Plant Cell Rep (2016) 35:2207–2225
DOI 10.1007/s00299-016-2041-8

REVIEW

Micropropagation and genetic transformation of *Tylophora indica* (Burm. f.) Merr.: a review

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Received: 19 June 2016 / Accepted: 15 August 2016 / Published online: 23 August 2016

Abstract
Key message **This review provides an in-depth and comprehensive overview of the in vitro culture of *Tylophora* species, which have medicinal properties.**
Abstract Tylophora indica (Burm. f.) Merr. is a climbing perennial vine with medicinal properties. The tissue culture and genetic transformation of *T. indica*, which has been extensively studied, is reviewed. Micropropagation using nodal explants has been reported in 25 % of all publications. Leaf explants from field-grown plants has been the explant of choice of independent research groups, which reported direct and callus-mediated organogenesis as well as callus-mediated somatic embryogenesis. Protoplast-mediated regeneration and callus-mediated shoot organogenesis has also been reported from stem explants, and to a lesser degree from root explants of micropropagated plants in vitro. Recent studies that used HPLC confirmed the potential of micropropagated plants to synthesize the major *T. indica* alkaloid tylophorine prior to and after transfer to field conditions. The genetic integrity of callus-regenerated plants was confirmed by RAPD in a few reports. Tissue culture is an essential base for genetic transformation studies. Hairy roots and transgenic *T. indica* plants have been shown to accumulate tylophorine suggesting that in vitro biology and transgenic methods are viable ways of clonally producing valuable germplasm and mass producing compounds of commercial value. Further studies that investigate the factors affecting the biosynthesis of *Tylophora* alkaloids and other secondary metabolites need to be conducted using non-transformed as well as transformed cell and organ cultures.

Keywords Asclepiadaceae · In vitro · Micropropagation · Morphogenesis · Somatic embryogenesis · Tissue culture · Transformed cultures

Introduction

There are currently 90 *Tylophora* species with accepted names, and a multitude of synonyms and species whose nomenclature is being revised (The Plant List 2016). *Tylophora indica* (Burm. f.) Merr. (syn *Tylophora indica* var. *glabra* (Decne.) H. Huber) (The Plant List 2016), of the Asclepiadaceae family and commonly known as Indian ipecacuanha in English, or as Antamul in India (see list of vernacular names in Table 1), is a climbing perennial vine. Kirtikar and Basu (1991) and Schmelzer and Gurib-Fakim (2013) offer a botanical description of this medicinal plant, and indicate that *T. indica* is a perennial climber that can reach up to 1.5–3 m in length, forms short stocky rhizomes (3–4 mm thick) and fibrous roots. The simple opposite leaves, which can be 2–10 cm long, have margins that can be entire, ovate or orbicular. Many green-yellow flowers (outside) with a purple inside form on an axillary umbel-like cyme, which is the inflorescence. The fruit is 5–10 cm long with many 2–2.5 cm long seeds. The plant is usually propagated by seed collected from plants in the wild and

Communicated by N. Stewart.

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Table 1 Vernacular names for *Tylophora indica*

Language/dialect	Common/vernacular name
Bengali	*Antamul*
Gujarati	*Damnivel*
Hindi	*Antamul*, *jangli pikvam*
Kannada	*Adumutadhagida*, *nipaladaberu*
Marathi	*Khodiki*, *pittakhadi*, *rasna*
Oriya	*Mendi*, *mulini*
Sanskrit	*Arkaparni*, *shwasaghni*
Tamil	*Asthma kodi*, *nanjaruppan*, *nancaruppan*, *naippalai*, *mirkkurinja*, *tellavidavela*, *kondachani*
Telugu	*Kakapala*, *verripala*

Modifed from Shahzad et al. (2016), and also based on Dhandapani and Balu (2002)

vegetative propagation is poorly explored (Dhandapani and Balu 2002). However, more recently, Mehandru et al. (2014) used stem cuttings of *T. indica* from field-grown plants for clonal propagation using an aeroponic system and found that 100 % of stem cuttings rooted with 2 g/l indole-3-butyric acid (IBA), performing better than cuttings rooted directly in soil (77 % of cuttings while only 6.65 % of control cuttings rooted in the absence of an auxin).

Tylophora indica has numerous medicinal properties, including antioxidant, antiallergic, anti-angiogenic, antibacterial, anticancer, antifeedant, anti-inflammatory, antimicrobial, antitumor, antiasthmatic, cardioprotective, diuretic, hepatoprotective, and displaying immunomodulatory activity, all of which have been recently reviewed by Shahzad et al. (2016) and will thus not be included in this review. Overexploitation and the lack of organized cultivation strategies underscore the importance of developing biotechnological approaches for the rapid and reproducible in vitro propagation of this medicinal plant species and the stable and improved in vitro and *in planta* production of its valuable pharmaceuticals that endow it with these widely reported medicinal properties.

Two *Tylophora* species are on the Red List of the International Union of Conservation of Nature, *Tylophora cameroonica* N.E.Br., listed as near threatened, and *Tylophora urceolata* Meve, listed as vulnerable International Union for Conservation of Nature and Natural Resources (IUCN 2015). Other than these two species, currently the use of biotechnology is not a tool for preservation of rare or endangered material, but rather a tool for mass propagation of clonal material, or as a stable base for creating a sterile in vitro *milieu* to engage in other applied biotechnologies for improvement of medicinal plants such as tissue culture (Yoshimatsu 2008), somatic hybridization (Murch and Saxena 2001), germplasm cryopreservation (Dixit et al. 2004), genetic transformation (Bajaj and Ishimaru 1999; Roychowdhury et al. 2013b), synthetic seed production (Sharma et al. 2013), or bioreactor production of secondary metabolites (Baque et al. 2012). This review highlights the advances that have been made thus far in the tissue culture of *Tylophora* species, although the analysis in Table 2 reveals that the majority (63/65 studies, or 97 %) of studies have focused on *T. indica* with only a single study on *T. ovata* (Lindl.) Hook. ex Steud. (syn. *T. ovata* var. *balansae* (Costantin) Tsiang, *T. ovata* var. *brownii* (Hayata) Tsiang & P.T. Li, and *T. ovata* var. *ovata*), by Jeyachandran and Bastin (2014) and one study on *T. subramanii* Henry (Murukan et al. 2015).

Morphogenesis and propagation of *Tylophora indica* in vitro

The explant is the central unit of plant tissue culture, and its choice depends on seasonal availability or quality of mother plant material, on the experimental objective, and on its responsiveness in vitro. The most popular explant used for the tissue culture of *Tylophora* species has been leaves (Fig. 1a) from field-grown and micropropagated plants.

T. indica has been extensively studied since 1970 when Rao et al. (1970) reported the induction of callus from stem explants which differentiated into roots, shoots and bipolar somatic embryos (SEs). Rao et al. (1970) also demonstrated SE development in a cell suspension culture while the histological basis of morphogenesis was described by Rao and Narayanswami (1972). The morphogenetic potential of dedifferentiated cells of *T. indica* in vitro was demonstrated using various types of explants, namely leaves (50 % of reports, Table 2; Fig. 1a), internodes or stems, petioles and roots. Micropropagation based on the use of shoot tips or nodal explants involving apical or axillary bud proliferation has been the method of choice in 25 % of published reports (Table 2).

Use of explants from ex situ plants

Leaves from ex situ plants have been the primary source for callus induction and indirect shoot regeneration from dedifferentiated callus, with only few reports on somatic embryogenesis, most of which have not been substantiated by suitable histological analyses.

The earliest report of direct shoot organogenesis from mature leaves of field-grown plants was by Bera and Roy (1993), inducing as many as 304 shoot buds/explant in optimized medium but no histology was performed. Manjula et al. (2000) induced embryogenic callus from mature

Table 2 Micropropagation and tissue culture of *Tylophora indica*, except for *Tylophora ovata* and *Tylophora subramanii* (chronological listing)

Explant conditions and disinfection procedures	Culture medium, PGRs and additives*,**	Culture conditions***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Young vines → 10 % NaOCl → SW → 5 mm explants	MS + 1 mg/l 2,4-D + 1 mg/l 2-BTOA (CIM). MS + 2 mg/l 2,4-D + 10 % CM + 200 mg/l CH + 100 mg/l *myo*-inositol (CPM). 2 % sucrose. pH, agar conc. NR	Continuous light. CWFT. LI NR. 25 ± 2 °C. 50–60 % RH	Shoots and somatic embryos formed	Rao et al. (1970)
Young vines → 10 % NaOCl → SW → 5 mm internodes = explants	White's + 1 mg/l 2,4-D + 1 mg/l 2-BTOA (CIM). pH 5.8. 2 % sucrose. Agar conc. NR	Continuous light. CWFT. LI NR. 25 ± 2 °C. 50–60 % RH	Callus developed within 4 w. Histology of shoots and somatic embryos described	Rao and Nayaranaswami (1972)
Stem from stem cuttings and roots from germinated seedlings	MS + 2 mg/l 2,4-D + 10 % CM + 200 mg/l CH + 5 mg/l AdS (CIM). Other conditions NR	Continuous light. Light source and LI NR. 25 ± 2 °C. 50–60 % RH	In the 1973 study, the addition of phenylalanine—an alkaloid precursor—to CIM did not change the level of steroids and alkaloids. In the 1976 study, the irradiation of callus with gamma rays decreased the content of β-amyrin, β-sitosterol, stigmasterol and campesterol	Benjamin and Mulchandani (1973, 1976)
Leaf, stem and root segments of field-grown plants (age and disinfection procedures NR)	MS + 2 mg/l 2,4-D + 0.2 mg/l Kin (CIM for 3 explants). MS + 2 mg/l NAA + 0.2 mg/l Kin (SIM for stem and roots). MS + 2 mg/l IAA (RIM). pH, carbohydrate source, agar conc. NR	PP and LI NR. CWFT. 25 ± 2 °C	Callus was induced within 2 w, shoot buds after another 3–4 w, and complete plantlets 4–6 w thereafter. Alkaloids could not be synthesized in in vitro callus cultures, even in the presence of alkaloid precursors. Tissue cultured plants showed a similar alkaloid profile as normal field-grown plants	Benjamin et al. 1979
Stems (ca. 5 mm long) of field-grown plants → 0.1 % $HgCl_2$ 10 min → 4× SDW	MS + 2 mg/l 2,4-D + 0.2 mg/l Kin (CIM). MS + low auxin + high cytokinin (shoot buds). MS + 10 % CM + 1 mg/l 2,4-D (SEIM). pH 5.5–5.8	Continuous light. Light source NR. 1000 lx. 25 ± 2 °C	Stem segments produced callus in 4 w. Protoplasts isolated from callus when incubated for 7 h in light 2 % cellulose + 1 % macerozyme + 1 % hemicellulose + 0.6 M mannitol. Embyoids and shoot buds developed from callus. Regeneration of whole plants was possible	Mhatre et al. (1984)
Nodes (1–1.5 cm) of ex situ plants → Teepol™ 15 min while stirring → RTW 2 h → 0.1 % $HgCl_2$ 7 min → SDW	MS + 5 mg/l BA + 0.5 mg/l NAA + 100 mg/l AA (SIM, SMM; 4–6 w subcultures). MS + 1 mg/l IAA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 2800–3000 lx. 25 ± 2 °C. 60 % RH	Axillary shoots did not develop in the presence of BA, Kin, 2iP, NAA, IAA, 2,4-D or GA_3 alone. Callus formed when 2,4-D (1-5 mg/l) and NAA (1–2 mg/l) were used in combination. In SIM, axillary buds sprouted within 10 d from 60 % of explants. Subculture on SIM formed 5.38 shoots/explant and 92 % of new shoots formed roots on RIM within 4 w. 90-100 % survival in Soilrite™ (Keltech Energies Ltd., Bangalore, India)	Sharma and Chandel (1992)

Table 2 continued

Explant conditions and disinfection procedures	Culture medium, PGRs and additives*,**	Culture conditions***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Leaves (2.5-3 cm long) of ex situ plants (age NR) → 70 % EtOH 1 min → 0.1 % $HgCl_2$ + 5 % Teepol 10–12 min (continuous shaking) → SW → whole or cut leaves = explants	MS + 5 mg/l BA + 0.5 mg/l AdS (SIM, SMM). MS + 3 mg/l IAA (RIM). pH 5.8. 3 % sucrose. 0.6 % agar	16-h PP. CWFL. 35.7 μE cm^{-2} s^{-1}. 25 ± 1 °C. 55–60 % RH	Direct shoot bud induction at apical and basal part of whole leaf or cut surfaces. Prolific shoot bud induction (304/explant). Shoot multiplication every 4-6 w. Plantlets transferred to soil in pots with 60-72 % survival	Bera and Roy (1993)
Mature leaves (6-m-old greenhouse plants) → RTW → 1 % Labolene™ → DW → 0.1 % $HgCl_2$ 5 min → SDW → 1 cm^2 explants	MS + 1-2 mg/l BA or 3–5 mg/l Kin or 2 mg/l Kin + 0.1 mg/l IAA (CIM; 30-d subcultures). MS + 2 mg/l Kin + 0.1 mg/l IAA (SIM/SEM/SEIM). pH 5.8. 3 % sucrose. 0.8 % agar	Continuous light. CWFT. 40 μmol m^{-2} s^{-1}. 25 ± 2 °C	30 embryoids/100 mg callus formed, with 27 % conversion into plantlets, which could be acclimatized in sand: soil (1:1) with 90 % survival	Manjula et al. (2000)
Mature leaves (greenhouse plant) → 1 % Teepol™ → RTW → 0.1 % $HgCl_2$ 5 min → 5× SDDW → 0.5 cm^2 explants	MS + 2 mg/l 2,4-D + 0.01 mg/l Kin (CIM; 14-d subcultures). MS + 2 mg/l 2iP (SEIM). PGR-free MS (SEGM). pH 5.8. 3 % sucrose. 0.2 % phytagel	Continuous dark (CIM) or 10-h PP + CWFT + 50 μmol m^{-2} s^{-1} (SEIM). 25 ± 1 °C	25 SEs/initial callus cluster (0.5 g) in 85 % of callus. No variation claimed using RAPD	Jayanthi and Mandal (2001)
First and second expanded leaves from terminal 1 cm → TW 30 min → 5 % Teepol™ 5 min → 0.1 % $HgCl_2$ 3 min → 4× SDW → 1 cm^2 explants	MS + 10 μM 2,4,5-T or 10 μM 2,4-D (CIM). MS + 5 μM Kin (SIM). ½ MS + 0.5 μM IBA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. Light source NR. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C	96 % (2,4-D) or 100 % (2,4,5-T) of leaf explants formed callus. 64.8 shoots/callus clump formed on SIM, with 85 % of callus explants being responsive. In RIM, 90 % of shoots rooted, forming 4.3 roots/shoot. Plantlets acclimatized in sterilized vermiculite, watered with ½ MS every 3 days for 2 w, then transferred to pots with garden soil (survival % NR)	Faisal and Anis (2003)
Young shoots (May–June growth of 15-y-old trees) → 5 % Teepol™ 10 min → RTW 30 min → DW → 0.1 % $HgCl_2$ 18-20 min → 4–5× SDW → shoot tips (10 mm) = explants. Roots of micropropagated plants used as explants	MS (initial shoot induction). MS + 26.8 μM BA (SIM). MS + 28.54 μM IAA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 48 μmol m^{-2} s^{-1}. 24 ± 1 °C. 50–60 % RH	Initial shoots formed plantlets that were then used to source two types of root explants: WRS and GRS. 78 % of GRS formed shoots in 24 weeks but 75 % could form shoots in response to 5.36 μM BA within 6 w. 92 % of WRS formed shoots in 12 w. 24.61 μM 2iP in SIM, rather than BA, could also induce shoots in 61 % of GRS in 6 w or in 89 % of WRS in 12 w. 100 % of GRS or WRS (pooled) shoots could form roots on RIM, with 10 roots/shoot after 4 w. 88 % (from SEs) or 96 % (from shoots) survival in soil + sand (1:1)	Chaudhuri et al. (2004, 2005, 2006), Roychowdhury et al. (2013a, 2015a, b)****
Young shoots → RTW 30 min → 5 % Teepol™ 5 min → DW → 0.1 % $HgCl_2$ 3 min → SDW several times → 0.5–1 cm long stem explants	MS + 10 μM 2,4,5-T (CIM). MS + 5 μM Kin (SIM). ½ MS + 0.5 μM IBA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. Light source NR. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C. 60 % RH	100 % of explants formed callus. 45 shoots/callus clump formed on SIM, with 80 % of callus explants being responsive. In RIM, 90 % of shoots rooted, forming 4.3 roots/shoot. Plantlets acclimatized in sterilized vermiculite, watered with ½ MS every 3 days for 2 w, then transferred to pots with garden soil (survival % NR)	Faisal and Anis (2005)

Table 2 continued

Explant conditions and disinfection procedures	Culture medium, PGRs and additives*,**	Culture conditions***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Petioles (3–5 mm long) (age of mother plants NR): same protocol as Faisal and Anis (2005)	MS + 10 μM 2,4-D + 2.5 μM TDZ (CIM; 21-d subcultures). MS + 2.5 μM TDZ (SIM). ½ MS + 0.5 μM IBA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 50 μmol m^{-2} s^{-1}. 24 ± 2 °C. 60 % RH	100 % of explants formed callus. 56 shoots/callus clump formed on SIM, with 90 % of callus explants being responsive. In RIM, 90 % of shoots rooted, forming 4.3 roots/shoot. Plantlets acclimatized in sterilized vermiculite after dipping cut ends of shoots in 150 μM IBA for 30 min, watered with ½ MS every 3 days for 2 w, then transferred to pots with garden soil (100 % survival). Photosynthetic parameters between in vitro plantlets and *ex vitro* acclimatized plants were comparable	Faisal et al. (2005)
15-d-old leaves (outdoor plants) → 70 % EtOH 30 s → 0.1 % $HgCl_2$ + 2 drops Tween-20 5 min → 3× SDDW → 1 cm^2 explants	MS + 7 μM 2,4-D + 1.5 μM BA (CIM). MS + 8 μM TDZ (SIM).½ MS + 3 μM IBA (RIM). pH 5.8. 0.8 % (w/v) agar. Carbohydrate NR	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 24 °C	92 % of explants formed callus. 100 % of callus cultures formed shoots, with 66.7 shoots/callus culture. 92 % of acclimatized plants survived in soil.	Thomas and Philip (2005)
Leaves (1-y-old plants) → DW 2× → 1 % Teepol™ 5 min → 0.1 % $HgCl_2$ 5 min → 4–5× SDDW → 8 × 20 mm explants	MS + 0.5 μM TDZ + 1.5 μM 2,4-D (SEIM). PGR-free MS (SEGM). 2 % Na-alginate → 5 % Ca-alginate (SE synseed). pH 5.7. 3 % sucrose. 0.8 % agar	16-h PP. CWFT. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C.	18.2 globular SEs/explant. 65 % of cultures formed plantlets. 90 % of SEs germinated. 65-70 % survival after acclimatization in garden soil + sand (1:1). No morphological variation claimed	Chandrasekhar et al. (2006)
Internodes (outdoor plants, age NR) → 1 % Savlon (detergent) 10 min → SDDW → 0.1 % $HgCl_2$ 5 min → 3× SDDW → 10 mm explants	MS + 4 μM 2,4-D (SEIM). MS + 6 μM Kin, 10 μM GA_3 or μM ABA (SEGM). pH 5.8. 200 mM sucrose. 0.8 % (w/v) agar	16-h PP. CWFT. LI NR. 24 °C	71 % of callus formed SEs (49 SEs/g callus). 94 % of acclimatized plants survived (substrate NR)	Thomas (2006)
Young shoots from 2-y-old plant → RTW 30 min → 5 % Labolene™ 5 min → 3× SDW → 0.1 % $HgCl_2$ 3 min → 4× SDW → stem segments with nodes (3-5 mm long) = explants	MS + 2.5 μM BA + 0.5 μM NAA (SIM). ½ MS + 0.5 μM IBA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 50 μmol m^{-2} s^{-1}. 24 ± 2 °C. 60 % RH	Synthetic seeds (encapsulated nodes) were produced with 3 % N alginate and complexed with 100 mM $CaCl_2 \cdot 2H_2O$ for 30 min. 72 % of beads converted into plantlets when plated immediately, or only 50.3 % when stored for 8 w at 4 °C. 43 % of synseeds germinated directly on sterilized Soilrite™ moistened with ½ MS after 6 w. After an initial transfer for 1 m to Soilrite™, 90 % of germinated plantlets survived in soil	Faisal and Anis (2007)
Same explants and protocol as Faisal and Anis (2007)	MS + 2.5 μM BA + 0.5 μM NAA + 100 mg/l AA (SIM). ½ MS + 0.5 μM IBA (RIM). Subcultures on all media every 2 w. pH 5.8. 3 % sucrose. 0.8 % agar (0.25 % gelrite for RIM)	16-h PP. CWFL. 50 μmol m^{-2} s^{-1}. 24 ± 2 °C. 60 % RH	On SIM, 93 % of nodal segments formed shoots (8.6/explant). explants being responsive. In RIM, 90 % of shoots rooted, forming 4.5 roots/shoot. 90 % of plantlets acclimatized in sterilized vermiculite survived. Acclimatized plants showed higher SOD (and other antioxidant) levels when placed at a higher light irradiance (300 μmol m^{-2} s^{-1})	Faisal et al. (2007), Faisal and Anis (2010)

Table 2 continued

Explant conditions and disinfection procedures	Culture medium, PGRs and additives*,**	Culture conditions***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Leaves (10-y-old plant) → 5 % Teepol™ 10 min → RTW → 70 % EtOH 2 min → 0.1 % $HgCl_2$ 2 min → SDDW → 1 cm^2 explants	MS + 2 mg/l 2,4-D + 0.5 mg/l Kin + 200 mg/l CH (CIM, CPM). MS + 1 mg/l Kin + 0.5 mg/l IAA (SIM). 4-w subcultures. pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. Light source NR. 2000 lx. 26 ± 2 °C	100 % of explants formed callus in 7 d and 35 shoots/callus cluster in 15–20 d. The level of amino acids increased in callus between day 10 and 20 (assessed by SDS-PAGE)	Singh et al. (2009, 2010)
Leaves from 2nd node (greenhouse plants, age NR) → 20 % NaOCl + 2 drops Tween-20 20 min → 5× SDW → leaf tissue without lower epidermis and midrib → protoplast isolation	Liquid MS + 0.4 M mannitol + 3 % sucrose + 2–6 µM 2,4-D (PCM). 0.4 ml of PCM with 0.2 M mannitol added each week. PCM – mannitol (SIM). MS + 5 µM TDZ + 0.4 µM NAA (SELM). ½ MS + 3 µM IBA (RIM). pH 5.8. 0.8 % (w/v) agar	Darkness + 25 °C (callus induction from protoplasts). All other cultures: 200 µmol m^{-2} s^{-1}. Temp., light source, PP NR	10.3 × 105 protoplasts yielded from 1 g of leaf tissue. Protoplasts showed 84 % viability after 4 h, but only 65 % after 24 h. 44.2 shoots formed per callus cluster. 80 % of acclimatized plants survived in soil	Thomas (2009)
Nodal explants (size, age NR) → TW → 0.5 % NaOCl 20 min → 0.01 % $HgCl_2$ 5 min → SDW	MS + 1 mg/l BA (SIM; subcultures NR). MS + 0.05 mg/l IAA (RIM). pH 5.6–5.8. 3 % sucrose. 0.8 % agar	18-h PP. 25 ± 1 °C. Light source, LI, RH NR	Shoots formed from 64 % of nodes and 70.5 % of shoots rooted. 99 % survival of acclimatized plants in cocopeat, soil and sand (ratio NR). Different extracts from in vitro vs in vivo leaves and roots showed weak antimicrobial activity relative to 0.01 g/l tetracycline, the positive control	Gami and Parmar (2010)
Nodal explants (size, age NR) → 5 % Teepol™ 20 min → 0.1 % $HgCl_2$ 5–10 min → 3–5× SDW	MS + 3 mg/l 2,4-D (CIM). MS + 3 mg/l BA (SIM; 3-w subcultures). MS + 2 mg/l IBA + 4 mg/l NAA (RIM). pH, carbohydrate source, agar conc. NR	12-h PP. CWFT. 3000 lx. 25 ± 2 °C. 70 % RH	Explant swelling after 6–8 d on CIM, and callus formation from cut ends. 80 % of explants formed callus. 80 % of explants formed shoots directly and 90 % of shoots induced roots in RIM. Chlorophyll, carbohydrate, protein and lipid content not different between in vivo and in vitro regenerants. Acclimatization not performed.	Kaushik et al. (2010)
Nodes (5-y-old plant) collected from Sept. to Nov. → 5 % Teepol™ 10 min → RTW 30 min → DW → 0.1 % $HgCl_2$ 10 min → 4–5× SDW (explant size NR)	MS + 2 mg/l BA + 0.2 mg/l GA_3 + 100 mg/l *myo*-inositol (SIM; 4-w subcultures). MS + 0.5 mg/l IBA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 48 µmol m^{-2} s^{-1}. 24 ± 1 °C. 60–65 % RH	95 % of nodes formed an average of 4.86 shoots/explant. 90 % of shoots rooted, forming 4.55 roots/shoot. 96 % of plantlets could be acclimatized in VC	Rani and Rana (2010)
Leaves (age of mother plant NR) → RTW → 5 % Teepol™ 16–20 min → SDW → 70 % EtOH 30 s → 0.1 % $HgCl_2$ 7 min → 3–4× SDDW → 0.5 cm^2 explants	MS + 2.5 µM BA + 3.5 µM 2,4-D (CIM). MS + 1 µM Kin (SIM). PGR-free ½ MS (RIM). Subcultures every 35 d. pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 60 µmol m^{-2} s^{-1}. 25 ± 2 °C	80–90 % of explants callused. 4.6 shoots/explant on SIM. 90 % of shoots rooted. 62 % acclimatization in sterilized peat soil and sand (1:1)	Rathinavel and Sellathurai (2010)

Table 2 continued

Explant conditions and disinfection procedures	Culture medium, PGRs and additives*,**	Culture conditions***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Leaves (5-y-old plant) → RTW 30 min → 5 % Teepol^{TM} 15 min → RTW → 0.1 % $HgCl_2$ 3 min → 3–4× SDW → 1 cm^2 explants (or 1.5 cm long green root segments: 2010b)	MS + 5 µM 2,4-D (CIM; Khatoon et al. 2013). MS + 5 µM BA (CIM; all other 3 studies). MS + 2.5 µM TDZ (CIM; 2010a). MS + 5 µM BA (SIM). ½ MS + 0.5 mg/l IBA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 40 µmol m^{-2} s^{-1}. 25 ± 2 °C. 70 % RH	Number of shoot buds/culture: 23.4 (BA) or 26.8 (TDZ) (2010a), 46.8 (2010b). Number of roots/shoot: 5.8 (2010a), 6.4 (2010b). 5–10 mg/l NAA also effective for root formation (2010a, 2010b). 90 % survival of acclimatized plants in soil + garden manure (1:1) (2010a), or vermiculite (2010b). Antibacterial (Jahan et al. 2013) and antifungal activity (Khatoon et al. 2013) claimed from 70 % ethanolic extract of leaves and callus.	Sahai et al. (2010a, b), Jahan et al. (2013), Khatoon et al. (2013)
Leaves (1-y-old plant) → RTW 15–20 min → 1 % Teepol^{TM} 2–4 min → 3–4× SDW → 0.1 % $HgCl_2$ 2–3 min → rinses and explant size NR	MS + 2 mg/l BA + 0.5 mg/l IBA (CIM). MS + 0.1 mg/l TDZ (SIM). ½ MS + 0.5 mg/l IBA (RIM). pH 5.8. Subcultures, carbon source, and gelling agent NR	16-h PP. CWFL. 2500 ± 500 lx. 26 ± 2 °C. 55 ± 5 % RH	95 % of leaves formed callus and 15.22 shoots/explant. 85 % of shoots rooted with 7.75 roots/shoot within 8 d. Internodes and petioles could also induce callus, but less than leaves. 75 % acclimatization in VC and autoclaved soil (1:3). 93 % genetic similarity shown with ISSR markers	Verma et al. (2010), Sharma et al. (2014)
1 cm leaf segments from in vitro plants	MS + 2 mg/l Kin + 1 mg/l NAA (SEIM, SEGM). pH 5.6. 3 % sucrose	16-h PP. CWFL. 50–60 µmol m^{-2} s^{-1}. 25 °C. 70 % RH	74.3 % of explants formed SEs on SEIM and 77.2 % germinated after subculture every 2 w. Synthetic seeds (encapsulated nodes) were produced with 3 % N alginate and complexed with 50 mM $CaCl_2{\cdot}2H_2O$ for 30 min. 20.2 % of beads germinated and 5.2 % of those converted into plantlets when 30-d-old SEs were used and plated on ½ MS. Acclimatization not performed	Davendra et al. (2011)
Leaves and stems (3-y-old plant) → RTW 30 min → 0.1 % Teepol^{TM} 5 min → TW → 0.1 % Bavistin 10–12 min → 0.1 % $HgCl_2$ 2–4 min → 3–4× SDW → 5–6 cm stem or intact leaves (explants)	MS + 4.65 µM Kin + 29.4 µM NAA (CIM). MS + 8.88 µM BA (SIM). ½ MS or ½ MS + 9.84 µM IBA (RIM). pH 5.8. 2 % sucrose. 0.8–1 % agar	16-h PP. CWFL. 50 µmol m^{-2} s^{-1}. 25 ± 2 °C	Green callus formed within 7–8 d and shoots formed within 6–8 w in about 90 % of cultures (2011a; 85 % from leaves in 2011b). 92 % acclimatization in soil + VC + *Azobacter* (N_2 fixer) + *Pseudomonas* (phosphate solubilizer) (1:1:1:1) (2011b), or 88–90 % acclimatization in soil + VC (1:1) (2011a, 2011b). HPTCL detected 80, 28.3 and 24.5 µg/ml tylophorine in the leaves of in vitro plants, suspension cultures and callus, respectively	Kaur et al. (2011a, b, 2014), Anand et al. (2012)
Internodes (age of mother plant NR) → RTW 30 min → 70 % EtOH 50 s → SDDW → 0.1 % $HgCl_2$ 2 min → 2× SDDW (5 min each)	MS + 3.25 µM BA + 1.5 µM IAA (CIM; SIM). MS + 12.25 µM Kin + 3.75 µM NAA (SIM). MS + 1.25 µM Zea + 2.75 µM IAA (RIM). pH 5.7. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 60 µmol m^{-2} s^{-1}. 26 ± 2 °C. 55–60 % RH	Callus was induced within 8 w. 100 % of explants formed callus, shoots, and roots (organogenesis not quantified). Acclimatization claimed, but not shown.	Mahesh et al. (2011)

Table 2 continued

Explant conditions and disinfection procedures	Culture medium, PGRs and additives*,**	Culture conditions***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Young leaves (plant age NR) → RTW + Teepol^{TM} 10–15 min → 0.1 % $HgCl_2$ 2–3 min → 3× DW → 1 cm^2 explants	MS + 0.2 mg/l BA + 2.0 mg/l NAA (CIM). MS + 1 mg/l BA + 1 mg/l Kin (SIM). 20-d subcultures (CIM, SIM). pH 5.8. 3 % sucrose. 0.8 % agar	Callus 7 d in darkness → light. PP, light source, LI, temp, RH NR	Callus formed after 15 d. 37.5 % band polymorphism in regenerants using RAPD (46 % using ISSR). Rooting and acclimatization not performed	Chaturvedi and Chowdhary (2012)
Young leaves (plant age NR) → TW 2× → 5 % Teepol^{TM} 10 min → TW → 1 % Bavistin (fungicide) 20 min → TW → 70 % alcohol 30 s → 0.12 % $HgCl_2$ 3 min → 3–4× DDSW	MS + 2.5 mg/l BA (CIM; SIM; SMM). MS + 0.5 mg/l IBA (RIM). pH 5.8–5.9. 3 % sucrose. 1 % agar	16-h PP. CWFL. 2000 lx. 25 ± 2 °C	Callus formed in 7 d on 90 % of explants. Shoots developed from callus within 20 d. 95 % survival after acclimatized in soil + sand + compost (1:1:1). Alkaloids tested by TLC	Kalimuthu and Jeyaraman (2012)
Shoot tips, leaves, nodes (plant age NR) → Tween-20 → NaOCl 2 min → 0.5 % $HgCl_2$ 3–4 min → water	MS + 1 mg/l 2,4-D + 1 mg/l Kin (CIM; shoot tips). MS + 1 mg/l 2,4-D + 1 mg/l BA (CIM; leaves, nodes). MS + 3 mg/l BA + 0.5 mg/l IBA (SIM). MS + 1 mg/l IBA (RIM). pH, carbon source, gelling agent NR	No conditions reported	80 % of shoot tips and leaves formed callus (60 % in nodes). 100 % of callus formed shoots and all shots rooted. Acclimatization not performed	Patel and Patel (2012)
Leaves of 1-y-old plant → 2× DW → 1 % Teepol^{TM} 5 min → 0.1 % $HgCl_2$ 5 min → 4–5× SDW → 5 mm^2 explants	MS + 1 mg/l IAA (RIM). pH 5.8. 3 % sucrose. 0.2 % gelrite	Constant darkness. 25 ± 1 °C	Adventitious roots formed within 2 w and profuse roots by 6 w (24.33 roots/explant; 2 mg/l IBA induced 15.33 roots/explant)	Rashmi et al. (2012)
Aged leaves (base of plants) and young leaves (from terminal 5th – 8th nodes) of 2-y-old plants → 2 % Bavistin 10 min → 5 % Tween-20 3 min → 0.1 % $HgCl_2$ 8 min → 3× DW → cut in half	MS + 2 mg/l BA (SIM; old leaves). MS + 2 mg/l BA + 0.2 mg/l IAA (SIM; young leaves). ½ MS + 0.2 mg/l IBA (RIM). 30-d subcultures. pH NR. 3 % sucrose. 0.7 % agar	16-h PP. CWFL. 50 µmol m^{-2} s^{-1}. 23 ± 2 °C	Shoot primordia formed from cut edge of old leaves within 26–30 d but within 40–45 d from youg leaves. Young leaves formed 25.2 shoots/explant (18.6 shoots/explant in old leaves). 100 % of shoots rooted on RIM, forming 8.3 roots/shoot. 93.3 % of acclimatized plantlets survived in soil + VC (3:1). No variation between micropropagated plants and mother plant shown by RAPD. Acclimatized plants flowered, formed fruit and R1 seed	Haque and Ghosh (2013)
Leaves from 1-y-old plant → cut into small pieces (size NR) → RTW 10 min → 0.1 % $HgCl_2$ 6 min → 4–5× SDW	MS + 1 mg/l BA + 1 mg/l 2,4-D (CIM). MS + 1 mg/l BA + 2 mg/l glutamic acid (SIM). ½ MS + 4 mg/l IBA (RIM). Subculture every 20 d. 3.5 % sucrose. pH and gelling agent NR	12-h (CIM) or 16-h (SIM) PP. Light source NR. 2000 lx. 25 ± 2 °C	Callus induced after 16 d. Shoot buds formed after 4 w (18 shoots/explant) in 90 % of explants. Callus and rooting not quantified. Acclimatization not performed	Sadguna et al. (2013)
Shoots from 1-y-old plant → 3× TW → leaves in SDW 30 min → 5 % Teepol^{TM} 5 min → DW → 70 % alcohol → 0.1 % $HgCl_2$ 5 min → 3× SDW	CIM (Chandrasekhar et al. 2006). Subculture every 3 w. pH 5.8. 0.8 % agar. Carbon source NR	16-h PP. CWFL. 36 µmol m^{-2} s^{-1}. 26 ± 2 °C. 55–60 % RH	Wide antimicrobial activity (growth inhibition) shown by callus extracts	Sellathurai et al. (2013)
Leaves, nodal segments (size NR) from 1-y-old plant → RTW → cetrimide → streptomycin sulfate → bavistin → $HgCl_2$ → 70 % alcohol → SDDW (in all cases, exact concentrations and exposure times NR)	MS + IAA + Kin (SIM from nodal segments; CIM from leaf segments; conc. NR). MS + 2 mg/l BA + 0.5 mg/l NAA (SIM from leaf segments)	PP, light source, LI NR. 25 ± 2 °C. 55 ± 5 % RH	Shoot formation after 4 w, plantlet formation after 8 w	Anjum et al. (2014)

Table 2 continued

Explant conditions and disinfection procedures	Culture medium, PGRs and additives*,**	Culture conditions***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
1-m-old leaves → Teepol™ (conc. time NR) → RTW → 0.1 % $HgCl_2$ 2 min → 3× DW → homogenized (protocol NR) → alginate beads (2 % Na-alginate → 50 mM $CaCl_2$ 30 min)	Beads → Zenk et al. (1975) basal medium + 5 % sucrose + 5 mg/100 ml cinnamic acid	Shaking at 90 rpm. 25 ± 2 °C. Light conditions NR	Relative to the control (0.16 %), kaempferol content increased to 2.07 % and 3.31 % after 2 and 3 w, respectively, when beads with agarized suspension culture were cultured in liquid medium	Chaturvedi et al. (2014)
Shoots (age of mother plants NR) → RTW 30 min → nodes 10× TW → 0.2–0.5 % Bavistin + 0.03 % streptomycin 10 min → SDW 2× → savlon (1.5 % chlorohexidine gluconate + 3 % cetrimide) 10 min → SDDW → 0.01 % $HgCl_2$ 1 min → SDW	MS + 2.5 mg/l BA + 3.5 mg/l NAA (SIM). ½ MS + 2.5 mg/l IBA + 2 mg/l IAA (RIM). Subcultures NR. pH 5.8. Carbon source and gelling agent NR	16-h PP. Light source, LI NR. 25 ± 2 °C	73 % of explants formed shoots. 100 % acclimatization in sterile soil, sand, and organic manure (1:1:1)	Jeyachandran and Bastin (2014)‡
Nodes → RTW 20 min → 0.1 % Tween-20 5 min → TW → 70 % EtOH 5 min → water → 0.1 % $HgCl_2$ 3–5 min → SDW	MS + 1 mg/l AdS + 0.5 mg/l 2,4-D (SIM). MS + 1 mg/l IAA (RIM). pH 5.8. 2 % sucrose. 0.8 % agar	16-h PP. Light source NR. 2000 lx. 22 ± 1 °C. 75 % RH	75 % of explants formed shoots and 65 % of shoots rooted on RIM. 70 % acclimatization	Mohan et al. (2014)
Adventitious roots (size NR) deried from in vitro plant leaves produced according to Rashmi et al. (2012) (RIM = MS + 1 mg/l IAA).	MS + 2 mg/l IBA → 0.1 mg/l BA (SIM). ½ MS + 0.2 mg/l IAA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 50 µmol m^{-2} s^{-1}. 26 ± 2 °C	25 shoots/root explant within 4 w. Acclimatization in sterile cocopeat, sand, and soil (1:1:1), with 80 % survival.	Nayeem et al. (2014)
Nodes → RTW 30 min → 1 % Teepol™ 10 min → wash → 0.1 % $HgCl_2$ 2–3 min → 4–5× DDW	MS + 2 mg/l 2,4-D + 0/1 mg/l Kin (CIM). MS + 0.4 mg/l BA + 0.1 mg/l Kin (SIM, RIM). 30-d subcultures. pH 5.8. 2 % sucrose. Gelling agent NR	16-h PP. Light source, LI NR. 25 ± 2 °C	85 % of explants formed callus within 25 d. 98 % of explants formed shoots (2.6/explant) within 7 d. 97 % acclimatization in soil + Soilrite™ (ratio NR) after a 5 min dip in Bavistin	Patel and Nadgauda (2014)
Nodes → 2× DW → 1 % Teepol™ 5 min → 0.1 % $HgCl_2$ 5 min → 4–5× SDW	MS + 1.5 mg/l BA + 0.5 mg/l NAA (SIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. LI NR. 25 ± 2 °C	Different alternative carbon sources were tested on the effect on shoot formation. 95.2 % of explants formed shoots with AR grade sucrose (94.8 % with white, refined sugar; 76.8 % with sugarcane juice; 73.8 % with unrefined, brown sugar; 67.6 % with jaggery), forming 16.5, 15.5, 12.4, 11.5 and 9.3 shoots/explant, respectively, when added at 3 % in all cases	Rajavel and Stephan (2014)
Leaves from mature plant (age NR) → RTW 30 min → detergent 5 min → TW → 0.1 % $HgCl_2$ 2 min → 4–5× SDW → 1 cm^2 explants	MS + 1 mg/l 2,4-D + 1 mg/l Kin (CIM). MS + 2 mg/l BA + 0.2 mg/l IAA (SIM). pH 5.8. 3 % sucrose. 0.8 % agar	PP and light source NR. 2000 lx. 25 ± 2 °C. 50–60 % RH	80 % of explants formed callus. 80 % of callus formed shoots (8.1 shoots/callus cluster). Protein, reducing sugar and total amino acids, as well as peroxidase and IAA oxidase were highest in the initial callus phase (among 4 in vitro stages tested)	Rathod et al. (2014)
Leaves (age NR) → 70 % EtOH 1 min → TW → 0.2 % $HgCl_2$ time NR → 3× SDW	MS + 2 mg/l BA + 2 mg/l 2,4-D (CIM). MS + 2 mg/l BA + 0.2 mg/l IAA (SIM). pH 5.8. Carbon source NR. 0.3 % clerigelas	16-h PP. Light source and LI NR. 25 ± 2 °C	Callus formed after 20 d in 80 % of explants. Shoot tips induced callus and shoots simultaneously. Acclimatization NR	Dhokrat et al. (2015), Jogdand et al. (2016), Shimple and Pandhure (2016)

Table 2 continued

Explant conditions and disinfection procedures	Culture medium, PGRs and additives*,**	Culture conditions***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Nodes from young shoots → RTW 30 min → 5 % labolene 5 min → 3× SDW → 0.1 % $HgCl_2$ 3 min → 3× SDW	MS + 1 mg/l Kin + 4 mg/l NAA (CIM). ½ MS + 2 mg/l BA (SIM). ½ MS + 4 mg/l IBA (RIM). pH 5.8. 3 % sucrose. 0.8 % agar	16-h PP. CWFL. 50 µmol m^{-2} s^{-1}. 25 ± 2 °C	Callus was induced within 6–8 d, and covered the explant after 3 w. 40–50 shoots/node formed. Leaves also formed callus and shoots, but in lower amounts and took longer. 80 % of shoots rooted in RIM	Murukan et al. (2015)†
Leaf and nodal segments → RTW (time NR) → 0.2 % Cetrimide (leaves: 5 min; nodes: 7 min) → 0.25 % Streptomycin sulphate → 0.5 % Bavistin (leaves: 15 min; nodes: 20 min) → 0.1 % $HgCl_2$ (leaves: 3 min; nodes: 5 min) → 70 % EtOH 1 min → 6× SW	MS + 2 mg/l Kin + 0.5 mg/l IAA (SIM: nodes). MS (liquid) + 1 mg/l BA + 0.5 mg/l IAA (CIM, SIM: leaves; precursor feeding). MS + 1 mg/l IBA (RIM). pH 5.7. Carbon source NR. 0.63 % agar	16-h PP. Light source and LI NR. 25 ± 2 °C. 55 ± 5 % RH	100 % of explants formed callus in 12 w. A maximum of 3.59 shoots formed/explant. 95.1 % of shoots rooted. Acclimatization in autoclaved Soilrite™ + soil (1:1). Increased levels of tylophorine were induced after feeding tyrosine in culture	Soni et al. (2015a)
Nodes → RTW 30 min → 5 % Teepol 8–10 min → SDW several times → Bavistin 1 h → 3× SDW → 0.1 % $HgCl_2$ 4–6 min → SDW	MS + 0.5 mg/l BA (SIM). ½ MS + 1 mg/l IAA (RIM). pH 5.8. Carbon source and gelling agent NR	16-h PP. Light source NR. 3000 lx. 25 ± 2 °C. 65–70 % RH	100 % shoot formation and 95 % root induction (organogenesis not quantified). Acclimatization in sand + garden soil (1:1)	Soni et al. (2015b)

2-BTOA 2-benzothiazoleacetic acid; *2,4-D* 2,4-dichlorophenoxyacetic acid; *2,4,5-T* 2,4,5-trichlorophenoxy acetic acid; *2iP* N^6-(2-isopentenyl) adenine; *AA* ascorbic acid; *ABA* abscisic acid; *AdS* adenine sulphate; *BA* N^6-benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original, according to Teixeira da Silva 2012a; *CH* casein hydrolysate; *CIM* callus induction medium; *CM* coconut milk; *CPM* callus proliferation medium; *CWFT* white fluorescent tube(s); *d* day(s); *DDW* double distilled water; *DW* distilled water; *EtOH* ethyl alcohol (ethanol); *GA_3* gibberellic acid; *GRS* gree-root-segment = green region of roots that is not WRS; *$HgCl_2$* mercury chloride; *HPTLC* high performance thin layer chromatography; *IAA* indole-3-acetic acid; *IBA* indole-3-butyric acid; *ISSR* inter simple sequence repeat; *Kin* kinetin (6-furfuryl aminopurine); *Labolene™* a detergent (Qualigens, Mumbai, India); *LI* light intensity; *m* month(s); *MS* Murashige and Skoog (1962) medium; *NAA* α-naphthaleneacetic acid; *NaOCl* sodium hypochlorite; *NR* not reported in the study, *PCM* protoplast culture medium; *PGR(s)* plant growth regulator(s); *PP* photoperiod; *RAPD* random amplified polymorphic DNA; *RH* relative humidity; *RIM* root induction medium; *rpm* revolutions per minute; *RTW* running tap water; *SDW* sterilized (by autoclaving) distilled water; *SDDW* sterilized (by autoclaving) double distilled water; *SDS-PAGE* sodium dodecyl sulfate polyacrylamide gel electrophoresis; *SE* somatic embryo; *SEGM* somatic embryo germination medium; *SEIM* somatic embryo induction medium; *SELM* shoot elongation medium; *SIM* shoot induction medium; *SMM* shoot multiplication medium; *SOD* superoxide dismutase (EC 1.15.1.1); *SW* sterile water; *Teepol™* a detergent (Reckitt and Colman, Slough, UK); *TDZ* thidiazuron (*N*-phenyl-*N*'- 1,2,3-thiadiazol-5-ylurea); *temp* temperature; *TLC* thin layer chromatography; *VC* vermicompost; *w* week(s); *White's* White's nutrient solution (White 1943); *WRS* white-root-segment = growing white apical region of the roots (15–20 mm); *Zea trans* zeatin

* Even though the term "calli" was used in the original, the term callus has been used here based on the recommendation of Teixeira da Silva 2012b

** All percentage values represent w/v (weight/volume) for solids or v/v (volume/volume) for liquids, unless otherwise specified

*** 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 lx; the sun, 1 µmol m^{-2} s^{-1} = 55.6 lx; high voltage sodium lamp, 1 µmol m^{-2} s^{-1} = 71.4 lx (Thimijan and Heins 1983)

**** For the 2005, 2006, 2013 and 2015 studies, the basic protocol to establish the initial cultures and to obtain the in vitro aseptic explants used for genetic transformation experiments, and eventually transgenic plants, used the Chaudhuri et al. (2004) protocol. Claims of somatic embryogenesis without sufficient proof (cytological, histological, genetic), i.e., only photos of macromorphology

‡ *Tylophora ovata*

† *Tylophora subramanii*

Fig. 1 The in vitro culture of *Tylophora indica*. **a** Direct shoot organogenesis from mature (thick, leathery, and *dark green*) leaves (from first 10 leaf pairs from the base) after 50 days of culture in Murashige and Skoog (MS) medium supplemented with 2 mg/l BA. Leaf explants were collected from a four-year-old plant maintained inside a shade-net house to avoid direct sunlight and exposed to 60–65 % relative humidity using a misting system. In vitro cultures were incubated inside a growth chamber maintained at 23 ± 2 °C with a 16-h photoperiod and at a photosynthetic photon flux density of approximately 50 µmol m^{-2} s^{-1} emitted by cool fluorescent tubes (Philips India Ltd.). **b** Direct shoot organogenesis from green root segments, excised from 4 to 6-week-old in vitro plant, on MS medium supplemented with 0.5 mg/l IBA, after 8 weeks of culture at 24 ± 1 °C with a 16-h photoperiod. **c** In vitro root induction from 12-day-old shoot (2.5 cm) derived from (**a**) on half-strength MS medium fortified with 0.1 mg/l IBA. **d** Three-month-old hardened plants kept in earthen pots containing a mixture of soil and vermicompost (3:1, v/v) and maintained inside a greenhouse (30 ± 2 °C, 14-h photoperiod, 60–65 % relative humidity). **e** Ex vitro plant (22 months old) maintained in the field under full sunlight and natural conditions without any additional care. **f** Flower of ex vitro plant. All photos (unpublished) provided with kind permission of Dr. B. Ghosh (**a**, **c**–**e**), and Dr. D. Roychowdhury (**b**)

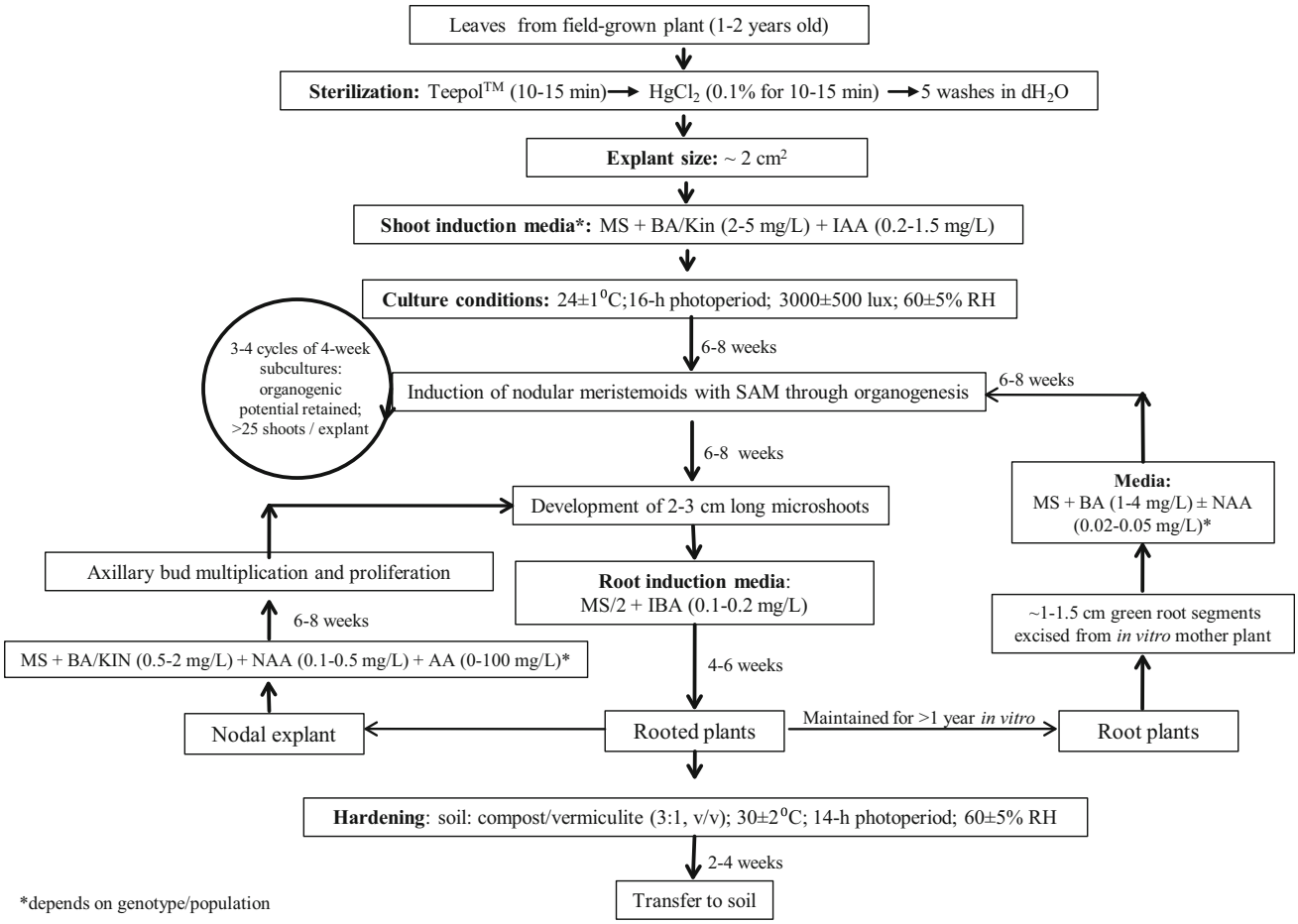

Fig. 2 In vitro plant regeneration in and mass propagation of *Tylophora indica* can be achieved in several ways, based on Table 2

leaves and subsequent development of SEs, but the conversion frequency to SEs depended on the concentration and combination of indole-3-acetic acid (IAA), 6-benzyladenine (BA) and kinetin. Jayanthi and Mandal (2001) induced SEs from embryogenic callus induced on mature leaf explants from ex situ plants, obtaining 50 plantlets/g of callus in 5 months. Somatic embryogenesis has also been reported from leaf explants by Chandrasekhar et al. (2006) and Sahai et al. (2010a), and from internodes (Thomas 2006).

Faisal and Anis (2003) induced shoots indirectly from callus in 85 % of leaves from field-grown plants, from stem explants (Faisal and Anis 2005) and from petiole explants (Faisal et al. 2005), a similar result being obtained by Verma et al. (2010) leaf, petiole and internode explants. Shoot induction was possible from mature leaf explants (Rathinavel and Sellathurai 2010; Anjum et al. 2014) of young leaves (Kalimuthu and Jeyaraman, 2012). Kaur et al. (2011a) developed a protocol for the induction of shoots from the stems of field-grown plants, and a subsequent protocol for the acclimatization and ex situ establishment of tissue cultured plants (Kaur et al. 2011b). Thomas and Phillip (2005) were the first to provide histological evidence of indirect shoot formation from immature leaves of field-grown plants, noting 100 % regeneration potential by long-term (up to 180 days) callus cultures. Haque and Ghosh (2013) showed a different morphogenic response by young and mature leaves of field-grown plants grown in vitro: while aged leaves formed shoots directly, young leaves first formed nodular meristemoids. The Haque and Ghosh (2013) study is the only publication in which micropropagated plants transferred to field flowered, and 28.5 % of plants produced fruit. The development of a successful protocol for the micropropagation of *Tylophora* plants (Fig. 1c–e) is a prerequisite for more advanced studies such as genetic transformation.

To induce callus using leaf and stem explants, MS medium supplemented with 2.5–7.5 µM 2,4-D or 2,4,5-T is optimal while shoot organogenesis from this callus can be induced in MS medium supplemented with 5 µM kinetin or BA, or 8 µM thidiazuron (Faisal and Anis 2003, 2005; Thomas and Philip 2005; Fig. 2). Microshoots can be rooted in half-strength MS medium containing 0.5–0.1 µM IBA.

Use of explants from in vitro plants

All studies that have used in vitro tissue to initiate in vitro cultures have employed leaf and root explants for whole plant regeneration. There are three reports on the use of root explants excised from in vitro plants to develop whole plants. Chaudhury et al. (2004) induced nodular shoot buds from green root segments in the presence of a cytokinin, and embryogenic callus from the same explants in the presence of BA and 2iP (N^6-(2-isopentenyl) adenine), with 42 % of explants converting to SEs. Sahai et al. (2010b) reported direct shoot organogenesis and callus-mediated somatic embryogenesis in green root segments, and provided a detailed histological assessment of shoot development. Nayeem et al. (2014) induced shoots from adventitious roots that developed from leaf explants, either directly or via callus formation. Devendra et al. (2011) claimed to induce SEs from leaf explants but provided no histological evidence.

Use of axillary buds, nodes and shoot tips

Sharma and Chandel (1992) first reported axillary bud proliferation and propagation from nodal stem segments in *T. indica* in response to cytokinins and auxin in basal medium supplemented with ascorbic acid. Faisal et al. (2007) used 100 mg/l ascorbic acid in addition to auxins and cytokinins to improve shoot number and length from nodes. Axillary bud multiplication and micropropagation were subsequently reported by Gami and Parmar (2010) and Kaushik et al. (2010). Rani and Rana (2010) collected nodal explants during different seasons and found that the frequency of bud break and shoot number/explant were maximum when collected in September–November. Micropropagation from nodal explants was also reported by Mohan et al. (2014) and Patel and Nadgauda (2014). While shoot multiplication using a pre-existing meristem has been the method of choice for clonal propagation of plants in many plant species, in *T. indica*, the fewest reports of shoot organogenesis involve nodal explants. The rate of multiplication using nodal explants is not mentioned in most publications and needs to be improved. It is also unclear whether a range of endophytic fungi isolated from leaves and stems (Kumar et al. 2011) may impact the effectiveness of organogenesis in vitro.

An optimum number of shoots per nodal explant with an average length of 3–4 cm can be obtained in MS medium supplemented with 2.5 μM BA or kinetin, 0.1 μM NAA, and 50–100 mg/l ascorbic acid after 6 weeks of culture (Faisal et al. 2007). Rooting of these shoots is optimum in half-strength MS medium supplemented with 0.5 μM IBA followed by acclimatization in vermiculite in a growth chamber under a 16-h photoperiod for 4 weeks (Fig. 2).

Protoplast culture and morphogenesis

Mhatre et al. (1984) were the first to report plant regeneration from protoplasts in *T. indica* from callus induced on stem explants. The yield of viable protoplasts, and induction of shoots, was higher when freshly induced callus was used than from five year old callus that had been regularly subcultured. Thomas (2009) regenerated *T. indica* plants from mesophyll-protoplast-derived callus that had been induced from the leaves of field-grown plants. Protoplasts have not yet been used to generate hybrids in *Tylophora*.

Optimal in vitro protocol

Based on the protocols described in Table 2, it is evident that *T. indica* is an interesting example of a plant species showing morphogenic potential from almost all vegetative parts. However, for the purpose of mass clonal propagation, the most commonly used explant, namely shoot tips or nodes, is not very suitable due to a low rate of multiplication. To maintain the fidelity of the genetic and chemical profile of the parent plant, it is necessary to avoid propagation protocols that involve dedifferentiation of explant cells to friable callus and indirect morphogenesis requiring auxin and/or cytokinin supplementation in the basal medium, which is very prevalent in *T. indica*. To induce *de novo* shoots from leaf explants of field-grown plants via the formation of nodular meristemoids, MS medium supplemented with 2–3 mg/l BA or kinetin, together with low levels of IAA (0.2–0.5 mg/l) can be used with subcultures every 4–6 weeks. Similarly, shoot organogenesis can be induced via the formation of nodular meristemoids in green root segments excised from 6 to 8 week old rooted in vitro plants. Thus, induction of nodular meristemoids from leaf explants is a suggested method of choice to establish and in vitro culture and to propagate plants for commercial purposes (Fig. 2).

Secondary metabolite production in vitro

Benjamin and Mulchandani (1973) first reported secondary metabolite production in *T. indica* in vitro callus induced from stem segments and roots from in vitro germinated seedlings. The callus did not form any phenanthroindolizidine alkaloids even after feeding precursors, but

phytosterols were detected. Benjamin et al. (1979) further investigated alkaloid synthesis in callus cultures and in vitro regenerated plants: while alkaloids were not detected in callus, the alkaloid profile of tissue culture-derived flowering plants were similar to field-grown plants. Jha et al. (2005) investigated the potential of differentiated or morphogenic root-derived callus cultures and plants regenerated from root segments of *T. indica*. The level of tylophorine—a phenanthroindolizidine alkaloid and the main alkaloid in *T. indica*—in nodular meristemoid and friable embryogenic cultures and in plantlets regenerated in vitro prior to and after transfer to the field was assessed by HPLC. Tylophorine was detected in all in vitro cultures, in the shoots and roots of in vitro plantlets as well as in the leaves, stems and roots of one-year-old plants after transfer to the field. Friable embryogenic cultures had double the tylophorine content when the culture period was extended from 4 to 12 weeks, and 12-week-old tissue cultured plantlets had a 21-fold higher tylophorine content than 4-week-old plants. The tylophorine content of one-year-old micropropagated plants growing in the field and wild plants was comparable. Kaur et al. (2011a) detected 71–80 μg/ml of tylophorine in tissue culture-derived plantlets, confirmed by Kaur et al. (2011b) study (80 μg/ml). Kaur et al. (2011b) also found that suspension cultures and callus produced 28.3 and 24.5 μg/ml of tylophorine, respectively. Soni et al. (2015a), using precursor feeding of 2 mg/l tyrosine, induced 27.7, ~12.5, ~9.5, and ~4.5 μg/ml of tylophorine in in vitro-derived plantlets, shoots, callus and mother plants, respectively.

Molecular verification of somaclonal variation

Molecular markers have not been extensively used in *Tylophora* biotechnology. While two studies (Jayanthi and Mandal 2001; Haque and Ghosh 2013) showed no variation (i.e., polymorphism) in random amplified polymorphic DNA (RAPD) banding between mother plants and in vitro regenerants, Chaturvedi and Chowdhary (2012) reported 37.5 % polymorphism while Pathak et al. (2013) showed 62.1 % polymorphism.

Genetic transformation, hairy root production, secondary metabolites and bioreactors

Tissue culture forms an important structural basis for genetic transformation studies. Several studies reporting on the development of transgenic *T. indica* exist. The first (Chaudhuri et al. 2005) documents the induction of hairy roots in excised leaf and stem explants infected with *Agrobacterium rhizogenes* strain A4. In that study, as many as 60 % of inoculated shoots formed hairy roots with different transformed root clones (Fig. 3a) that accumulated tylophorine at 0.16–0.29 mg in roots/Petri dish and 1.03–1.29 mg/g root dry weight. The transformed roots could be successfully cultured in liquid medium, forming higher biomass, yield and tylophorine content than in solid medium. This is a prerequisite for scale-up studies. The secretion of tylophorine in liquid root culture medium was a significant finding for large-scale production using bioreactors.

Spontaneous regeneration of plants from Ri (root-inducing)-transformed roots in plant growth regulator-free basal medium (Fig. 3b, c) was reported by Chaudhuri et al. (2006). The Ri-transformed *T. indica* plants had 160–280 % higher tylophorine content than untransformed plants, and an equivalent 350–510 % higher biomass (Chaudhuri et al. 2006). The same group (Roychowdhury et al. 2013a, 2015a, b) then assessed the morphological and genetic stability of long-term (4–6 years) in vitro hairy root cultures and plants derived from transgenic hairy roots (Fig. 3d). Among the most notable morphological variations observed were shorter shoots with more nodes and leaves/plant, both in in vitro plantlets and in one-year-old greenhouse-grown plants (Roychowdhury et al. 2013a). Despite this variation, no genetic variation (RAPD profiles) was detected (Roychowdhury et al. 2015a). The *rolA*, *rolB*, *rolC*, and *rolD* genes were stably inserted (Fig. 4), as confirmed by RT-PCR, in all clones and tylophorine content, as confirmed by HPTLC, was almost two-fold higher than in non-transformed plants (Roychowdhury et al. 2013a, 2015b).

The morphogenic potential of transformed hairy root cultures was not affected by the presence of *rol* genes of the Ri plasmid and plants regenerated both via direct (less common) and indirect (more common) organogenesis as well as via callus-mediated somatic embryogenesis (Chaudhuri et al. 2006; Roychowdhuri et al. 2015b). In these studies, since Ri-transformed plants showed enhanced tylophorine production, and since such high tylophorine-containing plantlets could be stably micropropagated as non-transformed plants, this technique can be commercialized for the production of *T. indica* secondary metabolites. A protocol for the induction of Ri-transformed roots, regeneration of plants via direct organogenesis and indirect somatic embryogenesis in *T. indica*, does not require exogenous supplementation of phytohormones at any stage thereby ensuring the genetic stability of Ri-transformed plants.

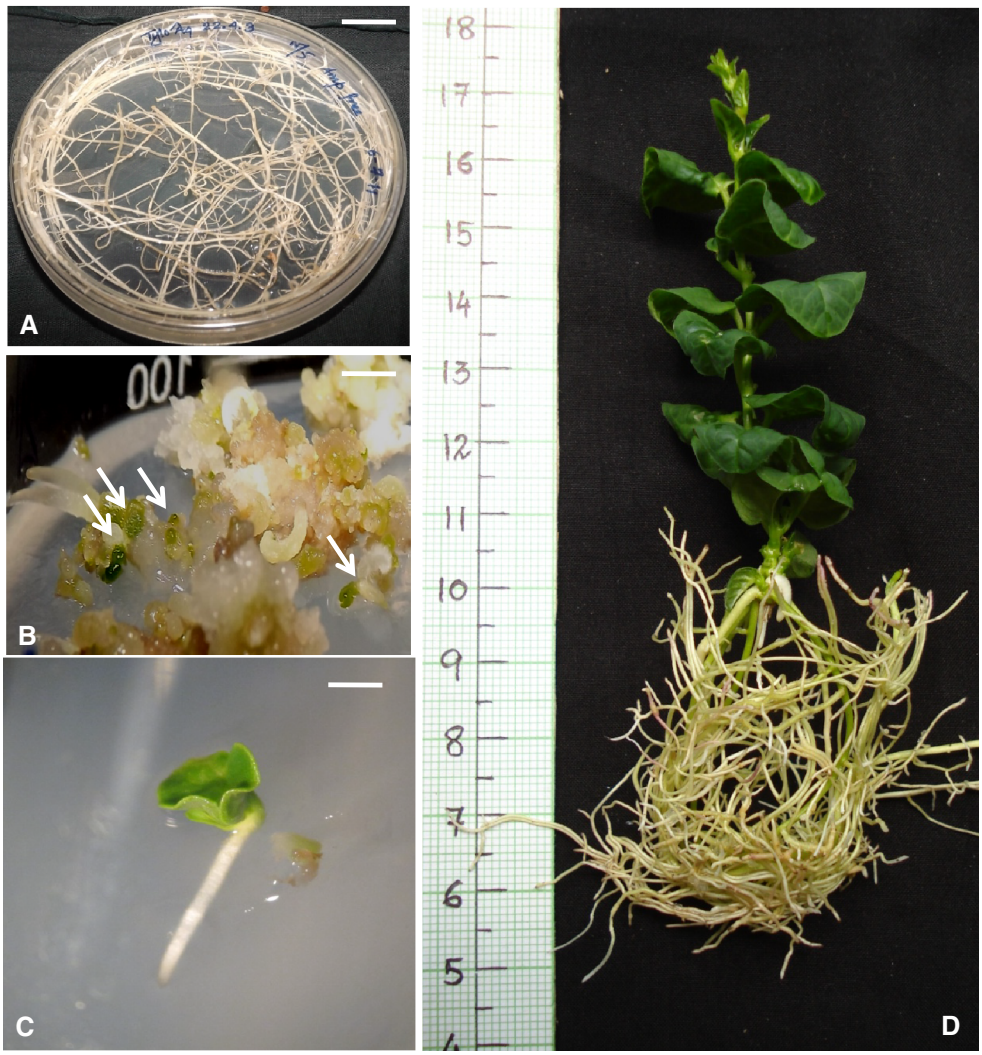

Fig. 3 Spontaneous regeneration of Ri-plant from hairy root culture of *Tylophora indica.* **a** Ri-transformed root culture on MS medium (*bar* 1.3 cm). **b** Spontaneously regenerating Ri-transformed callus cultured under 16-h photoperiod on MS medium showing somatic embryogenesis (*bar* 0.5 cm). **c** Single germinated somatic embryo on MS medium (*bar* 0.3 cm). **d** Ri-transformed plant

Conclusions and future perspectives

The development of an economically viable scale-up culture system using transformed root cultures is a pre-required for the large-scale production of tylophorine (Roychowdhury et al. 2013b), although there are several other means of establishing in vitro cultures (Fig. 2). Hairy roots serve as a continuous source of target metabolites of parent plants due to their genetic stability, and ability for rapid growth in plant growth regulator-free liquid medium in bioreactors (Stiles and Liu 2013; Mehrotra et al. 2015), allowing for hairy root-mediated biotransformation (Banerjee et al. 2012). These technologies would allow for the use of *T. indica* hairy roots to further improve the biosynthetic potential of superior clones. Studies on the factors affecting the biosynthesis of *T. indica* alkaloids and other secondary metabolites need to be conducted using transgenic cultures via exogenous and endogenous elicitation (Chaudhuri et al. 2009; Ramirej-Estrada et al. 2016) and biotransformation (Banerjee et al. 2012).

The following topics still need to be explored in *Tylophora* in vitro biotechnology: anther culture (e.g., Teixeira da Silva et al. 2015), the use of thin cell layers technology (Teixeira da Silva and Dobránszki 2013, 2015), in vitro flower induction (e.g., Teixeira da Silva et al. 2014), or CO_2 enrichment for increasing biomass (e.g., Norikane et al. 2013). More recently, an aqueous extract of *T. indica* leaves was used to synthesize silver nanoparticles (Oke et al. 2015), but that procedure would need to be improved to make it more economically viable than for

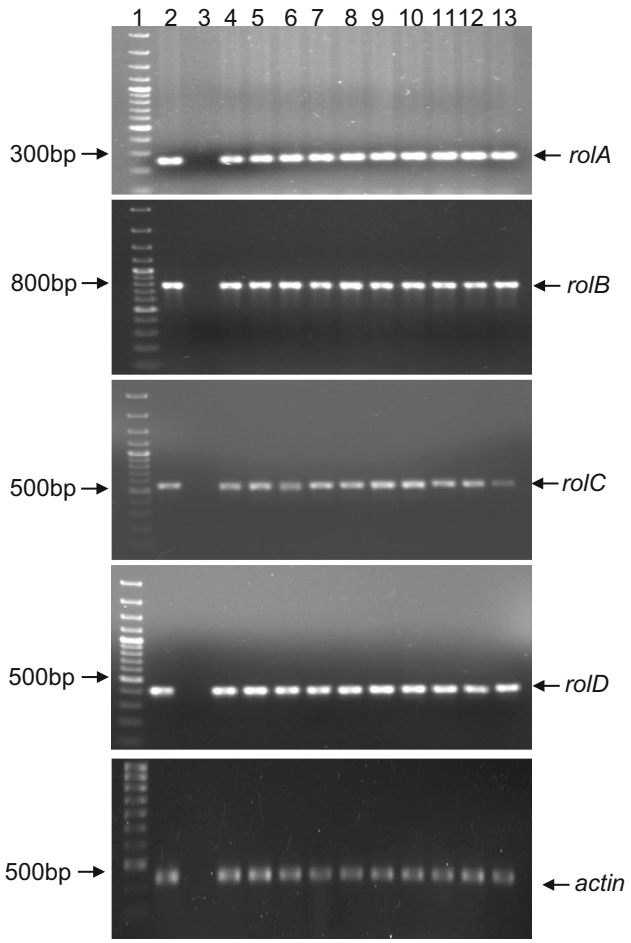

Fig. 4 Molecular confirmation of integration of the *rolA*, *rolB*, *rolC*, and *rolD* genes at the transcription level. The β-actin gene served as the internal control (see primers and additional evidence of integration in Roychowdhury et al. 2015a, b) using RT-PCR of Ri-transformed *Tylophora indica* plant derived using procedures explained in Figs. 1 and 2 (Chaudhury et al. 2006 protocol; Table 2). *Lane 1* molecular markers (100-bp plus DNA ladder); *lane 2* positive control (pLJ1, which carries TL-DNA); *lane 3* negative control (genomic DNA from non-transformed plant); *lanes 4–13* amplified cDNAs of Ri-transformed plants lines. Unpublished photos

tylophorine production. The use of these detailed protocols and advice may also be useful for the in vitro propagation of other *Tylophora* species, such as *T. ovata* (Jeyachandran and Bastin 2014) and *T. subramanii* (Murukan et al. 2015).

Author contribution statement Both authors contributed equally to all aspects of the development and writing of this review.

Acknowledgments The authors thank Mafat M. Kher (Sardar Patel University, India) for providing some difficult-to-access literature and for assistance with organizing the references in the first draft of the paper. We are also very thankful to Dr. B. Ghosh (RKMVC College, Rahara, West Bengal, India) for providing unpublished photos of micropropagation in *T. indica* (Fig. 1a, c–f) and Dr. D. Roychowdhury (Calcutta University) for help in organizing Fig. 2.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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