of the plant tissue culture protocols for *Vitex* spp. to allow plant biotechnologists to design new and detailed experiments. This review, which provides a detailed analysis of reports from 1986 to 2016 of the micropropagation of six *Vitex* species (*V. agnus-castus* L., *V. doniana*, *V. glabrata* R. Br., *V. leucoxylon* L., *V. negundo* L., *V. trifolia* L.) (Tables 1 and 2), provides a solid foundation for the sustainable social and economic use of valuable members of this genus. A brief background of the socio-economic importance of *Vitex* species for which micropropagation protocols exist is provided next.

V. agnus-castus (chast berry) is a deciduous shrub native of Mediterranean Europe and Central Asia. The fruit extract of *V. agnus-castus* is used to treat menstrual disorder (amenorrhoea, dysmenorrhoea), premenstrual syndrome, corpus luteum insufficiency, hyperprolactinaemia, infertility, acne, menopause and disrupted lactation [30]. The fruits and leaves of *V. doniana* (black plum) are either consumed raw or after processing while the leaves, fruits, roots, barks and seed of the plant are used in traditional medicinal in Africa to treat a wide range of ailments [28,29,32], and references therein]. *V. glabrata* is a tree commonly known as "Kai Nano" in Thailand whose bark and roots are used as an astringent because the bark accumulates high levels of ecdysteroids, primarily 20-hydroxyecdysone or β-ecdysone [110]. The former compound, 20-hydroxyecdysone, can be synthesized in cell suspension cultures of *V. glabrata* [83,84,23]. *V. leucoxylon* is a large deciduous tree found in India and is commonly known in Marathi as *Songarbhi*. The crude alcoholic extract of its leaves possesses anti-psychotic, anti-depressant, analgesic, anti-parkinsonian, anti-microbial, anti-inflammatory and wound-healing properties [100]. *V. negundo* is a woody, aromatic shrub used in Ayurveda, Unani, Chinese, and folk medicine [109,56], and has mainly anti-inflammatory, analgesic, anti-hyperglycaemic, hepato-protective, anti-microbial and snake venom neutralization activity [71]. *V. trifolia* is a component of a number of commercially available herbal formulations that employ its leaves, and which have antiseptic, aromatic, febrifuge, anodyne, diuretic, and emmenagogue activity, fruits, which have nervine, cephalic, emmenagogue, amenorrhoea-treating and anthelmintic activity, roots, which are used to treat febrifuge, painful inflammation, cough and fever, and flowers, which are used to treat fever [76].

This review highlights the advances made in the micropropagation (including synthetic seed technology), *in vitro* flowering, and production of secondary metabolites of *Vitex* species. Emphasis is also given to the use of molecular markers to detect variation arising from *in vitro* propagation. This review is useful for conservation biologists, plant physiologists and biotechnologists that aim to explore other unexplored *Vitex* species or to expand the repertoire of research existent for the currently investigated species.

2. Selection of suitable starting material and disinfection

The choice of explant often depends on the material that is available, and sometimes even on the season. The selection of explants in *Vitex in vitro* studies tends to be from young and actively growing shoots or branches, with shoot tips and nodal explants with dormant axillary buds being the first choice (Table 2) due to the presence of a predetermined meristem which allows for true-to-type clonal propagation.

2.1. Alternative explant sources

There are several studies available in which other explants were used. For instance, internodes or stem segments were used for callus induction and regeneration studies in *V. negundo* [103,77,26,79], *V. leucoxylon* [25], and *V. trifolia* [15]. Stem-induced callus of *V. glabrata* produced 20-hydroxyecdysone (20-HES) in a liquid culture system [83,84,98]. *V. negundo* leaves were used for callus culture and

Table 1 Disinfection procedures of tissues for *in vitro* use of *Vitex* species (alphabetical listing).

Species	Disinfection protocol	References
V. agnus-castus	After removal of testa by mechanical treatment → 1.25% NaOCl (5% available chlorine) 20 min → SDW 4–5×	[22]
V. agnus-castus	Shoot tip/nodal explant → RTW → 0.05% Bavistin® 5 min → SDW 3–4 × → SDW + 2–3 drops Tween-20 10 min → SDW 3–4 × → 70% EtOH 30 s → SDW 3–4×	[16]
V. agnus-castus	Apical bud or stem with nodes from seedlings → 75% EtOH 15 s → 0.1% $HgCl_2$ 3–5 min → SDW 5–6×	[59]
V. doniana	Second pair of young leaves from 2–4 year old tree → cleaned with cotton wool soaked in liquid soap → 0.5% fungicide (Ridomil®) + 2 drops of Tween 20 1 h → 70% EtOH 30 min → SDW 2 × → $CaCl_2$ (1, 1.5, 2%) + 2–3 drops of Tween 20 (duration NR) → 2% $CaCl_2$ 30 min → SDW 2 × → quick dip in 70% EtOH → 2% $CaCl_2$ 15 min → SDW 2 × → 70% contamination-free cultures obtained	[29]
V. leucoxylon	Explants → 10% NaOCl 10 min → SDW 4–5×	[25]
V. negundo	Young shoots → 1% Teepol® (detergent) → 0.1% $HgCl_2$ (2 min) → repeated wash with SDW (node with one axillary bud 1 cm)	[108]
V. negundo	Shoot apices and nodal segments → RTW 45 min → 5% Laboline® (detergent) + 7% NaOCl (7–10 min) → 5–7 × SDW → 0.1% $HgCl_2$ 8 min → SDDW 6–7×	[80]
V. negundo	Shoot apices and nodal segments → RTW → 1% Teepol® 10 min → RTW → 80% EtOH 30 s → 0.1% $HgCl_2$ 3 min→ SDW 3×	[102]
V. negundo	Internode segment (1.0–1.50 cm) from 10-year-old plant → RTW → 1%Teepol® 10 min → RTW → 80% EtOH 30 s → 0.1% $HgCl_2$ 3 min	[103]
V. negundo	Shoot apices and nodal segments → RTW → 1% Teepol® 10 min → RTW → 50% EtOH 30 s → 0.1% $HgCl_2$ 3 min→ SDW 3×	[104]
V. negundo	Actively growing healthy shoot with 3–4 nodes from adult plant (0.5–1.0 cm node with dormant axillary bud) → RTW 30 min → 5% Laboline® 7–8 min → DW → 0.05% $HgCl_2$ 20 min → SDW 5–6×	[24]
V. negundo	Young leaves (< 3 months) → 2% detergent + 2.5% commercial bleach + 0.01% NaOCl → 70% EtOH → SDW	[67]
V. negundo	Shoot tip/nodal explant with one dormant axillary bud (1.0–1.50 cm) from 6-month-old plant in March → RTW 30 min → 10% Laboline 10 min → washed thoroughly with SDW → 0.1% $HgCl_2$ 5 min → SDDW 6–7×	[77]
V. negundo	Explants → RTW → 5% Extran® (detergent) 10 min → 1% Bavistin (fungicide) 20 min → SDDW → 0.05% $HgCl_2$ 4 min → several rinses in SDDW	[107]
V. negundo	Young shoots → RTW 30 min → 5% Laboline® (2007, 2008, 2013b) or Teepol® (2011) 10 min → SDW 3–4 × → 0.1% $HgCl_2$ 4 min (2007, 2013) or 5–7 min (2008, 2011) → SDW 5–8×	[3,5,7,6]
V. negundo	Shoot tips (1.0–1.5 cm) → 0.5% Tween-20 → 70% EtOH 10 s → 0.1% $HgCl_2$ 3 min → SDW 4–5×	[106]
V. negundo	Young leaves from 3–5-month-old plant → RTW → 5% Tween-20 10 min → 70% EtOH 10–15 s → SDW 5–10 min → 0.1% $HgCl_2$ 2–3 min → SDW 4–5×	[49]
V. negundo	Young shoots → RTW → 0.1% $HgCl_2$ 1 min → SDW 3–4×	[50]
V. negundo	Explants → 5% Teepol® 10–15 min → 0.1% $HgCl_2$ 5 min → SDW 3×	[68]
V. negundo	Twigs → RTW → DW → shoot tips excised → 0.1% $HgCl_2$ 7 min → SDW 3–5×	[47]
V. negundo	Explants → RTW → detergent 30 min → 0.1% $HgCl_2$ 7 min → SDW 5×	[48]
V. negundo	Explants → water 10–15 min → 0.1% $HgCl_2$ 7–10 min → SDW 5–7×	[85]
V. negundo	Young stems 15–20 mm → RTW 30 min → Tween-80 → SDW 4×	[26]
V. negundo	Twigs → RTW → 1% Savlon® + liquid soap 5–10 min with shaking → SDW 3–4 × → 70% EtOH < 1 min → 0.1% $HgCl_2$ 5–7 min → SDW 4–5×	[75]
V. negundo	0.1% Bavistin® 10–15 min → 0.1% antibiotics (streptomycin and tetracycline) 5–10 min → 0.1% $HgCl_2$ 3–4 min → SW 5–6 × → 0.1% AA + 0.05% CA	[78]
V. negundo	RTW → Tween-20 (2 drops/100 ml) → 0.1% $HgCl_2$ 3–5 min → dip in 70% EtOH → SDW 4–5×	[41]
V. negundo	RTW 3 min → cut into 3–5 cm sections with 2–3 internodes → 2 g/l Blitox® (fungicide) + Tween-20 45 min → DW 3 × → SDW 1 min → AA + CA 2 min → SDW 3 × → 70% EtOH 30 s → SDW 2× → 0.12% $HgCl_2$ + 1 drop Tween-20 8 min → SDW 3×	[79]
V. negundo Dhaka 1205	Shoot tip/nodal explant → RTW → detergent 30 min → 70% EtOH 1 min → 0.1% $HgCl_2$ 5 min → SDW 5×	[2]
V. rotundifolia	Nodal explant → RTW → 70% EtOH 10 s → NaOCl (2% active chlorine) 15 min → SDW 3×	[72]
V. trifolia	1-cm long nodal explants → RTW → 0.1% Laboline® 10 min → 70% alcohol 5 min → 0.1% $HgCl_2$ 5 min → several rinses in DW	[44]
V. trifolia	RTW → Bavistin® + Tween-20 10–15 min → 70% EtOH 1 min → 0.1% $HgCl_2$ 4 min → SDDW	[15]
V. trifolia	5–7 cm long young shoots → RTW 20 min → 5% Laboline® 5 min → DW 3–4 × → 0.1% $HgCl_2$ 4 min → SDW repeatedly	[10,11,12,13]
V. trifolia	Shoot tips → RTW 10 min → 5% Tween-20 15 min → DW → 0.5% NaOCl 5 min → DDW → 0.01% $HgCl_2$ 10 min → 0.1% $HgCl_2$ 5 min → Bavistin® 30 min → DDW 1×	[64]
V. trifolia	5–7 cm long shoots → 0.1% $HgCl_2$ 20 min → rinse 4–5 × in SDW	[81]

AA, ascorbic acid; CA, citric acid; DDW, double distilled water; DW, distilled water; EtOH, ethyl alcohol (ethanol); $HgCl_2$, mercuric chloride; NaOCl, sodium hypochlorite; NR, not reported; RTW, running tap water; s, second(s); SDW, sterilized (by autoclaving) distilled water; SDDW, sterilized (by autoclaving) double distilled water; SW, sterile water.

Table 2 Micropropagation and tissue culture of *Vitex* species (alphabetical listing).

Species and/or cultivar	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions**	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
V. agnus-castus	Shoot tips, nodal segments (5–10 mm long) of mature plants	MS + 8.88 μM BA + 4.65 μM Kin. 3% sucrose. 0.8% agar (SIM). MS + 4.44 or 6.66 μM BA + 0.28 μM GA_3. 3% sucrose. 0.8% agar (shoot elongation). ½ MS + 0.49 μM IBA. 2% sucrose + 0.4% phytagel (RIM). After 25 d, PGR-free ½ MS (root elongation). Subculture every 20 d. pH 5.8.	16-h PP. CWFT. 35–50 μmol m^{-2} s^{-1}. 25 ± 2 °C.	94.5% and 90.3% regeneration and 7.7 and 6.7 shoots/explant (shoot tips and nodal explants, respectively). 90.4% of shoots formed roots after 30–35 d of culture. 80% survival in autoclaved sand combined with either organic manure, VC, or garden soil (1:1) and watered with ½ MS every 4 d for 2 w. Acclimatization at 16-h PP, CWFT, 35–50 μmol m^{-2} s^{-1}, 25 ± 2 °C.	[16]
V. agnus-castus	Apical buds or stem with nodes from seedlings	WPM + 2.22 μM BA + 0.1 μM NAA (SIM). WPM + 3.33 μM BA + 0.1 μM NAA + 3% sucrose (SMM). WPM + 0.13 μM NAA (shoot elongation; RIM). 0.5% agar. pH 5.8	10-h PP. CWFT. 50–60 μmol m^{-2} s^{-1}. 25 ± 1 °C	Axillary buds only elongated about 3 cm within 4 w; little callus formation at base of buds. 7–8 shoots/node, 90% of shoots formed roots after 20 d of culture, 85% survival in disinfectant peat soil at 25 °C, 85% RH.	[59]
V. doniana	Second pair of leaves from 2–4 year old tree	½ MS + 0.50 μM TDZ + 5.55 μM *myo*-inositol + 49.74 μM $AgNO_3$ or 6.25 μM tryptophan. 2% sucrose. Gelling agent NR. pH 5.7–5.8.	Darkness. 25 ± 2 °C.	Average of 6.5 somatic embryos/explant with either $AgNO_3$ or tryptophan. Conversion of somatic embryos to plantlets NR.	[29]
V. glabrata	Stem-induced callus (10-y-old cultures)	MS + 8.88 μM BA + 4.52 μM 2,4-D + 3% sucrose + 0.8% agar (CIM). B_5 + 8.88 μM + 4.52 μM 2,4-D (cell suspension culture), rotary shaker 120 rpm. pH NR.	Continuous light. 2000 lux, 25 °C.	20-hydroxyecdysone (ecdysteroid) production was improved with the addition of precursors: 7-dehydrocholesterol (10 mg/l; 1.31 mg/l/d; 1.36-fold higher than control), ergosterol (10 mg/l; 1.12-fold higher than control), and cholesterol (5 mg/l; 1.11-fold higher than control [98].	[83,84,98,23]
V. leucoxylon	Internodes from young shoots of 2-y-old plants	MS + 2.2 μM BA + 5.4 μM NAA (CIM). MS + 13.3 μM BA + 5.4 μM NAA or 8.9 μM Kin + 2.7 μM NAA (SIM). MS + 7.2 μM BA + 8.6 μM GA_3 (SMM). ½ MS + 4.9 μM IBA (RIM). Explant subculture NR. 3% sucrose, 0.8% agar, pH 5.8	16-h PP. CWFT. 20 μmol m^{-2} s^{-1}. 25 ± 2 °C	91% of explants formed callus, and shoots from callus (4.3/explant) after 8 weeks. 96% of shoots formed roots (8.3/shoot). Acclimatization in garden soil, red soil and sand (1:2:1) and watered with ½ MS for 2 w, but survival not quantified.	[25]
V. negundo	1 cm node with one axillary bud (1 cm) from young shoots	MS + 17.76 μM BA + 0.18 μM Kin + 3% sucrose. pH 5.8. 0.8% agar (SIM). ½ MS + 10.72 μM NAA + 3% sucrose + 0.8% agar (RIM).	10-h PP. 3000 lux. 25 ± 2 °C.	Multiple shoot buds formed within 15 d of inoculation, which developed into well-developed shoots within 30 d. 6.66 shoots per node and 8.89 roots per shoot. Acclimatization not performed.	[108]
V. negundo	Young nodal segments (0.6–0.8 cm)	MS + 4.44 μM BA + 0.11 μM GA_3 + 5.55 μM *myo*-inositol (SIM) subcultured every 4 w. ½ MS + 4.9 μM IBA + 5.71 μM IAA (RIM). pH 5.8. 3% (SIM) or 2% (RIM) sucrose. 0.8% (SIM) or 0.7% (RIM) agar.	16-h PP. CWFT. 35–50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–65% RH.	Shoots formed within 30 d and roots after another 30 d. Plant material collected in June–August showed higher shoot responsiveness *in vitro*. 6–8 shoots per nodal segment in first three subcultures, free of callus. 98–100% shoot induction (direct) and 94% of shoots induced roots. Plants placed at 25 ± 1 °C, 80–85% RH and 16-h PP of 50 μmol m^{-2} s^{-1} CWFT for 30 d. 93% survival of acclimatized plants in VC.	[80]
V. negundo	Shoot tips (0.3–0.5 cm) and nodes (1–1.5 cm)	MS + 6.66 μM BA (SIM). MS + 6.66 μM BA + 0.5 μM NAA (SMM). MS + 4.9 μM IBA (RIM). pH 5.8. 3% sucrose 0.8% agar.	16-h PP. CWFT. 80 μE m^{-2} s^{-1}. 26 ± 1 °C.	Frequency and number of shoots per explant obtained from both explants but nodes produced more shoots/explant. At shoot multiplication stage, 84.3% of nodal explants and 65% of shoot tips could form multiple shoots. 5–7 roots/shoot. Rooted plants	[102]

				transferred to sterile vermiculite + soil (ratio NR). 95% plant survival.	
V. negundo	Internode (1.0–1.5 cm)	MS + 8.05 μM NAA + 2.22 μM BA (CIM). MS + 4.44 μM BA + 2.69 μM NAA (SIM). MS + 2.46 μM IBA (RIM).3% sucrose. 0.8% agar. pH 5.8	16-h PP. CWFT. 80 μE m^{-2} s^{-1}.	Nearly 85% of cultures induced callus which proliferated. 67.3% of cultures regenerated shoots from callus: 10 shoots/callus, 51% of *in vitro* shoots rooted. Rooted plants transferred to vermiculite + soil (1:2). Survival% NR.	[103]
V. negundo	Shoot tips and nodes (size NR)	MS + 4.44 μM BA + 0.5 μM NAA (SIM, FIM). 3% sucrose. pH 5.8. 0.8% agar.	16-h PP. CWFT. 80 μE m^{-2} s^{-1}. 25 ± 2 °C.	About 95% of cultures flowered *in vitro*. Acclimatization (vermiculte and soil mixture). Kept in green house.	[104]
V. negundo	Nodal explants (0.5–1.0 cm)	MS + 17.8 μM BA + 2.15 μM NAA + 100 mg/l Na_2SO_4. Subculture on same medium at 25 d (SMM). ½ MS + 4.9 μM IBA (RIM). MS + 9.9 μM BA + 1.61 μM NAA (FIM). 5% sucrose. 0.8% agar (RIM) or 0.9% agar (SIM, SMM). pH 5.7–5.8. 5% sucrose.	16-h PP. CWFT. 35–50 μmol m^{-2} s^{-1}. 25 ± 2 °C.	97% of explants formed multiple shoots (20.7 per explant). 95% of shoots induced roots (9.0 per shoot). Plants acclimatized in autoclaved garden soil or red soil + clay + sand (3:2:1) or soilrite, 96% survival of acclimatized plants in VC. No morphological variation observed. *In vitro* flowering results NR.	[24]
V. negundo	Callus from *ex vitro* leaves	MS + 2.22 μM BA + 2.26 μM 2,4-D (CIM) subcultured every 4 w for 2 y. No other details reported.	NR	Total of 475 g of callus obtained. The profile of 7 oleanane-type triterpenes identified in callus culture extracts stayed constant over several (number unspecified) subcultures assessed by TLC. Acclimatization not performed.	[67]
V. negundo	Two studies: 1) Axillary shoot multiplication: nodal explants (1.0–1.5 cm)/shoot tip (0.5–0.8 cm) of 6-m-old plant (March); 2) Callus-mediated regeneration: leaf segments (7 × 10 mm) with midrib (adaxial surface on medium) + stem segment (1–1.5 cm) from 6-m-old plant (March)	Two studies: 1) Axillary shoot multiplication: MS + 10% CW + 1.8 μM TDZ + 3% sucrose. 0.8% agar (SIM). 2) MS + 1% PVP + 0.1–5.0 μM IAA or 0.1–5.0 μM 2,4-D alone or in combination with 0.1–4 μM TDZ or 1–25 μM BA. (CIM, SIM). 3. SEM: MS + GA_3 2.4 μM. MS + 1.71 μM IAA + 1.62 μM NAA (RIM).	12-h PP. CWFT. 36 μmol m^{-2} s^{-1}. 25–28 °C and 70–90% RH.	96% of explants formed multiple shoots (14.6 per node) but shoot length not determined due to stunted growth due to TDZ. Methods about acclimatization details is not available and data on acclimatization is not available. Resultant plantlets were without any external defects.	[77]
V. negundo	Nodal explants (4–10 mm long) of mature plant	MS + 5.0 μM BA (SIM). MS + 4.4 μM BA + 0.53 μM NAA (SMM, FIM). ½ MS + 0.5 μM NAA (RIM). pH 5.9. 3% sucrose. 0.8% agar.	14-h PP. CWFT. 3500 lux. 25 °C.	4 shoots/node (25 shoots/node on SMM after 4 subcultures) and 7.44 roots/shoot formed. 10-cm long shoots formed in 30 d. 80% of cultures formed flowers *in vitro* when the C/N ratio was 7.1; 90% survival of acclimatized plants in VC and cocopeat (1:1) at 90% RH. No phenotypic variation observed.	[107]
V. negundo	Nodal explants (5–10 mm long) from young shoots of 15-y-old tree	MS + 1.0 μM TDZ (SIM). MS + 1.0 μM BA + 0.5 μM NAA (SMM). All explants subcultured every 2 w. pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–65% RH.	No rooting *in vitro*; shoot (4–5 cm long) base treated with 500 μM IBA for 10 min then planted directly in Soilrite, with 97% shoots forming roots. Acclimatization at 16-h PP and 25 ± 2 °C; 24.8 shoots/node with SIM in 4 w and 13.6 roots/shoot after 4 w. 90% survival of acclimatized plants. No phenotypic variation observed.	[3]

(*continued on next page*)

Table 2 (*continued*)

Species and/or cultivar	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions**	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
V. negundo	Shoot tips (10–15 mm long) from 2-y-old plants	MS + 8.87 μM BA + 2.69 μM NAA (SIM, SMM). ½ MS + 4.9 μM IBA + 2.85 μM IAA + 3 g/l AC (RIM). Subculture every 4 w. pH 5.8. 3% (SIM, SMM) or 2% (RIM) sucrose. 0.8% agar.	16-h PP. CWFT. 55 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–65% RH.	6.3 shoots/shoot tip within 4 w. 85% survival after 4 w in soil, sand and farmyard manure (1:1:1), with true-to-type flowering.	[106]
V. negundo	Shoot tips, nodal segments (10–15 mm long) of mature plants	MS + 4.44 μM BA (SIM). ½ MS + 1.6 μM NAA (RIM). Subculture every 2 w. pH 5.8. 3% sucrose. 0.7% agar.	16-h PP. CWFT. LI NR. 24 ± 2 °C.	96% of explants formed shoots (21.8/node) after 3 w. 93% of shoots formed roots. 80% survival after 4 w in soil, compost and sand (1:1:1). Acclimatization at 12-h PP, 32 ± 2 °C, and 80% RH.	[2]
V. negundo	Nodal segments (5–10 mm) from 15-y-old tree	MS + 5.0 μM BA + 0.5 μM NAA (SIM, SMM). MS + 1.0 μM IBA (RIM). Subcultures NR. pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 50–60% RH.	16.4 shoots/explant. 97% survival of acclimatized plants in Soilrite watered with ½ MS without vitamins. No variation observed among micropropagated plants using 4 ISSR primers.	[6]
V. negundo	Leaves of 3–5-m-old plants	MS + 1.3 μM BA + 1.7 μM IAA (CIM, SIM). MS + 2.46 μM IBA (RIM). Explant subculture NR. pH 5.8. 3% sucrose. 0.7% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C.	80% of explants formed callus and 13 shoots/explant (indirect), and 11.6 roots/shoot. Plants acclimatized in vermiculite and garden soil (3:1) at 16-h PP, 25 ± 2 °C, but survival not quantified.	[49]
V. negundo	Nodal segments	MS + 4.44 μM BA (SIM). ½ MS + 4.92 μM IBA (RIM). Explant subculture NR. pH 5.8. 3% sucrose. 0.6% agar.	14-h PP. CWFT. 2000 lux. 25 ± 2 °C.	75.3% of explants formed shoots (8.2 per node) and 7.5 roots per shoot, 90% survival after 4 w in sterile garden soil and sand (3:1) watered with 10-fold dilution of MS.	[50]
V. negundo	Nodal segments (5–10 mm long) of adult plant	MS + 16.8 μM BA + 2.25 μM IBA + 589 μM $AgNO_3$ (SIM). Subculture every 20 d. pH 5.7–5.8. 5% sucrose. 0.9% agar.	16-h PP. CWFT. 35–50 μmol m^{-2} s^{-1}. 25 ± 2 °C.	98.6% of explants formed shoots (22.45/node) after 25–30 d. Acclimatization not performed.	[68]
V. negundo	Shoot tips	MS + 4.44 μM BA + 0.5 μM NAA (SIM). ½ MS + 2.46 μM IBA (RIM). Explant subculture NR. pH 5.8. 3% sucrose. 0.36% phytagel.	16-h PP. CWFT. 1500 lux. 26 ± 2 °C.	95% of explants formed shoots (6.8/explant) with callus, and 7 roots/shoot. Acclimatization claimed but no data or methodology provided.	[47]
V. negundo	Nodal segments (3 mm) from *in vitro* plants [6]	MS + 2.5 μM Kin + 1.0 μM NAA (synseed germination and plant growth). pH 5.8.Synseeds of 3% Na_2-alginate with 100 mM $CaCl_2$ (1 explant/synseed)	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 55–65% RH	92.6% synseed to plantlet conversion. Conversion% decreased as storage period at 4 °C from 1 to 8 w. Encapsulated explants showed higher conversion (71%) than unencapsulated explants (43%).	[4]
V. negundo	Shoot tips, nodal segments (3–4 cm)	MS + 13.32 μM BA (SIM). MS + 11 .43 μM IAA (RIM). Explant subculture NR. pH 5.8. 3% sucrose. 1.0% agar.	16-h PP. LI NR. 25 ± 2 °C	87.5% of explants formed shoots (6/shoot tip or 7/node). 87.5% of shoots formed roots. 85% survival in soil, compost and sand (1:1:1).	[48]
V. negundo	Nodal segments from mature plants	MS + 4.44 μM BA + 792.95 μM PG + 117.73 μM $AgNO_3$ (SIM). ½ MS + 2.46 μM IBA (RIM). Explant subculture NR. pH 5.8. 3% sucrose. 0.8% agar	16-h PP. LI NR. 25 ± 1 °C	95.3% of buds broke when collected in March–May. 95% of explants formed shoots (15.1 per node) after 45 d, 85% of shoots formed roots within 2 w, 80% survival after 3 m in soil, sand and VC (6:2:1) after dipping cut ends of shoots in 400 mg/l IBA.	[85]
V. negundo	Nodal segments (1–2 from the shoot tip) from a single 3-y-old micropropagated plant [6]	MS + 5.0 μM BA + 0.5 μM NAA (SIM, SMM). MS + 1.0 μM IBA (RIM). Explant subculture NR. pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}, 25 ± 2 °C	16.4 shoots/explant. 97% survival of acclimatized plants in Soilrite. No variation observed among micropropagated plants using 10 RAPD primers.	[5]

V. negundo	Leaves, internodes of 4-y-old plants	MS + 9.04 μM 2,4-D (CIM). Explant subculture NR. pH 5.8. 3% sucrose. 0.7% agar.	16-h PP. CWFT. 20 μmol m^{-2} s^{-1}. 26 ± 2 °C.	100% of explants formed callus in 8 d. Organogenesis and acclimatization not performed.	[26]
V. negundo	Shoot tips, leaf and nodal segments (5–10 mm long)	MS + 8.88 μM BA + 2.69 μM NAA (SIM). MS + 8.88 μM BA + 5.71 μM IAA (shoot elongation).½ MS + 5.37 μM NAA (RIM). Explant subculture NR. pH 5.8. Carbon source NR. 0.8% agar.	14-h PP. LI NR. 25 ± 2 °C.	Only nodes produced shoots. 93% of explants formed shoot buds (3.6 per node) after 8–12 d. 95% of shoots formed roots (35.6 per shoot). 82% survival in soil and compost (1:1) with no morphological abnormalities.	[75]
V. negundo	Shoot tips, nodal explants (4–5 cm long) collected in June-July	MS + 8.88 μM BA (SIM). MS + 4.44 μM BA + 283.5 μM AA + 130.12 μM CA + 0.57 μM IAA (pre-SMM) for 3–4 subcultures. Pre-SMM + 1.16 μM Kin (SMM). ¼ MS + 16.66IBA + 0.01% AC (RIM). Subculture every 20–25 d. pH, carbon source, gelling agent NR.	PP, LI, temperature NR.	Shoots emerged after 5–6 d on SIM. 9–10-m-old cultures with a subculture delay of 6–7 d flowered *in vitro*. 100% of explants formed shoots (6.1 per node), reaching 29.1 after 3–4 subcultures on SMM. 95% of shoots formed roots *in vitro* (6.1 per shoot) or 100% *ex vitro* after dipping cut ends of shoots in 300 mg/l IBA (5.5 roots/shoot). 85–90% survival in sand, garden soil and organic manure (3:1:1).	[78]
V. negundo	Shoot tips from young shoots of mature plant	MS + 5.0 μM BA (SIM). MS + 5.0 μM BA + 0.5 μM NAA (SMM). Subculture every 3 w. pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–65% RH.	3.6 shoots/shoot tip in SIM and 4.8 shoots/shoot tip in SMM after 8 w. No rooting *in vitro*; rather shoot (4–5 cm long) base treated with 500 μM IBA for 10 min then planted directly in Soilrite, with 95% survival. Extracts of *in vitro* plants used for antimicrobial assays and to measure total phenolic content. No variation observed among micropropagated plants and mother plant using TLC of phytoextract.	[7]
V. negundo	Nodes from mature plant	MS + 8.88 μM BA + 2.69 μM NAA + 30% sugarcane juice (SIM). ½ MS + 4.69 μM IBA (RIM). 0.8% agar. pH 5.8.	16-h PP. 40 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–70% RH.	93% of explants formed shoots (4.1 per/node). 66% of shoots formed roots (7.4 per shoot). Acclimatization in sterile soil and sand (3:1): plantlets covered with transparent plastic bags and watered with ½ MS medium every 2 d for 2 w then bags removed. After 4 w plants transferred to pots containing garden soil and maintained under normal daylength. Plantlet survival NR.	[41]
V. negundo	Internodes (3–4 cm)	MS + 8.88 μM BA + 9.04 μM 2,4-D + 5.37 μM NAA (CIM). MS + 8.88 μM BA + 4.0 μM NAA (SIM).½ MS + 2.46 μM IBA (RIM). All explants subcultured every 3–4 w. pH 5.7. 3% sucrose. Agar conc. NR.	16-h PP. CWFT. 3000 lux. 24 ± 2 °C. 55% RH.	Callus induction within 7–18 d. 95% of callus formed shoots (7.0 per explant). 87% of shoots formed roots (6.4 per shoot). Acclimatization in soil and vermiculite (1:1) but survival not quantified.	[79]
V. rotundifolia	Nodal explants from incubator-grown plant at 25 °C	Nitsch + 4.44 μM BA (SIM). ½ Nitsch + 2.69 μM NAA (RIM). Carbon source, gelling agent NR. pH 5.8.	16-h. PPFD NR. 25 ± 1 °C.	Results not available (only abstract available).	[72]
V. trifolia	Nodal explants (8 mm long) from 8-y-old tree	MS + 5.0 μM BA (SIM). ½ MS + 0.5 μM NAA (RIM). pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 40 μmol m^{-2} s^{-1}. 25 ± 2 °C.	Nine shoots/explant and 6.9 roots/shoot formed, 90% survival of acclimatized plants in autoclaved soil and vermiculite (1:1) at 16-h PP, 50 μmol m^{-2} s^{-1}, 25 ± 2 °C, 80% RH, irrigated with Hoagland's solution (Hoagland and Amon, 1950) once a week. No phenotypic variation observed.	[44]
V. trifolia	Leaf; internode (1–1.5 cm)	MS + 6.78 μM 2,4-D + 0.13 μM Kin (CIM). MS + 8.88 μM BA + 0.16 μM NAA (SIM). MS + 2.46 μM IBA (RIM). pH 5.8. 3% sucrose. 0.6%	12-h PP. LI NR. 25 ± 2 °C.	88% callus induction, 95% shoot induction (indirect) and 85% of shoots induced roots. 75% survival of acclimatized plants in soil and compost (1:1).	[15]

(*continued on next page*)

Table 2 (*continued*)

Species and/or cultivar	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions**	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
		agar.			
V. trifolia	Nodal explants (5–10 mm long) from young shoots of 3-y-old tree	MS + 5.0 μM TDZ (SIM). MS + 1.0 μM BA + 0.5 μM NAA (SMM). MS + 0.5 μM NAA (RIM). Subculture every 3 w. pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–65% RH.	90% of explants formed shoots (22.3 per node) after 8 w when plant material collected from mid-Sept-Oct. 87% of shoots formed roots (4.4 per shoot), 92% survival after 4 w in sterile Soilrite, then vermiculite and garden soil (1:1) with true-to-type morphology.	[10]
V. trifolia	Nodal explants (5–10 mm long) from young shoots of 3-y-old tree	MS + 5.0 μM BA + 0.5 μM NAA (SIM). Subculture every 3 w. pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–65% RH.	97% of explants formed shoots (16.8 per node) after 8 w. No rooting *in vitro*; shoot (4–5 cm long) base treated with 500 μM IBA for 10 min then planted directly in sterile Soilrite, and watered with ½ MS without organics: 95% survival after 4 w. Acclimatization conditions NR. Genetic stability claimed by RAPD.	[8]
V. trifolia	Shoot tips	MS + 9.90 μM BA (SIM). ½ MS + 9.84 μM IBA (RIM). pH, carbon source, agar conc. NR.	16-h PP. LI NR. 24 ± 2 °C.	15 shoots/shoot tip. 90% survival of acclimatized plants in Soilrite, cocopeat and vermiculite (1:1:1). *In vitro* flowers obtained after 20 d in response to BA, but flowering not quantified.	[64]
V. trifolia	Stems, leaves and petioles	MS + 0.44 μM BA + 16.11 μM NAA (CIM) subcultured every 4 w. MS + 11.1 μM BA + 0.54 μM NAA + 271.5 μM AdS + 1.44 μM GA_3 (SIM). ½ MS + 1.43 μM IAA or 1.23 μM NAA (RIM). pH 5.8. 2% sucrose. 0.8% agar.	16-h PP. 61 μmol m^{-2} s^{-1}. 25 ± 2 °C.	Stem and petiole explants more receptive than leaves. Globular callus formation in 3–4 w, shoots in 4 w, and roots in 11–12 d. 87%, 77% and 58% shoot induction (indirect) from stem, petiole and leaf explants, respectively. 86% of shoots rooted.∼90% survival of acclimatized plants in soil, sand and well decomposed manure (1:1:1). Genetic uniformity of regenerated plantlets of subculture 2–5 confirmed by RAPD and ISSR.	[81]
V. trifolia	Shoot tips (5–8 mm long) from young shoots of 3-y-old tree	MS + 5.0 μM TDZ (SIM). MS + 1.0 μM BA + 0.5 μM NAA (SMM). ½ MS + 0.5 μM NAA (RIM). Subculture every 3 w. pH 5.8. 3% sucrose. 0.7% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–65% RH.	95% of explants formed shoots (22.2/shoot tip) after 8 w. 87% of shoots formed roots (4.4 per shoot) after 4 w. 90% survival after 8 w in sterile Soilrite in the *in vitro* culture room conditions. Several antioxidant enzymes activated 28 d after transplantation.	[12]
V. trifolia	Shoot tips (5–8 mm long) from young shoots of 3-y-old tree	MS + 5.0 μM TDZ (SIM). MS + 1.0 μM BA + 0.5 μM NAA (SMM). ½ MS + 0.5 μM NAA (RIM). Subculture every 3 w. pH 5.8. 3% sucrose. 0.7% agar.	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60–65% RH.	94% of explants formed shoots (19.2 per shoot tip) after 12 w. No rooting *in vitro*; shoot (4–5 cm long) base treated with 500 μM IBA for 10 min then planted directly in vermiculite and garden soil (1:1): 95% survival and 7 roots/shoot after 4 w.	[11]
V. trifolia	Nodal segment derived from 2 month old *in vitro* raised shoots Same as [10]	Encapsulation: 3% sodium alginate and 100 mM calcium chloride. Germination: MS + 5 μM BA + 0.5 μM NAA. Rooting, MS + 0.5 μM, bacteriological agar 0.8%.pH 5.8.	Same as [10]	84.9% synthetic seed formed shoots after 6 w. Encapsulated seeds were stored at 4 °C up to 8 w with 42.5% regeneration efficiency. 90% rooting after 4 w with avg 5.7 roots with 2.1 cm length. Plants with fully expanded leaves were transferred to soilrite with 92% survival rate.	[13]

V. trifolia var. *Simplicifolia*	Young shoot tips of mature plants	(1) White's + 4.44 µM BA + 2.46 µM IBA; (2) ½ White's + 0.4 µM BA + 2.46 µM IBA; (3) MS + 4.44 µM BA + 3.69 µM IBA; (4) MS + 3.33 µM BA + 4.9 µM IBA (SIM). White's + 2.46 µM IBA (RIM)	8–10-h PP. 25–30 °C	Much callus formed on media (1) and (2), callus differentiated to 7–8 cm tall shoots after 50 d culture. Little callus formed on media (3) and (4), and few adventitious buds formed in these media. 3 cm long shoots formed roots after 20 d of culture.	[35]

2,4-D, 2,4-dichlorophenoxyacetic acid; AA, ascorbic acid; AC, activated charcoal; AdS, adenine sulphate; $AgNO_3$, silver nitrate; B_5 medium, or Gamborg medium, [38]; BA, N^6-benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original [86]; CA, citric acid; CIM, callus induction medium; CW, coconut water; CWFT, white fluorescent tubes; d, day(s); FIM, flower induction medium; GA_3, gibberellic acid; Hoagland medium [45]; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; ISSR, inter simple sequence repeat; Kin, kinetin (6-furfuryl aminopurine); LI, light intensity; m, month(s); MS, Murashige and Skoogs [63] medium; NAA, α-naphthaleneacetic acid; Nitsch medium [66]; NR, not reported in the study; PG, phloroglucinol; PGR, plant growth regulator; PP, photoperiod; PVP: polyvinyl pyrrolidone; RAPD, random amplified polymorphic DNA; RH, relative humidity; RIM, root induction medium; rpm, revolutions per minute; SEM, shoot elongation medium; SIM, shoot induction medium; SMM, multiplication induction medium; TDZ, thidiazuron (*N*-phenyl-*N*'- 1,2,3-thiadiazol-5-ylurea); TLC, thin layer chromatography; w, week(s); VC, vermicompost; WPM, woody plant medium [60]; White [111]; y, year(s).

* Even though calli was used in the original, the term callus has been used here based on recommendation of Teixeira da Silva [87].

** The original light intensity reported in each study has been represented since the conversion of lux to µmol m^{-2} s^{-1} is different for different illumination (main ones represented): for fluorescent lamps, 1 µmol m^{-2} s^{-1} = 80 lux; the sun, 1 µmol m^{-2} s^{-1} = 55.6 lux; high voltage sodium lamp, 1 µmol m^{-2} s^{-1} = 71.4 lux [101].

to produce triterpenes [67]. Leaf segments of *V. negundo* with a midrib and adjacent stem segment were used to study callus-mediated organogenesis, and their findings were compared with shoot tips and nodal explants [77]. Explants from *in vitro* germinated seedlings (hypocotyls, cotyledons, roots and shoot tips) can be employed [22], although seed-derived material is genetically dissimilar and is thus not suitable for clonal tissue culture experiments. There is one report on somatic embryogenesis but none on direct organogenesis (adventitious shoot regeneration) in the *Vitex* genus, although there are reports on callus-mediated organogenesis in *V. trifolia* [15,35,81], *V. negundo* [103,26,79], *V. rotundifolia* [72] and *V. leucoxylon* [25]. The chances of somaclonal variation are greater in the case of callus-mediated organogenesis or somatic embryogenesis [52,62], although the latter can provide a useful platform for genetic transformation studies. Somaclonal variation during *in vitro* cultures of *Vitex* species has not been reported yet, and this topic should be explored in future studies.

2.2. Influence of season

The impact of season for collecting nodal explants from *V. negundo* from Bhubaneswar, Orissa, in India was compared (1. March to May; 2. June to August; 3. September to November; 4. December to February), and collection between June and August showed higher shoot multiplication [80]. In other studies [10,11,12], nodes of *V. trifolia* collected from Aligarh, Uttar Pradesh, in India, from January to December, or shoots collected in March, September and October showed best bud break. None of these studies [80,10,11,12] reported the effects of season on explant contamination but such information is essential as explant disinfection is the most important step in the establishment of a tissue culture experiment [93].

2.3. Optimized disinfection procedures

Once the explant has been selected, surface disinfection is an important step to establish contamination-free cultures. Based on the *Vitex* tissue culture literature (Table 1), explants are generally washed under running tap water followed by a wash with a detergent used in a concentration range of 0.1–5%. Laboline® applied at 0.1% for 10 min was effective for *V. trifolia* [44,3,5], 5% Laboline® or Teepol® for 10 min for *V. negundo* [7] and *V. trifolia* [8], while 5% Extran® for 10 min effectively disinfected *V. negundo* explants [107]. Explants are then surface disinfected with 0.01% (10 min)-0.1% mercury chloride ($HgCl_2$) (1–8 min; Table 1), although 0.1% $HgCl_2$ for 3 min was sufficient to obtain a 90% contamination-free culture for *V. negundo* nodes and shoot tip explants [102]. After treatment with $HgCl_2$, explants were treated with sterile distilled water (SDW) or sterile double-distilled water (SDDW) three to seven times and then inoculated on basal medium supplemented with various plant growth regulators (PGRs). In some reports, $HgCl_2$ was omitted from the protocol. *V. rotundifolia* nodal explants were sterilized with 70% EtOH for 10 s followed by treatment with sodium hypochlorite (NaOCl; 2% active chlorine) for 15 min and rinsed three times with SDW [72]. Only 10% NaOCl for 10 min followed by 4–5 washes of SDW was effective for *V. leucoxylon* [25] but the percentage of contamination-free cultures was not reported. In summary,

surface disinfection and explant preparation can be divided into three stages: (1) removal of dirt and surface dust by washing under running tap water for 10–30 min followed by treatment with liquid detergent; (2) disinfection under a laminar air hood: treatment with EtOH, preferably 70%, which can penetrate bacterial cell walls (by disrupting hydrogen bonding) for at most 30 s otherwise explants can be damaged [70] and then 0.1% $HgCl_2$ for 1–3 min depending on the explant (juvenile explants can be treated for a maximum of 1 min and mature explants for as long as 3 min); (3) 2–3 washes with SDW. NaOCl is a much more environmental-friendly disinfectant then $HgCl_2$ [19]. The presence of endophytic microorganisms can cause problems during the establishment of *in vitro* cultures [57].

In our experiments, we observed contamination by bacteria in *V. negundo* nodal explants (Fig. 1) that were disinfected with (1) 0.1% $HgCl_2$ for 3 min followed by three rinses with SDW, (2) 70% ethanol for 2 min followed by treatment with 0.1% $HgCl_2$ for 3 min and three rinses with SDW, and (3) 0.1% $HgCl_2$ for 3 min followed by disinfection with 70% ethanol for 2 min and three rinses with SDW. Browning of explants was observed when $HgCl_2$ was used as the disinfectant (Fig. 1A–C) while surface disinfection with 70% ethanol for 2 min followed by three rinses with SDW allowed explants to remain green although contamination was still observed in more than 50% of cultures. However, 95% of contamination could be controlled by adding filter-sterilized antibiotics (41.28 μM kanamycin, 59.81 μM penicillin, or 34.39 μM streptomycin) to Murashige and Skoog (MS) medium [63] (Fig. 1E–H). Nanoparticles have been shown to control disinfection [17] and could be used in the future to disinfect explants from other *Vitex* species. Contamination by endophytes has not yet been reported in any *Vitex* species and could be controlled through meristem culture.

3. Light conditions

Most culture conditions for the *in vitro* growth of *Vitex* species require a 16-h photoperiod (more rarely 10, 12, or 14 h [77,15,107,108,50,75] under cool white fluorescent tubes at a photosynthetic photon flux density of 35–50 μmol m^{-2} s^{-1} (Table 2). However, a continuous supply of light was useful for the production of 20-HES from stem-induced callus of *V. glabrata* [83,84,98].

Light-emitting diodes (LEDs) and cold-cathode fluorescent lamps (CCFL) have seen expanded scientific applications [112], including in plant physiological studies [69,37]. To maximize plant production *in vitro*, or even to alter morphogenesis, changes in the intensity, type of light source, spectral range and photoperiod can be explored in the future for *Vitex* tissue culture and secondary metabolite production.

4. Medium composition

MS medium was most frequently used for tissue culture studies of *Vitex* species (Table 2) although Li et al. Li et al. [59] used woody plant medium (WPM) [60] for *V. agnus-castus*. Park et al. Park et al. [72] used Nitsch medium [66] for *V. rotundifolia*. Since there are no comparative studies between basal media, such studies are needed to improve the efficiency of *in vitro* culture of different *Vitex* species.

For shoot tip and axillary shoot multiplication, the most frequently used cytokinins were 6-benzyladenine (BA), kinetin (Kin), and thidiazuron (TDZ) (Table 2). In *V. agnus-castus*, Balaraju et al. Balaraju et al. [16] showed that BA or Kin, usually in the presence of an auxin, α-naphthaleneacetic acid (NAA), could induce shoots from shoot tips and nodal explants. In *V. negundo*, NAA induced callus formation when used alone, but organogenesis was observed when callus was transferred to medium containing BA and NAA [103]. Groach et al. Groach et al. [41] studied the effect of various carbon sources (sucrose, table sugar, sugarcane juice) and gelling agents (agar, sago powder) and found that sugar cane juice was a better carbon source than sucrose for axillary shoot multiplication of *V. negundo*.

Additives such as sodium sulphate, silver nitrate and phloroglucinol were used for tissue culture studies of *V. negundo* [24,85]. Silver nitrate is frequently used in *in vitro* plant propagation as it is a well-known ethylene inhibitor [55]. The addition of amino acids can improve the *in vitro* culture of many plants [105]. We conducted an experiment that employed nodal explants of *V. negundo* that were inoculated on MS medium supplemented with 4.44 μM BA, 41.28 μM kanamycin, 59.81 μM penicillin, and 34.39 μM streptomycin. When this medium, or medium containing 4.44 μM BA and 57.41 μM arginine, 68.43 μM glutamine or 86.86 μM proline, was used, shoot growth improved (Fig. 1A–D). Therefore, the effects of various additives on the morphogenic responses of different explants of *Vitex* species need to be explored. For example, Dadjo et al. Dadjo et al. [29] found that the number of somatic embryos induced from the leaves of *V. doniana* could be increased when tryptophan was added to MS medium.

Successful rooting of *in vitro*-raised shoots prior to their *ex vitro* establishment is an important aspect of plant tissue culture that ultimately renders a protocol efficient, or not. Rooting of shoots can be performed both under *in vitro* and *ex vitro* conditions, although the former is most common. *In vitro* rooting of most *Vitex* species was carried out using a basal medium (half-strength MS, Nitsch or WPM) with a low salt concentration in combination with an auxin [indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or NAA, alone, or in combination (Table 2)]. Phloroglucinol can induce a wide range of organogenic responses in *in vitro* plants, the most common of which is associated with improved rooting [91]. *Ex vitro* rooting of *in vitro*-raised shoots was performed by dipping the cut ends of *V. negundo* shoots in 1466–2442 μM IBA [7,8,85,78].

Several studies did not complete an acclimatization stage while others did not assess the success (plantlet survival) of acclimatization (Table 2). In general, well-rooted plantlets are washed under running tap water to remove adhering agar from the roots and transferred to a suitable substrate and plantlets in containers that are held under high humidity, followed by conditions [18,40] which differ widely depending on the species (Table 2).

5. Synthetic seeds

The use of synthetic seeds (synseeds) to transport or preserve important germplasm, or even to serve as a module for cryopreservation is well established in many plants [82,54]. Only two reports are available on synseed production in *Vitex* species.

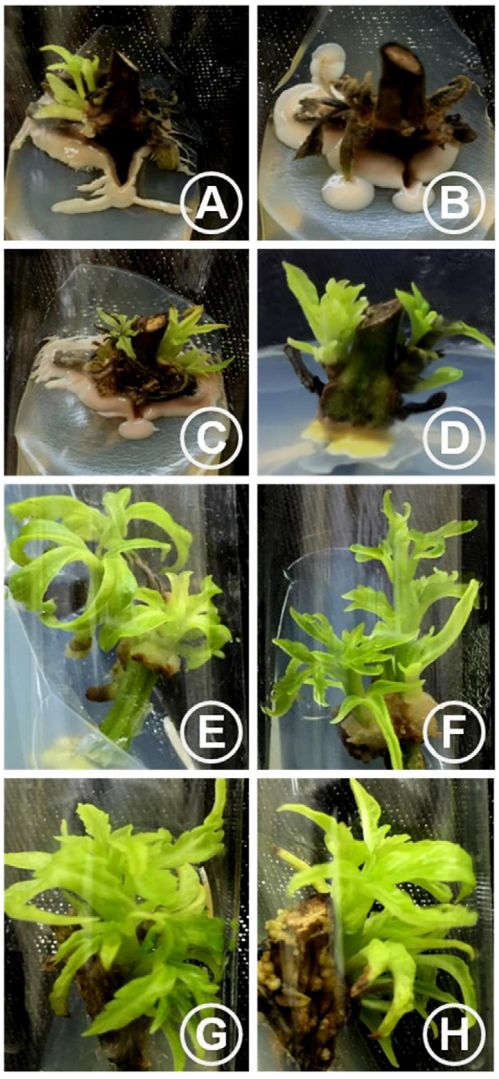

Figure 1 Endophytic contamination during *in vitro* culture of *Vitex negundo*. (A–D) Nodal explants of *V. negundo* after 15 days of culture. A. Surface disinfection with 0.1% $HgCl_2$ for 3 min followed by a rinse with sterilized distilled water (SDW) three times. (B) Surface disinfection with 70% ethanol for 2 min followed by treatment with 0.1% $HgCl_2$ for 3 min followed by a rinse with SDW three times. (C) Surface disinfection with 0.1% $HgCl_2$ for 3 min followed by disinfection with 70% ethanol for 2 min followed by a rinse with SDW three times. (D) Surface disinfection with 70% ethanol for 2 min followed by a rinse with SDW three times. (E–H) Axillary shoot multiplication of *V. negundo* on MS medium supplemented with 41.28 μM kanamycin, 59.81 μM penicillin, and 34.39 μM streptomycin following disinfection with 70% ethanol for 2 min and three washes with SDW. (E) 4.44 μM BA. F. 4.44 μM BA with 57.41 μM arginine. (G) 4.44 μM BA with 68.43 μM glutamine. (H) 4.44 μM BA with 86.86 μM proline. Culture conditions in all cases (culture period 45 days, growth temperature 25 °C, photoperiod 16-h, light intensity 35 μmol m^{-2} s^{-1}).

Optimum synseeds were produced when *V. negundo* nodal explants were encapsulated in 3% sodium alginate (Na-alginate) with 100 mM calcium chloride ($CaCl_2$). MS medium containing 2.5 μM kinetin (Kn) in combination with 1.0 μM α-naphthaleneacetic acid (NAA) resulted 92.6% conversion to plantlets *in vitro* [4]. More recently, Ahmed et al. Ahmed et al. [13] reported 92% survival of *V. trifolia* nodal segments encapsulated according to the Ahmad and Anis [4] method. This indicates that 3% Na-alginate with 100 mM $CaCl_2$ could be used to develop synthetic seeds of other *Vitex* species in the future. Encapsulated nodal segments could be stored at 4 °C up to 8 weeks with 42.5% regeneration efficiency and plantlets, which rooted best on full-strength MS medium containing 0.5 μM NAA, were successfully acclimatized (92%) to field conditions [13].

6. *In vitro* flowering

One of the most captivating events in the lifecycle of a flowering plant is the shift from the vegetative phase to the reproductive phase, a complex developmental shift that can be determined by several factors. Under natural conditions, flowering duration varies widely, at least in *V. rotundifolia* and *V. agnus-castus* [43]. Flowers that are induced *in vitro* are an ideal source of explants for the production of haploids since the chance of contamination is very low and since flower induction can take place independent of the season [95,97]. *In vitro* flowering is also a useful strategy to investigate flowering physiology. Only two studies have examined *in vitro* flowering in *V. negundo* [107,104]: details of flowering conditions are described in Table 2). Data about *in vitro* flowering are not available in Thiruvengadam and Jayabalan [104]. Ahmad et al. Vadawale et al. [107] studied different C/N ratios (they increased the C/N ratio by increasing the sucrose concentration and maintaining the concentration of NH_4NO_3 at 20.6 μM) and observed that the addition of 146.06 mM sucrose (C/N ratio = 7.08) resulted in *in vitro* flowering of 80% of cultures within 24 days on MS medium supplemented with 4.44 μM BA and 0.53 μM NAA. In that study, 90% of plants formed an inflorescence with an average of 5.1 flowers per plant [107]. Only one report is available on *in vitro* flowering, in *V. trifolia* [64], but details about *in vitro* flowering induction, specific culture conditions or data were not reported. This leaves a wide scope for plant physiologists and biotechnologist to explore this technique for other *Vitex* species.

7. Somatic embryogenesis

Somatic embryogenesis is the process by which somatic cells differentiate into somatic embryos. Somatic embryos, which morphologically resemble zygotic embryos, are used as a model system in embryological studies [58]. However, the greatest importance of somatic embryos, in medicinal plant biotechnology, is their practical application in large-scale vegetative propagation; in some cases, somatic embryogenesis is favoured over other methods of vegetative propagation because of the possibility of scaling up propagation by using bioreactors while somatic embryos or embryogenic cultures can be cryopreserved, allowing gene banks to be established while embryogenic cultures are also an attractive target for genetic modification [61]. Dadjo et al. Dadjo et al. [29] induced somatic embryos from leaf explants of *V. doniana* on ½ MS medium supplemented with 0.50 μM thidiazuron, 5.55 μM *myo*-inositol, and 49.74 μM silver nitrate or 6.25 μM tryptophan, but the conversion of somatic embryos to plantlets

and field transfer and survival were not reported. Therefore, an efficient protocol should be developed for the propagation of *V. doniana* and other unexplored *Vitex* species using somatic embryogenesis.

8. Production of secondary metabolites

The medicinal properties of medicinal plants are derived from the presence of single or multiple secondary metabolites [39]. *V. glabrata* produces sterols like 7-dehydrocholesterol (7-DHC), α-ecdysone and 20-HES [110]. 20-HES has multiple uses, including as a growth stimulator for shrimp culture [21], an insecticide [33], an anabolic steroid in sports and body-building, and as a tonic supplement for male and female reproductive systems [34]. Ten-year-old callus induced from stem explants (the exact explant source, i.e., node or internode, was not indicated) used [84] of *V. glabrata* (as suggested in Thavornnithi [99]; culture conditions described in Table 2) to study the effects of cholesterol, 7-DHC, and ergosterol fed to callus cultures for 0, 12, 24, 72, 96 and 120 h. They found that cholesterol did not increase 20-HES content but instead inhibited cell growth while 7-DHC and ergosterol increased 20-HES production 1.36-fold more than the control without affecting cell growth. Sinlaparaya et al. Sinlaparaya et al. [83], using the same callus as in two other studies [84,99], tested ½ MS, MS, ½ B5 and B_5 medium (syn. Gamborg medium [38] supplemented with 8.88 µM BA and 9.04 µM 2,4-dichlorophenoxyacetic acid (2,4-D) for 1 to 5 weeks (detailed culture conditions in Tables 1 and 2). They found that highest cell dry weight (DW) (12.1 g/L) and maximum production of 20-HES (0.038% DW) was possible in B_5 medium in the third week. Thanonkeo et al. Thanonkeo et al. [98] used the same explant as that used in two other studies [83,84], namely callus from a 10-year-old *V. glabrata* culture, to test basal media, temperatures and sucrose concentrations (MS, ½ MS, B_5 and ½ B_5; 25 vs 30 °C; 20, 30, 40 g/L sucrose) for 0–12 days at 2-day intervals. Thanonkeo et al. Thanonkeo et al. [98] also observed that when cells were cultured in suspension cultures at 30 °C on B_5 or ½ MS medium with 30 and 40 g/L sucrose, the production of 20-HES increased 1.09-fold more than the control. Feeding cholesterol at 5 mg/L as a precursor to biosynthesize 20-HES accumulated 1.11-fold more 20-HES than control cells. In another study [23], callus cultures were initiated using the same explants as in the three previous reports. Elicitation with chitosan at 50 mg/L resulted in 17.16 g/L biomass and 377.09 mg/100 g DW 20-HES, which were 1.62 and 8.33 times higher than the control cultures, respectively. Likewise, the addition of methyl jasmonate at 100 µM also enhanced growth and production of 20-HES. The highest growth and 20-HES production reached 14.44 g/L and 621.76 mg/100 g DW, which were 1.35- and 14.54-fold higher than the control cultures [23]. Noel and Darit [67] isolated triterpenes from callus cultures derived from leaf explants of *V. negundo* but triterpenes were not quantified nor was the method used to induce callus described.

9. Molecular markers to detection somaclonal variation

Molecular markers play an important role in studies related to genomics, evolution, and phylogeny of medicinal plants [42]. Only three reports (Table 2) are available on the use of molecular markers in the *Vitex* genus, specifically in *V. negundo* and *V. trifolia*, primarily to verify the homogeneity of *in vitro*-derived plant material. Ahmad and Anis [5] isolated DNA from micropropagated and field-grown plants (plant parts and other conditions were not reported) by the Doyle and Doyle [36] method and used random amplified polymorphic DNA (RAPD) markers (10 GC-rich decamer primers, OPA1-10) to verify the genetic fidelity of two-year-old micropropagated *V. negundo* plants versus the mother plant (control), and detected no genetic variation. Samantaray et al. Samantaray et al. [81] isolated DNA from fresh leaves of micropropagated and a field-grown mother plant by the cetyltrimethyl ammonium bromide (CTAB) method [20] with minor modifications; 1% polyvinylpyrrolidone (PVP) was added to remove polyphenols and RAPD and inter simple sequence repeats (ISSR) were used to test the genetic fidelity of *V. trifolia*; they found no genetic variation relative to the mother plant. Ahmad et al. Ahmad et al. [8] also carried out RAPD analysis of *V. trifolia* plantlets developed from axillary shoots and extracted DNA from young leaves using the [36] protocol. No phenotypic variation was observed in micropropagated plants of *V. trifolia* [81,8] and *V. negundo* [5]. A wider selection of molecular markers could assist in the discrimination of adulterants from pure sources, and to screen out somaclonal variants derived from bioreactors, synthetic seeds, or cryopreservation.

10. Conclusions and future perspectives

Even though there are an estimated 707 plant species known as *Vitex* sp. worldwide, 230 species have a taxonomically accepted name, 455 names are synonyms and 22 names are unresolved [74]. Protocols for the *in vitro* propagation of only six species have been established (Table 2), and most have been dedicated to *V. negundo* (27 articles from a total of 46 papers dedicated to the genus) and *V. trifolia* (9/46). The only four reports that exist for *V. glabrata* are related to the production of secondary metabolites [83,84,23,98] but a micropropagation protocol is not available for this species yet. Despite these studies, ironically, none of these studies was performed on the 15 *Vitex* species listed on the IUCN Red Data list [46]. Seven species are vulnerable (*V. acunae*, *V. ajugaeflora*, *V. amamiensis*, *V. keniensis*, *V. parviflora*, *V. urceolata* and *V. zanzibarensis*), five are endangered (*V. cooperi*, *V. evoluta*, *V. gaumeri*, *V. kuylenii*, *V. lehmbachii*), one is critically endangered (*V. yaundensis*), one is of low risk and least concern (*V. longisepala*) and data are deficient about *V. heptaphylla* IUCN Red Data list [46]. It is essential to provide useful, centralized and detailed information about protocols and *in vitro* experimental conditions that could urgently be applied to the remaining species, including the endangered ones, to establish *in vitro* propagation protocols suitable for clonal propagation, cryopreservation and genetic transformation. Photoautotrophic micropropagation, the use of bioreactors, or the use of thin cell layers [88], sonication and ultrasound [89], or magnetic fields [90] to alter or improve tissue growth *in vitro* are all aspects that merit investigation in *Vitex* species. Recent progress was made in the induction of *V. agnus-castus* polyploids using 0.05% colchicine while irradiation with 50 Gy of γ-rays

resulted in a single-stemmed plant type [14], emphasizing the importance of mutation technology as an applied breeding technique to induce variation.

This implies that the use of biotechnology for this genus is still at a nascent phase of development, and that there is still ample room for future exploration of the great majority of *Vitex* species. For example, the use of molecular techniques such as RFLP to authenticate *V. glabrata* [73], or the examination of explants for fungal or bacterial contaminants, e.g., in *V. trifolia* [113], prior to *in vitro* culture, indicate that biotechnology has pure and applied applications for *Vitex* research. Given the economic importance of these species, the *in vitro* protocols outlined in this review provide a platform for pure and applied studies to be conducted, including the use of bioreactors for mass production of important secondary metabolites or compounds of pharmaceutical and medicinal importance.

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