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THE *IN VITRO* CULTURE OF *HOLOSTEMMA* SPECIES: A CRITICAL ANALYSIS

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Abstract. The taxonomic status of *Holostemma* R.Br (Apocynaceae (formerly Asclepiadaceae), Asclepiadoideae) has recently (2014) been revised, allowing for biotechnology on this genus to assume a new and focused direction. This mini-review aims to cover what is known thus far about the tissue culture of *Holostemma* species, which includes medicinal species with ornamental potential. A post-publication peer review of the literature is provided, a critical assessment is made of the faults and weaknesses of the literature, and advice is offered as to how better to improve the research objectives to benefit researchers of *Holostemma* species.

Key words: *Holostemma*, Apocynaceae, biotechnology, medicinal properties, plant growth regulator, tissue culture

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Abbreviations

2,4-D – 2,4-diclorophenoxyacetic acid
2iP – N^6-(2-isopentenyl) adenine
BA – 6-benzyladenine
IAA – indole-3-acetic acid
IBA – indole-3-butyric acid
Kin – kinetin
MS – Murashige and Skoog medium
NAA – α-naphthaleneacetic acid
PGR – plant growth regulator

Introduction. A new taxonomy, a new vision

There is still considerable debate about the taxonomy within the genus *Holostemma* R.Br (Apocynaceae, Asclepiadoideae) even though it was recently revised by Surveswaran *et al.* (2014; Tab. 1) using molecular markers (plastid sequences of *rbcL*, *rps16*, *trnL* and *trnLF* regions), and thus serving as an update of the last revision which only employed morphological characters (Swarupanandan *et al.* 1996). In West Bengal, *H. ada-kodien* Schult. is used as a febrifuge (Dey & De 2012), the ethanolic extract of the roots of *H. annulare*, (Roxb.) K. Schum. commonly used in Ayurvedic medicine, showed antidiabetic activity in Wistar rats with diabetes mellitus (Shirwaikar *et al.* 2007). Reddy *et al.* (2010) reached the same conclusion when C57BL/6J *ob/ob* diabetic mice were used. Both the ethanolic extract and the ethyl acetate extract of dried and powdered *H. ada-kodien* leaves were able to paralyze and kill adult Indian earthworms (*Pheretima posthuma*) at 200 and 400 μg/mL, i.e., they displayed anthelmintic activity (Sadasivam *et al.* 2014). The hexane, ethyl acetate and methanolic extracts of *H. ada-kodien* root tubers showed antioxidant and radical (DPPH, superoxide, nitric oxide) scavenging activity (Mallikarjuna *et al.* 2011). The ethanolic and methanolic extracts of *H. ada-kodien* leaves and stems were able to exert antibacterial activity against *Staphylococcus aureus, Bacillus subtilis* and to a lesser extent *Salmonella typhymurium*, when compared with the control antibiotic gentamicin, but were ineffective against *Klebsiella pneumoniae* and *Escherichia coli* (Irimpan *et al.* 2011). Older texts in somewhat obscure or difficult-to-access textbooks (e.g., Warrier *et al.* 1995) point towards the use of *H. ada-kodien* as an antidiabetic, rejuvenative, aphrodisiac, expectorant, galactogogue, stimulant, and to treat ophthalmic disorders in traditional Indian

Tab. 1. Taxonomy of *Holostemma* R.Br (modified from SURVESWARAN *et al.* 2014).

Species	Currently accepted name
H. ada-kodien Schult. *[1]	*H. annularium* (Roxb.) K. Schum.
H. annularium (Roxb.) K. Schum.	*H. annularium* (Roxb.) K. Schum.
H. brunonianum Royle	*H. annularium* (Roxb.) K. Schum.
H. candolleanum Spreng.	*Fischeria scandens* DC.
H. chilense Phil.	*Diplolepis mucronata* (Decne.) Hechem & C. Ezcurra
H. fragrans Wall.	*H. fragrans* Wall.
H. laeve Blume	*Cynanchum ovalifolium* Wight [2]
H. muricatum Blume	*Cynanchum muricatum* (Blume) Boerl.
H. pictum Champ. ex Benth.	*Graphistemma pictum* (Champ. ex Benth.) Benth. & Hook. f. ex Maxim.
H. rheedei Wall.	*H. annularium* (Roxb.) K. Schum.
H. rheedianum Spreng.	*H. annularium* (Roxb.) K. Schum.
H. sinense Hemsl.	*Metaplexis hemsleyana* Oliv.
H. tuberculatum Blume	*Cynanchum tuberculatum* (Blume) Boerl.

* Claimed on the Plant List (http://www.theplantlist.org/tpl1.1/search?q=Holostemma) and Wikipedia (https://en.wikipedia.org/wiki/Holostemma) to be the only species within this genus, while other species names listed by SURVESWARAN *et al.* (2014) are considered to be synonyms of *H. ada-kodien* (The Plant List, updated in 2012).

[1] Common names (according to http://flowersofindia.net/catalog/slides/Holostemma%20Creeper.html). Holostemma creeper or adapathiyan; Hindi: Chhirvel; Marathi: Dudruli, Shidodi; Tamil: Palay kirai; Malayalam: Ada kodien; Telugu: Palagurugu; Sanskrit: Jivanti, Arkapushpi.

[2] Confirmed by LIEDE & KUNZE (2002).

Ayurvedic medicine (according to GEETHA *et al.* (2009) and IRIMPAN *et al.* (2011), and local references therein).

Are *Holostemma* species rare or endangered?

In 1997, the Foundation for Revitalisation of Local Health Traditions listed *H. ada-kodien* as being rare due to over collection (FRLHT 1997), although the IUCN does not red list any *Holostemma* species (http://www.iucnredlist.org/), thus bringing into question any claim of rare or endangered by several of the authors of the tissue culture studies covered next (Tab. 2) as one of the supporting claims of "novelty" of their study. For example, DECRUSE & SEENI (2002) describe *H. annulare* as being rare yet two years later, in DECRUSE *et al.* (2004), they describe the plant as being endangered, without any supporting evidence. One common tactic used by authors publishing studies on medicinal and aromatic plants to increase their chances of acceptance to a journal is by exaggerating (or inventing) the rare or endangered status of that plant, to sway the editors and reviewers opinions regarding the importance and originality of the study. Although I am not suggesting that this may be the case in any of the studies listed in Tab. 2, readers should be aware of this unpalatable issue when they assess the novelty of such studies.

In the light of this new taxonomic revision, and the questionable claims of the rarity or endangered status of several *Holostemma* species, this mini-review aims to revise what work exists on the tissue culture of this genus – in fact very few studies – with the objective of giving some direction for future experiments, and, based on a critique of the already published literature, advice on what should or should not be done in future research to make results more meaningful and robust, thus increasing confidence in the reported outcomes.

The tissue culture of *Holostemma* species

The earliest known study on the tissue culture of *Holostemma* species that could be identified in the published literature, after consulting at least

Tab. 2. Classification of the rarity of *Holostemma* species in different tissue culture studies *.

Reported species	Status	Status supported by sources?	Reference
H. annulare	Rare	Yes **	Sudha *et al.* 1998, 2000
H. annulare	Not indicated		Decruse & Seeni 2002 ***
H. ada-kodien	Rare + endangered	Yes ** (2002); No (2003)	Martin 2002, 2003
H. annulare	Endangered	No	Decruse *et al.* 2004
H. ada-kodien	Endangered	No	Geeta *et al.* 2009
H. ada-kodien	Rare	Yes **	Pushparajan & Surendran 2014
H. ada-kodien	Endangered	Yes **/No	Jaya Chandra *et al.* 2015

* As indicated in the text, the IUCN Red List does not list any *Holostemma* species as being either rare, or endangered.
** Only local, old, impossible-to-access, or anonymous sources provided.
*** Fails to cite Sudha *et al.* 1998 in the text, constituting a case of snub publishing (Teixeira da Silva 2013).

7 data-bases/web-sites (Elsevier's sciencedirect.com/Scopus; Springer-Nature's SpringerLink; Taylor & Francis Online; Wiley Online; Oxford University Press; NIH's PubMed; Google and Google Scholar), was from 1998.

In that first study, Sudha *et al.* (1998) claimed that tissue culture of *H. annulare* was necessary for three reasons: 1) conventional vegetative propagation through stem or root cuttings is impeded by herbivores and insects who are attracted to the sweet latex during monsoon months, but without providing proof; 2) since roots are used for Ayurvedic medicine, the use of roots for propagation is impractical; 3) flowering takes place once a year and little fruit is set, but no evidence was provided. With this set of limitations in hand, the authors propagated mother plants by nodal cuttings or shoot tips. Although the authors attempted liquid culture, they observed hyperhydricity. Although basal nodes regenerated more shoots than shoot tips (3.8 and 1.0 per explant, respectively) in optimized conditions specified in Tab. 3, the former explant showed higher levels of contamination, although contamination levels were not quantified. Although most shoots per explant and longest shoots formed in the 6-benzyladenine (BA; see notes in Teixeira da Silva 2012) + α-naphthaleneacetic acid (NAA) combination, shoots could also be induced in the presence of BA + indole-3-butyric acid (IBA) / indole-3-acetic acid (IAA), N^6-(2-isopentenyl) adenine (2iP) + NAA / IBA / IAA, or kinetin (Kin) + NAA / IBA / IAA. After the first subculture of shoots, shoot production could be increased to 7.6 shoots/explant. A maximum of 75% of shoots could root when IBA was used as the auxin and a maximum of 82% of rooted plants could be successfully acclimatized in the greenhouse without any visible morphological changes. Sudha *et al.* (2000) followed up their 1998 study with the induction of shoots from chlorophyllous roots with as high as 82% response without callus induction.

Martin (2002) was able to induce shoots from 100% of *H. ada-kodien* nodes, forming at most 8.1 shoots/explant. Although shoots could also form in the presence of coconut water without any plant growth regulators (PGRs), with BA or Kin alone, with BA + IAA / NAA, the ideal shoot induction medium involved BA + IBA (Tab. 3). When 1.5 mg/L BA was used, callus could be induced from leaves, nodes and internodes and subsequently shoots (i.e., indirect callogenesis), forming a maximum of 15.5 shoots/node and 5.2 shoots/internode. Even though leaf abscission could be avoided by adding $AgNO_3$ or $CoCl_2$ to medium, shoot number was compromised. The authors claimed 90% survival of acclimatized plants. Even though Martin (2003) claimed to have induced somatic embryos from roots, internodes and leaves, with as many as 43.8/10 mg of callus, no histological or cytological proof was provided. The author also claimed 90% plantlet survival, an identical value to the 2002 study. Geetha *et al.* (2009) claimed

Tab. 3. Basic culture conditions for the tissue culture of *Holostemma* species.

Species	Disinfection process	Culture conditions *	Optimal medium **	Reference
H. annulare	Shoots with 6-8 nodes removed from 6-8 week-old mother plants. Shoots stripped of leaves; the remaining fourth to sixth nodes from the apex were cut and washed in detergent (1% Labolene) for 10 min then rinsed in RTW until the detergent had been thoroughly removed. Terminal cuttings and older basal nodes were immersed in 15% bleach for 7-10 min or 12-15 min, respectively, then in 0.1% $HgCl_2$ for 5-7 or 8-10 min, respectively, each step followed by five rinses with SDW. After trimming damaged termini, explants were cut into 0.5-0.8 cm (shoot tips) or 1.0-1.5 cm (basal nodes). One final rinse in SDW before plating on medium.	PP, light source, LI, and temp. NR. Subcultured every 5-6 w. pH 5.8. 3% sucrose (RIM). 0.5% agar. Plants acclimatized in sand : soil : CD (1:1:1).	MS + 0.54 µM NAA + 4.43 µM BA (SIM). MS + 0.27 µM NAA + 2.21 µM BA (SMM). ½ MS + 1.48 µM IBA + 556.06 µM myo-inositol + 0.05% AC (RIM).	Sudha *et al.* 1998
H. annulare	Chlorophyllous roots (3-4 cm) of *in vitro* plants.	12-h PP. CWFT. 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$. 24 ± 2°C. Other conditions as for Sudha *et al.* (1998).	½ MS + 0.3 mg/L IBA (RIM). MS + 0.2 mg/L BA (SIM). MS + 0.5 mg/L BA + 0.05 mg/L NAA (SMM).	Sudha *et al.* 2000
H. annulare	Seeds were germinated (substrate and conditions NR) and nodes from seedlings were disinfected using the Sudha *et al.* (1998) protocol.	12-h PP. 30-50 $\mu mol \cdot m^{-2} \cdot s^{-1}$. 25 ± 2 °C. 87.7 mM sucrose. Other conditions as for Sudha *et al.* (1998).	MS + 2.22 µM BA + 2.6 mM $NH_4H_2PO_4$ and without NH_4NO_3 (cryopreservation and shoot tip recovery media). Recovery medium also contained 1.44 µM GA_3.	Decruse & Seeni 2002; Decruse *et al.* 2004
H. ada-kodien	Leaf, internode and node explants from tender parts of mature plants washed under RTW, then 5% Extran (detergent) for 5-10 min, then rinsed in SDDW. Explants placed in 0.5% $HgCl_2$ for 12-14 min then rinsed in SDDW.	16-h PP. CWFT. 2000 lux. 25 ± 2 °C. pH 5.8. 3% sucrose. 0.8% agar. Plants acclimatized in solrite : sand (1:1).	MS + 1.5 mg/L BA (CIM → SIM; all explants). MS + 2 mg/L BA + 0.5 mg/L IBA (SIM; nodes). ½ MS + 0.05 mg/L IBA (RIM).	Martin 2002
H. ada-kodien	Leaf, internode and root explants from tender parts of mature plants surface disinfected as for Martin (2002). Leaves cut into 1 cm^2 and internodes and roots 1 cm explants.	16-h PP. 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$. 25 ± 2 °C. pH 5.8. 3% sucrose. 0.8% agar. Plants acclimatized in solrite : sand (1:1).	MS + 1 mg/L 2,4-D (CIM). ¼ MS + 0.1 mg/L 2,4-D (SEIM).	Martin 2003

H. ada-kodien	Shoots washed in RTW for 15 min. After removing leaves, naked stem washed in dilute Tween-20 (2 drops in 100 mL) and cut into 3-4 cm long explants which are treated with 4% NaOCl and Tween-20 (2 drops/100 mL) for 2 min and rinsed 4-5× with SDW. Trim to 1.5 cm long segments, treat with 4% NaOCl for 2 min under constant agitation, and wash 5–6 times in SDW.	PP, LI, temp. NR ***. pH 5.8. 2% (SIM) or 3% (SMM) sucrose. 0.7% (SIM) or 0.8% (SMM) agar. Plants acclimatized in sand.	PGR-free MS (SIM, RIM). MS + 4.90 µM IBA + 0.46 µM Kin (SMM).	Geetha *et al.* 2009
H. ada-kodien	Leaves, shoot tips and nodal segments collected from fresh, young, healthy mother plants were washed thoroughly in RTW then with Salvon for 5 min. Explants treated with 5% Teepol for 5-10 min and thoroughly washed with DW. Explants immersed in 70% EtOH for 1 min, washed with SDW 3×, then treated with 0.1% $HgCl_2$ for 1-10 min. Washed with autoclaved DW 5× and cut into 0.5-1.0 cm explants.	16-h PP. 3000 lux. 25 ± 2 °C. 55 ± 5% RH. pH 5.8. 3% sucrose. 0.8% agar. Plants acclimatized in sterile soil : sand (1:1).	MS + 2.0-2.5 mg/L BA + 1 mg/L NAA or 2.5 mg/L Kin + 1 mg/L NAA (CIM). MS 1.5 mg/L Kin + 0.5 mg/L NAA (SIM).	Pushparajan & Surendran 2014
H. ada-kodien	Stem cuttings with three apical nodes treated with 5% detergent + Tween-20 for 5 min, 3-4 rinses in SDW, 70% EtOH for 30 s, 0.1% $HgCl_2$ for 5 min and 4× with SDW after each step.	16-h PP. 2500 lux. 25 ± 3 °C. pH 5.8. 3% sucrose. 0.8% agar. Plants acclimatized in garden soil : forest humus (1:1) (*in vivo* cuttings) or soil : vermicompost : sand (1:1:1) for *in vitro*-derived plantlets.	MS + 11.09 µM BA (SIM). MS + 8.87 µM BA + 4.92 µM IBA + 2.32 µM Kin (SMM). MS + 4.92 µM IBA (RIM, *ex vitro*).	Jaya Chandra *et al.* 2015

2,4-D – 2,4-dichlorophenoxyacetic acid; **AC** – activated charcoal; **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 2012); **CD** – cow dung; **CIM** – callus induction medium; **CWFT** – white fluorescent tubes; **DW** – distilled water; **EtOH** – ethyl alcohol (ethanol); **$HgCl_2$** – mercury chloride; **IBA** – indole-3-butyric acid; **Kin** – kinetin (6-furfuryl aminopurine); **LI** – light intensity; **MS** – Murashige & Skoog (1962) medium; **NAA** – α-naphthaleneacetic acid; **NaOCl** – sodium hypochlorite; **NR** – not reported in the study; **PGR** – plant growth regulator; **PP** – photoperiod; **RH** – relative humidity; **RIM** – root induction medium; **RTW** – running tap water; **SDW** – sterilized (by autoclaving) distilled water; **SDDW** – sterilized (by autoclaving) double distilled water; **SEIM** – somatic embryo induction medium; **SIM** – shoot induction medium; **SMM** – multiplication induction medium; **w** – week(s).

* 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; for the sun 1 µmol · m^{-2} · s^{-1} = 55.6 lux; for high voltage sodium lamp 1 µmol · m^{-2} · s^{-1} = 71.4 lux (Thimijan & Heins 1983).

** Percentage values of solids as w/v (weight/volume) and of liquids as volume/volume (v/v).

*** https://pubpeer.com/publications/19521837. Claims of somatic embryogenesis without sufficient proof (cytological, histological, genetic), i.e., only photos of macromorphology.

the ability to produce "300,000 plantlets from a single explant after 10 subculture cycles of 4 to 5 wk intervals with 90% field establishment."

PUSHPARAJAN & SURENDRAN (2014) induced callus and shoots from shoot tips and leaves of *H. ada-kodien* with a roughly 50% callus induction response in both explants when the BA + NAA combination was used, or closer to 60% when Kin was used with NAA (Tab. 3). The shoot induction response was 68% when Kin + NAA were employed. JAYA CHANDRA *et al.* (2015) showed that 700 mg/L IBA could induce roots in 88% of shoots in a greenhouse when rooted in vermiculite. *In vitro*, these authors induced shoots from nodes in 84% of explants, and multiplies to 8.14 shoots/shoot when subcultured (Tab. 3).

The ability to cryopreserve important medicinal plant germplasm has advantages, but only if the material can be regenerated post storage in liquid N_2. DECRUSE *et al.* (1999) noted that when NH_4NO_3 was removed from MS medium and replaced with 2.6 mM $NH_4H_2PO_4$, *H. annulare* shoot tips could be successfully cryopreserved using encapsulation-dehydration. Moreover, when NH_4NO_3 was omitted from post-cryopreservation shoot tip regeneration medium, no callus formed (DECRUSE & SEENI 2002; DECRUSE *et al.* 2004). In their experiment, DECRUSE & SEENI (2002) and DECRUSE *et al.* (2004) first subcultured shoot tips derived from seedling six times on MS medium with 0.2 M sucrose and 2.85 μM IBA for a total of 30 days (preconditioning period). Shoot tips 1-3 mm long were dissected then encapsulated in 2.5% sodium alginate and precultured in liquid medium containing 0.5 and 0.75 M sucrose for one day each at 25±2 °C and 0.75 M sucrose + 3% DMSO for 3 days at 4 °C. Precultured beads were dehydrated for 4 h in a sterile airflow hood until dry weight was 0.17-0.2 g water/g, based on DECRUSE *et al.* (1999) and stored in LN overnight. Encapsulated shoot tips (i.e., within synseeds, an essential tool in cryopreservation; SHARMA *et al.* 2013) were then rewarmed at 40 °C. Only 56.8% of cryopreserved shoot tips could regenerate, but without callus formation while control (uncryopreserved) shoot tips showed 90% recovery, but 8.3% formed callus (DECRUSE & SEENI 2002). DECRUSE *et al.* (2004) observed highest percentage recovery (90%) when MS medium free of NH_4NO_3 was used for pre- and post-cryopreservation procedures.

There are only two studies on the genetic transformation of *H. ada-kodien* conducted by KARMARKAR & KESHAVACHANDRAN (2001) and KARMARKAR *et al.* (2001). In those studies, 25% of hypocotyls could be induced to form hairy roots in the dark in response to *Agrobacterium rhizogenes* strain PcA4, although hairy root induction was also possible when strains 15834 and A4, in both darkness and light conditions, and using either shoots or hypocotyls, albeit at lower levels. Strains 8196 and 2659 could not induce hairy roots. However, apparent figure duplication and/or manipulation undermines the validity of these results.

Conclusions

To date, even though the number of studies on the tissue culture of *Holostemma* species is limited, the following has already been achieved: callus induction, shoot induction, root induction and cryopreservation. These processes from the basic steps for additional *in vitro* studies which should examine a much wider range of factors, both biotic and abiotic, to increase shoot production. Even though there are two studies on genetic transformation, the results are doubtful and thus more comprehensive trials are needed. The genetic stability of *in vitro*-derived plants needs to be tested with molecular markers. If *in vitro* flowering (TEIXEIRA DA SILVA *et al.* 2014) can be achieved, then floral tissue for novel *in vitro* studies can be developed. Ultrasound or sonication (TEIXEIRA DA SILVA & DOBRÁNSZKI 2014), the use of thin cell layers (TEIXEIRA DA SILVA & DOBRÁNSZKI 2013), or the use of magnetic fields (TEIXEIRA DA SILVA & DOBRÁNSZKI 2015) to study growth and development or to maximize organogenesis are recommended.

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