Direct regeneration and Genetic transformation studies in Hemidesmus indicus (L.) R. Br. (Indian Sarasaprilla)

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Research Article

Keywords: Hemidesmus, Sarsaparilla, Regeneration, Transformation, Multiple shoots, Callus

Posted Date: June 28th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3018443/v1

Abstract

Since ages, plants continue to provide new remedies to mankind. *Hemidesmus indicus* L. R. Br. is one such plant belonging to family Apocynaceae, showing potent medicinal properties known through traditional knowledge. *Hemidesmus* is also explored for the presence of flavoring compound namely 2-hydroxy-4-methoxybenzaldehyde (HMB) which is used in pharmaceutical and nutraceutical industries. Due to anthropogenic activities, the plant has been exploited till the ridge for its ethnobotanical properties for mankind. Biotechnological intervention to conserve this endangered sps through in vitro plant cultures, micropropogation and genetic transformation studies is the pre-requite to maintain it from extinction. The objective of the study is to improve the regeneration potential and optimize the genetic transformation in *Hemidesmus indicus.* The direct regeneration of *Hemidesmus indicus* through leaf explants, nodal explants with subsequent plant regeneration using Murashige and Skoog (MS) medium supplemented with various plant growth regulators (auxins, cytokinins, and gibberellic acid), adenine sulphate, TRIA. The *Agrobacterium tumefaciens* mediated genetic transformation studies in *Hemidesmus indicus* was carried out in callus cultures using the plant expression vector pCAMBIA 1301. The caulogenic response of 78.8%, 73.3% and 71.4% was observed when the leaf explant was inoculated on MS media containing 2.3 mgL^{-1} BAP + 0.2 mgL^{-1} 2,4-D, 0.02 mgL^{-1} TRIA + 2 mgL^{-1} BAP, 1 mgL^{-1} KIN + 1 mgL^{-1} NAA respectively with creamish yellow nodular friable callus by the 4 weeks. The initiation of shoot bud was observed within three days after inoculation of nodal explant on media supplemented with 1 mgL^{-1} BAP + 0.1 mgL^{-1} NAA, 1 mgL^{-1} BAP + 0.1 mgL^{-1} NAA + 40 mgL^{-1} $AgNO_3$, 1 mgL^{-1} BAP + 0.1 mgL^{-1} NAA + 40 mgL^{-1} $AgNO_3$ + 40 mgL^{-1} adenine sulphate respectively and incubated in the dark for 2 weeks. Shoot regeneration from the leaf explants was also observed within 4 weeks after inoculation in MS medium with 1 mgL^{-1} BAP + 0.1 mgL^{-1} NAA. *Agrobacterium* mediated genetic transformation was carried out successfully in callus cultures of *H. indicus*. The transformation efficiency was found to be 26%. The efficient shoot regeneration was observed within 4 weeks and transformation study can be further applied for over expression of biosynthetic genes to enhance the bioactive components that have immense significance in pharmaceutical, nutraceutical and cosmetic industries.

Key points

- A competent, reproducible in-vitro micropropogation protocol using nodal explant from *Hemidesmus indicus* L. R. Br.have been established,
- Optimization of the *Agrobacterium* mediated genetic transformation in *H. indicus* has been achieved, which will be the prerequisite for the gene manipulation using reverse genetic approach

Introduction

Hemidesmus indicus (L.) R. Br. is an indigenous medicinal herb of Apocynaceae family known with many vernacular names such as Indian Sarsaparilla, Anantamool or Nannari or Sariva. Commonly found in India and *H. indicus* is native of South Asia. *Hemidesmus* is well studied for the presence of many

bioactive compounds in roots, such as flavonoids, pregnane glycosides, 2-hydroxy-4-methoxybenzaldehyde, 4-hydroxy 3-methoxy benzaldehyde (Nagarajan and Rao, 2003). The presence of lupeol, hemidesmine, camphor, vanillin, and rutin are also reported in these plants which are known for its antioxidant properties (Banerjee and Ganguly, 2014). The immense medicinal properties of *Hemidesmus* has been explored, the roots are rich source of ethano-medicine which are used in the treatment of fever, diarrhea, dysentery, skin ailments, respiratory problems, syphills, epileptic fits in children and as tonic in biliousness. Root, bark and stem has been used to cure urinary disorders, burning sensation and rheumatism (Das et al. 2017). Since few decades the plant is being exploited for phytochemical, clinical investigations and pharmacological studies (Sreekumar et al. 2000). The presence of 2-hydroxy-4-methoxybenzaldehyde in roots of *H. indicus* is used commercially as flavoring agents in soft drinks (Neelima et al. 2017). The *in vitro* studies using root extracts of *Hemidesmus* are known to inhibit lipid peroxidation and scavenge hydroxyl and superoxide radicals (Mary et al. 2007).

The anthropogenic exploitation of the medicinal plants has resulted in listing many of medicinally important plants as endangered. Several strategies exist to conserve the germplasm and the one of the key conservation techniques is *in vitro* tissue culture or micropropagation. In addition targeting the biosynthetic gene through gene manipulation will be a value addition for enhancing the bioactive compounds. Plant genetic transformation techniques are used agronomically to enhance traits like enhanced nutritional value, providing tolerance to abiotic or biotic stresses in several crops plants (Vain 2007; Untergasser et al. 2012).

Genetic transformation through *Agrobacterium tumefaciens* is routinely used for genetic manipulation. The exploitation of *Hemidesmus indicus* for its various pharmaceutical and nutraceutical benefits has increased risk for its extinction; hence developing an efficient regeneration protocol should be a primary focus (Umesh et al. 2014). Plant tissue culture based techniques using various explants has been employed for mass production of plants (Joshi et al. 2010). The leaf and nodal explants have been used for *in vitro* regeneration, genetic transformation and protoplast fusion studies (Kumar et al. 2010; Umesh et al. 2013; Shukla et al. 2016; Ranganatha et al. 2020). The *in vitro* regeneration studies in *Hemidesmus indicus* are meager and many a times are non-reproducible. This manuscript deals with the development of a reproducible regeneration system using various explants and optimization of the *Agrobacterium* mediated genetic transformation techniques. The study will have its avenues for various applications specially in pharmaceutical, nutraceutical and cosmetic industries.

Materials and Methods

Plant source and explants

Hemidesmus indicus plants were procured from The Foundation for Revitalization of Local Health Traditions (FRLHT) (Bengaluru, India) and are being maintained in college garden were used as plant source. The nodal explant and the young tender leaves from first to 4th node from the shoot tip of *Hemidesmus indicus* were selected as explants for regeneration. The explants were washed initially with

Tween 20 in running tap water for 20 minutes. The explants were treated with 1% Bavistin for 1h with continuous shaking. After 1h, explants were washed thoroughly and subsequent sterilization by 0.1% mercuric chloride treatment for 2 min followed by sterile water washes. The explants were later treated with 70% alcohol for 20 seconds and washed thoroughly with sterile water. The sterilized explants were inoculated on MS (Murashige and Skoog 1962) basal media supplemented with different combination and concentration of plant growth regulators and adenine sulphate (ADS) and silver nitrate ($AgNO_3$) supplemented in the medium as an additive.

In vitro callusing and regeneration

The basal MS (Murashige and Skoog, 1962) medium consisted of MS mineral salts and organics with 3% sucrose and 0.8% agar (HIMEDIA) for solidification. The basal MS medium was supplemented with different plant growth regulators viz. benzyladenine (BAP), kinetin (KIN), indole-3-Acetic Acid (IAA), 1-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), gibberellic acid (GA_3) and triacontanol (TRIA), either alone or in combination (BAP + NAA, Kinetin + NAA) and pH of all the prepared medium was adjusted to 5.8. The supplements like ADS (40 mgL^{-1}) and $AgNO_3$ (40 mgL^{-1}) were used in combinations with BAP and NAA. The medium was sterilized at 121° C (15 psi) for 30 min. The surface sterilized explants were inoculated on MS medium with various combinations and concentration for induction of callus (Table 1) and incubated in dark for 2 weeks. After 2 weeks, the cultures were exposed to light with 16/8 h photoperiod at 26 ± 2° C and maintained for 4–8 weeks with regular sub-culturing with an interval of 3 weeks. The percentage response was calculated using the following formulae.

Table 1

In vitro callusing response in nodal segments of *Hemidesmus indicus* on MS basal medium supplemented with different plant growth regulators in varying combinations.

Sl.No.	Hormonal combinations (mgL^{-1})	Degree of callusing in %	Callus response	Remarks
1	2 BAP	71.4	+++	Greenish yellow compact callus
2	4 BAP	58.3	++	Greenish yellow compact callus
3	7 BAP	53.1	+++	Yellowish green fragile callus
4	2 BAP+ 40 ADS	45.3	++	Yellowish green compact callus
5	2 BAP+ 40$AgNO_3$	38.1	++	Greenish yellow compact callus
6	2 BAP + 0.6AgNO3 + 40 ADS	28.5	++	Yellowish green compact callus
7	2 BAP + 0.1 NAA	50.0	++	Greenish yellow compact callus
8	1 BAP + 0.1 NAA	72.3	++	Greenish compact callus
9	1 BAP + 0.1 NAA + 40$AgNO_3$	68.4	++	Greenish yellow compact callus
10	1 BAP + 0.1NAA + 40 ADS	76.5	++	Greenish yellow compact callus
11	1 BAP + 0.1NAA + 40 $AgNO_3$ + 40 ADS	33.2	++	Greenish yellow compact callus
12	1BAP + 0.1NAA + 0.5GA_3	61.2	++	Green compact / fragile callus
13	1.5 BAP + 0.1NAA + 40ADS	45.3	++	Yellowish green compact callus
14	1.5 BAP + 0.1NAA + 40$AgNO_3$	40.3	++	Greenish yellow compact callus
15	2.3 BAP +0.2 2,4-D	78.8	+++	Creamish yellow fragile callus
16	1KIN + 1NAA	71.8	+++	Greenish compact callus
17	1 TDZ + 2 BAP	52.8	++	Greenish yellow compact callus good

+++; indicates excellent callusing response, ++; indicates callus induction

Sl.No.	Hormonal combinations (mgL^{-1})	Degree of callusing in %	Callus response	Remarks
18	0.02 TRIA + 2 BAP	73.3	+++	Greenish yellow friable callus
19	0.25 Zeatin + 2–2,4,D	38.4	++	Yellowish green compact callus
+++; indicates excellent callusing response, ++; indicates callus induction				

No. of organogenic cultures

$$\text{Organogenic response \% (callus/ shoots)} = \frac{}{\text{Total cultures inoculated}} \times 100$$

The surface sterilized nodal and leaf explants were inoculated for *in vitro* regeneration (Table 2). The cultures were initially incubated in dark for 10 days and shifted to light condition. The two week old regenerated multiple shoots from nodal cultures were transferred to shoot elongation medium containing various concentration and combinations of BAP, NAA, IBA, GA_3 and $AgNO_3$ (Table 2). The cultures were maintained under 16/8 h photoperiod at 26 ± 2° C, for 4–8 weeks with regular subculturing.

Table 2

In vitro shooting response in nodal segments of *Hemidesmus indicus* on MS basal media supplemented with various combinations and concentrations of plant growth regulators in varying combinations.

Sl.No	Hormonal combination (mg L^{-1})	Shooting response	Shoot Elongation response (%)	Shoot Length (cm) by 4 wks	Total no. of shoots by 4 weeks	Internodal Length by 4 wks (cm)
1	1BAP + 0.1NAA	+++	57	3.0 ± 0.2^{b}	6.0 ± 0.9^{b}	0.5 ± 0.07^{c}
2	1BAP + 0.1NAA + 40 $AgNO_3$	++	76	4.2 ± 0.83^{b}	8.0 ± 0.74^{a}	1.5 ± 0.11^{b}
3	1BAP + 0.1NAA + 40 ADS	+++	55.5	2.5 ± 0.97^{c}	4.0 ± 0.36^{c}	0.32 ± 0.09^{c}
4	1BAP + 0.5NAA + 20 $AgNO_3$	++	53	2.1 ± 0.84^{cd}	2.0 ± 0.18^{d}	0.28 ± 0.04^{c}
5	1.5BAP + 0.1NAA + 20 ADS	++	52	2.0 ± 0.79^{cd}	3.0 ± 0.26^{cd}	0.26 ± 0.034^{c}
6	1.5BAP + 0.1NAA + 40 $AgNO_3$	++	53	2.0 ± 0.67^{cd}	2.0 ± 0.15^{d}	0.25 ± 0.034^{c}
7	1BAP + 0.1NAA + 0.1 GA_3	++	73.5	4.0 ± 0.76^{b}	2.0 ± 0.14^{d}	1.2 ± 0.09^{b}
8	1BAP + 0.1 IBA + 0.1 GA_3	++	82	6.5 ± 1.3^{a}	8.0 ± 0.79^{a}	2.5 ± 0.25^{a}
9	2BAP + 20 ADS	++	54.6	2.3 ± 0.17^{c}	5.0 ± 0.68^{bc}	0.25 ± 0.02^{c}

+++; indicates excellent shooting response, ++; indicates shoot induction response. The figures represent mean ± SE (Standard Error) with 0.05% level of significance

Rooting and Acclimatization

After elongation for two weeks the individual regenerated shoots from nodal and leaf cultures were separated and transferred to rooting medium containing half strength MS supplemented with (0.5- 4 mgL^{-1}) IBA solidified with 0.7% agar. The well rooted plants were transferred to plastic pots containing sterile soil rite mixture for 1–3 weeks. The plants after hardening were transferred and are being maintained in green house.

Agrobacterium tumefaciens mediated genetic transformation

Agrobacterium tumefaciens EHA105 harboring pCAMBIA 1301 (containing GUS gene driven by 35SCAMV promoter with NOS terminator sequences) were grown on Luria Bertini (LB) (HIMEDIA) Agar plates (20 mgL^{-1} Rifampicin + 50 mgL^{-1} Kanamycin) for 48hr at 28^0 C. The *Agrobacterium* cells were inoculated in MS liquid containing MES (2-(N-morpholino) ethanesulfonic acid, buffer (2 gL^{-1}) supplemented with 2.3 mgL^{-1} BAP + 0.2 mgL^{-1} 2,4-D (co-cultivation medium) and acetosyringone (100µM). The actively growing nodular callus obtained from leaf explants were selected for *Agrobacterium* transformation and were pre-cultured for 48h in the MS media containing 2.3 mgL^{-1} BAP + 0.2 mgL^{-1} 2,4-D. This pre-cultured callus was suspended in co-cultivation medium with *Agrobacterium* harboring pCAMBIA1301. The callus was incubated at 25^0 C in orbital shaker at 70 rpm for 20 min. After 20 min, the calli were blotted on sterile tissue papers and inoculated on co-cultivation medium with 100 µM acetosyringone. The co-cultivated calli were maintained in dark for 2 days at 23^0C. After 2 days, callus was thoroughly washed with sterile distilled water and finally washed with cefotaxime (250 mg L^{-1}) containing liquid medium for an hour. The callus was inoculated on MS basal medium with (2.3mgL^{-1} BAP + 0.2 mgL^{-1} 2,4-D) along with cefotaxime (250 mgL^{-1}) and hygromycin (30 mgL^{-1}) for selection. The co-cultivated callus was incubated in dark at 25° C ± 2° C for 4 weeks. Browning of callus tissue was the major intrusion during *Agrobacterium* infection and was reduced by augmentation with different additives like activated charcoal (AC); ascorbic acid (AsA); $AgNO_3$; Amino Oxyacetic Acid (AOAA); citric acid (CA) and poly vinyl pyrrolidone (PVP) to MS basal medium containing 2.3 mgL^{-1} BAP + 0.2 mgL^{-1} 2, 4-D (Table 3).

Table 3
Augmentation of various adjutants to combat tissue browning during *Agrobacterium* infection after 48 h of dark incubation

Sl. No.	MS basal with 2.3 mg L^{-1} BAP + 0.2 mg L^{-1} and 2, 4-D (M) supplemented with	Browning of the tissue after 48h of *Agrobacterium* co-cultivation
1	Maltose18%	+++
2	0.1%PVP	+++
3	0.1% activated charcoal(AC)	++
4	50 mg L^{-1} Ascorbic acid(AsA)	++++
5	0.1% $AgNO_3$	++
6	1 mg ml^{-1} Amino oxyacetic acid(AOAA)	++
7	50 mg L^{-1} Citric acid(CA)	+++
8	0.1%PVP + 0.1% AC	++
9	0.1%PVP + 50 mg L^{-1} AsA	+++
10	0.1%PVP + 0.1% $AgNO_3$	+++
11	0.1%PVP + 1 mg ml^{-1}AOAA	+++
12	0.1%PVP + 50 mg L^{-1} CA	++++
13	0.1%PVP + 0.1% AC + 50 mg L^{-1} AsA	++++
14	0.1%PVP + 0.1% AC + 50 mg L^{-1} CA	++++
15	0.1%PVP + 50 mg L^{-1} AsA + 50 mg L^{-1} CA	++++
16	0.1%PVP + 0.1% AC + 0.1% $AgNO_3$	++
17	0.1%PVP + 0.1% AC + 50 mg L^{-1} AsA + 50 mg L^{-1} CA	++++
18	0.1% PVP + 50 mg L^{-1} AC + 50 mg L^{-1} AsA + 0.1% $AgNO_3$	++++
19	0.1%PVP + 0.1% AC + 50 mg L^{-1} AsA + 50 mg L^{-1} CA + 0.1% $AgNO_3$	++++

++ Less browning of tissue; +++More browning tissue; ++++Maximum browning tissue with leaching to media

Sl. No.	MS basal with 2.3 mg L^{-1} BAP + 0.2 mg L^{-1} and 2, 4-D (M) supplemented with	Browning of the tissue after 48h of *Agrobacterium* co-cultivation
20	0.1%PVP + 50 mg L^{-1} CA + 50 mg L^{-1} AsA + 0.1% $AgNO_3$ + 1 mg ml^{-1}AOAA	+++

++ Less browning of tissue; +++More browning tissue; ++++Maximum browning tissue with leaching to media

Histochemical assay / β-Glucuronidase (GUS) activity assay

Histochemical *GUS* assay was carried out as per Jefferson et al. (1987). The actively dividing transformed *Hemidesmus* callus cultures after three successive sub-culturing that were maintained on MS media supplemented with hygromycin (20 mgL^{-1}) was used for GUS assay. The calli was washed with ice cold 80% acetone and subsequently rinsed with *GUS* buffer followed by treatment with *GUS* substrate, 5-bromo-4-chloro-3-indolyl- β-D-glucuronide (X-Gluc) in the *GUS* staining buffer (100 mM sodium phosphate, 1 mM X-Gluc, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10mM ethylene diamine tetraacetic acid disodium salt (Na_2EDTA), maintained at pH 7.0). The cells were incubated for 12 h at 37^0C. After incubation, the callus was rinsed with 70% ethanol and images were captured. Untransformed calli were used as control.

Confirmation for the integration of the transgene

The integration of transgene was confirmed by polymerase chain reaction (PCR) of genomic DNA using modified CTAB method from the callus culture after three successive sub-culturing on antibiotic medium (modified Keb-Llanes et al. 2002). PCR was carried out with genomic DNA as the template using *GUS* gene specific primers, FP (5'-TAGAGATAACCTTCACCCGG-3') and RP (5'-CGCGAACTGTGGAATTGA-3'). The PCR reaction contained genomic DNA as a template, Taq 2X Master mix red (AMPLIQON, Denmark) containing 1X reaction buffer, 1.5mM $MgCl_2$, 0.2mM each dNTPs, DMSO 2.5%, 0.5µM of forward primer and reverse primer each and 0.2 $µl^{-1}$ *Taq* Polymerase. The conditions for PCR was as follows : denaturation at 94° C for 1 min, annealing at 55° C for 1 min followed by extension/elongation at 72° C for 1 min, final extension at 72° C for 5 min and the PCR was carried out for 25 cycles. The PCR amplified products were resolved on agarose electrophoresis (1.2% agarose gel) and image was captured in gel imager system (Bio Rad).

Statistical analysis

All the experiments were carried out in triplicates with all the combination and results were calculated by Analysis of variance (ANOVA) and mean separations were carried out using Tukey's test with 0.05% level of significance.

Results

Hemidesmus indicus is an endangered recalcitrant medicinal plant with less regeneration potential. Present study deals with the optimization of tissue culture mediated regeneration and genetic transformation through *Agrobacterium tumefaciens* for *Hemidesmus indicus*.

Response of H. indicus leaf explants to various plant growth regulators

Callus induction was observed in *H. indicus* maintained on MS basal medium with varied concentration and combination of plant growth regulators (Table 1). The leaf explant dedifferentiated and initiated proliferation of callus within 8–10 days after culturing on MS media supplemented with different combinations of plant growth regulators, with varying degree of the callusing response. The type of callus formed in tissue culture is explant and species dependent, but can be altered by changing the concentration and combination of plant growth regulators in the medium. Both friable and compact callus were produced from the leaf explants of *H. indicus* in response to plant growth regulators (Fig. 1).

The caulogenic response was higher in 2.3 mg L^{-1} BAP + 0.2 mg L^{-1} 2,4-D (78.8%) with creamish yellow nodular friable callus by 4 weeks, followed by 0.02 mg L^{-1} TRIA + 2 mg L^{-1} BAP (73.3%), 1 mg L^{-1} KIN + 1 mg L^{-1} NAA (71.8%) and 2 mg L^{-1} BAP (71.4%) combinations (Table 1). BAP individual and its combination with NAA, ADS produced yellowish green compact callus, whereas BAP, NAA with $AgNO_3$ formed greenish yellow callus (Table 1). 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA (Fig. 1a), 1 mg L^{-1}KIN + 1 mg L^{-1} NAA (Fig. 1b) and 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA + 0.5 mg L^{-1} GA_3 (Fig. 1c) combinations produced more greenish callus compared to other plant growth promoters. But in 2, 4-D combinations creamish yellow or yellowish friable (2.3 mg L^{-1} BAP + 0.2 mg L^{-1} 2, 4-D) callus was observed which were highly suitable for cell suspension and genetic transformation studies (Fig. 1h). However no apparent regeneration was observed from callus produced by various growth regulators studied. However, these calli did not showed any morphogenetic differentiation even after 2 months with regular sub-culturing. The addition of plant growth modulators such as ADS and $AgNO_3$ together with 1 mg L^{-1}BAP + 0.1 mg L^{-1} NAA showed less callusing (33.2%) in both nodal and leaf explants, compared to 1 mg L^{-1} BAP + 0.1 mg L^{-1}NAA + 40 mg L^{-1}ADS (76.5%), 1 mg L^{-1}BAP + 0.1 mg L^{-1} NAA + 40 mg L^{-1} $AgNO_3$ (68.4%). 2 mg L^{-1} BAP + 40 mg $L^{-1}$$AgNO_3$ (38.1%) are less effective compared to 2 mg L^{-1} BAP + ADS 40 mg L^{-1}(45.3%) (Table 1 ; Fig. 1d-e).

In vitro regeneration of plantlets and its acclimatization

For *in vitro* regeneration from the nodal explants, response was observed in all the combinations studied. The initiation of shoot bud was observed within three days after inoculation on MS media supplemented with 1mg L^{-1} BAP + 0.1 mg L^{-1} NAA (Fig. 2a-b), 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA + 40 mg L^{-1} $AgNO_3$, 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA + 40 mg L^{-1} $AgNO_3$ + 40 mg L^{-1} ADS when incubated in the dark for 2 weeks. However, in the combination of 2 mg L^{-1} BAP + 0.5 mg L^{-1} GA_3 showed delayed response compared to other combinations. By two weeks multiple shoot induction with 3–4 nodes were observed. After 2 weeks of dark incubation the multiple shoot were transferred to medium containing 1 mg L^{-1} BAP

+ 0.1 mg L^{-1} NAA + 40 mg $L^{-1}AgNO_3$ (Fig. 2d) for a week. After a week there was an increase in internodal length along with leaf expansion (Table 2). The sub-culturing of multiple shoots to media containing 1 mg L^{-1} BAP + 0.1 mg L^{-1}IBA + 0.1 mg L^{-1} GA_3 for 2 weeks showed an increased internodal length of 2–3 cm with broad leaves along with lateral branching (Fig. 2e). The repeated sub-culturing of the regenerated shoots to MS medium with 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA resulted in multiple shooting.

Shoot regeneration from the leaf explants was also observed by 4 weeks after inoculation in MS medium 1 mg L^{-1} BAP + 0.1 mg L^{-1}NAA with dark incubation for 10 days and later shifted to light that enhanced good regeneration and callusing. The eight week old regenerated shoots were shifted to rooting media containing half strength MS medium supplemented with 0.5 mg L^{-1} IBA (Fig. 2f-g). The root initiation was observed within 10 days in 0.5 mg L^{-1} IBA and with increased concentration of IBA (4 mg L^{-1}) rooting was delayed by a week. Addition of plant growth and dark incubation increased shoot morphogenesis and elongation of the shoots. The plant lets were acclimatized and hardened in soilrite mixture (Fig. 2h).

Optimization of Agrobacterium tumefaciens mediated genetic transformation in H. indicus callus culture

Optimization of *Agrobacterium* mediated genetic transformation was successfully carried out in actively growing friable calli that were obtained from leaf explants inoculated on MS basal medium supplemented with 2.3 mg L^{-1} BAP + 0.2 mg L^{-1} 2,4-D. *Agrobacterium tumefaciens* EHA 105 strain harboring binary vector pCAMBIA 1301 with 35S CAMV promoter driving β-glucurodinase (*GUS*) gene expression and hygromycin phosphotransferase (*hptII*) gene as antibiotic selection marker was used in the study. The actively growing calli were mobilized with *Agrobacterium tumefaciens* harboring pCAMBIA 1301 plasmid, for 20 min and co-cultivated in the 2.3 mg L^{-1} BAP + 0.2 mg L^{-1} 2,4-D media with acetosyringone (100 µM) and maintained in dark for 48h at 23° C. The dark incubation at 23^0C was found to be more effective in controlling tissue browning.

After co-cultivation, the callus were thoroughly washed with sterile distilled water and finally washed with cefotaxime (250 mg L^{-1}) containing liquid MS basal medium for an hour. Putative transformed calli were transferred to hygromycin (30 mg L^{-1}) and cefotaxime (250 mg L^{-1}) containing MS basal media (2.3 mg L^{-1} BAP + 0.2 mg L^{-1} 2, 4 -D) for selection and sub-cultured regularly for 3–4 weeks.

The putative transformed callus cultures were assayed for histochemical *GUS* gene expression. The transformed and non-transformed callus tissues of *Hemidesmus* were immersed in ice cold 80% acetone solution, followed by wash with *GUS* buffer and then treated with 5- bromo, 4-chloro,3- indolyl D –glucuronide (X-Gluc -GUS substrate) and incubated at 37° C in dark for 12 h. After 12h of incubation the transformed callus had turned blue and showed positive for *GUS* histochemical assay (Fig. 3). During the histochemical reaction, the substrate 5- bromo, 4-chloro,3- indolyl D –glucoronide. X-Gluc is hydrolyzed into the product 5,5'-dibromo-4,4'-dichloro-indigo (diX-indigo) in presence of enzyme β-glucuronidase, (GUS) and DiX-indigo will appear blue. The *H. indicus* transformed cells harboring the GUS gene express

the protein GUS, that hydrolyze X- Gluc to diX-indigo and cells turn blue which is a conformation for the transformantion. To confirm the presence of *GUS* transgene, PCR was carried out with *GUS* specific primer using callus genomic DNA as template. Genomic DNA was isolated and PCR was carried out with denaturation at 94°C for a minute followed by annealing at a temperature of 55°C and elongation/ extension at 72°C. The cycle was repeated for 24 cycles. The PCR products were resolved on 1.2% agarose gel to check the presence of partial GUS gene for the transformation efficiency. The amplified PCR products of 650bp were observed in callus transformed with *Agrobacterium* (Fig. 4). The percentage of *Agrobacterium* mediated genetic transformation in callus was observed to be 26%.

Discussion

Since five decades, the extensive use of *Hemidesmus indicus* in pharmacological research have been reported which has been supported by the wide array of its ethno-medicinal applications (Bonvicini. et al. 2018). The major constraint in its improvement through *in-vitro* micropropagation of medicinal plants has been their recalcitrant nature. *In vitro* regeneration of recalcitrant medicinal plant like *H. indicus* is meager hence regeneration method was developed which can be harnessed for mass multiplication of the plant and to prevent the plant becoming extinct. The optimization of genetic transformation through *Agrobacterium* mediated genetic transformation technique can be applied to express biosynthetic genes so as to enhance the economically important bioactive compounds from the cultures.

In general high auxins and low cytokinins concentrations are used in callus induction and high cytokinins and low auxins in shoot bud initiation. However the callus induction and shoot regeneration potential vary with genotype, selection of explant and also the growth regulator concentration and combinations used in the media. In the present study the *in vitro* callus formation and regeneration response to various cytokinins, auxins, gibberellins with supplements like ADS and $AgNO_3$ were studied in *Hemidesmus indicus*. However, the different explants sub-cultured on MS basal medium without any growth hormones did not show any response. In the current investigation, induction of callus was initiated in leaf explants within 8–10 days after culturing on MS medium supplemented with different combinations of plant growth regulators (Table 1). A synergistic effect of auxin and cytokinin based growth regulators showed higher degree of caulogenic response of 78.8% in 2.3 mg L^{-1} BAP + 0.2 mg L^{-1} 2,4-D with creamish yellow nodular friable callus within 4 weeks (Table 1; Fig. 1). In contrary to this similar combination with 13.50 µM 2, 4-D and 4.50 µM BAP resulted in 93.75% of callus induction percentage with yellow friable callus and was an ideal medium for callus induction in *Hymenocallis littoralis* (Jeevandran et al. 2012). Similarly Manisha and Rajesh, (2013) also obtained maximum callus response of 91.2% on MS medium supplemented with 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} 2,4-D from leaf explants of *Tecomella undulate* (Sm.) Seem. Induction of the somatic embryogenesis was observed in Saffron (*Crocus sativus* L.) on MS media supplemented with 2 mg L^{-1} 2, 4-D + 1 mg L^{-1} BAP (Rajaboor et al. 2007).

The use of additives like silver nitrate and adenine sulphate is known to inhibit ethylene action, thence widely used to induce formation of somatic embryos in plant tissue culture (Table. 1) and rooting, which

are the prerequisite for successful genetic transformation (Vinod et al. 2009). The effect of ADS and $AgNO_3$ as plant growth modulators, on callus induction was studied in *H. indicus* leaf explants. Addition of ADS/$AgNO_3$ to basal MS media with 1 mg L^{-1}BAP + 0.1 mg L^{-1} NAA combinations induced 76.5% and 68.4% callus respectively. However the ADS and $AgNO_3$ together with 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA showed reduced callus induction (33.2%) in explants studied. An efficient callus induction and proliferation was observed from *Rosa hybrida* leaf explants using MS medium, in combination with 19.60 µM IBA + 4.65 µM KIN + 108.6 mM ADS with enhanced callus (Mohan et al. 2015). The frequency of callus induction from leaf explants was high when it was cultured on MS medium supplemented with 2.0 mg L^{-1} NAA + 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} $AgNO_3$ in *Solanum nigrum* (L.) (Geetha et al. 2016). The nodal explants are used for *in vitro* propagation of various medicinal plants such as *Salvadora persica* (Phulwaria et al. 2011), *Caralluma edulis* (Patel et al. 2014) and *Pluchea lanceol* (Kher et al. 2014). Cytokinins are known to activate the axillary bud exogenously, and among all cytokinin based growth regulators BAP is reported to induce shoot bud initiation and proliferation and has been well studied in several aromatic and medicinal plants such as *Menthavirdis* (Raja et al. 2008), *Eclipta alba* (Hussain and Anis, 2006) and *Prosaleacorydi folia* (Jebakumar and Jayabalan 2000). In the present study, *in vitro* regeneration of nodal explants was observed in all combinations studied with varying degree of response (Table 2). Among the combinations studied, 1mg L^{-1} BAP + 0.1 mg L^{-1} NAA alone and in combination with 40 mg L^{-1}ADS were found to induce better shooting response. However, addition of 40 mg L^{-1} $AgNO_3$ and 40 mg L^{-1}ADS in basal MS media along with 1mg L^{-1} BAP + 0.1 mg L^{-1} NAA showed multiple shoot induction (Table 2; Fig. 2). The 2–3 successive sub-culturing of the original explant to fresh media initiated multiple shoots. Riphi and Anis (2012) observed 5.0 µM BAP with 0.26 µM NAA was the most effective medium for maximum shoot regeneration (81.3%) in *Althaea officinalis* L. Many research findings demonstrated that combination of BAP + NAA with varying concentrations induces axillary shoot/multiple shoot regeneration from nodal explants. Karuppusamy and Pullaiah (2007) observed highest frequency of lateral shoots of 74% for nodal and 70% for shoot tip explants in *Bupleurum disticho-phyllum* Wight, when inoculated on the MS medium containing 1.0 mg L^{-1}BAP + 0.1 mg L^{-1}NAA. Inoculation of nodal and leaf explants of *Centella asiatica* L on MS media supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} NAA combination resulted in maximum number of multiple shoots (Mohapatra et al. 2008). *In vitro* propagation studies with nodal segments as explants in *Catharanthus roseus* L. G. Don. showed maximum number of multiple shoot with 0.5 mg L^{-1}BAP + 1.0 mg L^{-1}NAA (Mohammed et al. 2011). In contrast to our study, Sindhu et al. (2016) observed maximum shoot multiplication of *H. indicus* (10.25 ± 1.02) on MS medium supplemented with 1.0 mg L^{-1}BAP and 1.0 mg L^{-1} KIN.

The effect of ADS and $AgNO_3$ as additives with plant growth regulators on multiple shoot induction in nodal explants of *H. indicus* was assessed in the present study. Shooting response of 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA + 40 mg L^{-1}ADS was in line with 1mg L^{-1} BAP + 0.1mg L^{-1}NAA; with respect to shoot elongation, 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA + 40 mg $L^{-1}$$AgNO_3$ showed 76% response compared to 1 mg

L^{-1} BAP + 0.1 mg L^{-1} NAA + 40 mg L^{-1} ADS (55.5%) (Table 2). Several findings demonstrated the use of ADS and/or $AgNO_3$ in shoot differentiation and multiple shoot proliferation. Similarly, Pooja et al. (2017) obtained maximum regeneration frequency in MS media augmented with 2.5 mg L^{-1} BAP + 0.1 mg L^{-1} NAA + 1 mg L^{-1} ADS in petiole segments of *Pelargonium graveolens*. The combination of BAP (1–4 mg L^{-1}) with 40 mg L^{-1}ADS in combination with NAA/IAA gave better shoot differentiation in *Simmondsia chinensis* L. Schneider (Raman et al. 2015). Exogenous application of $AgNO_3$ to axillary buds of *Cichorium intybus* L. cv. Lucknow proved to increase shoot numbers and shoot multiplication (Bais et al. 2000). In contrast to our findings a combination of ADS and $AgNO_3$ produced enhanced number of multiple shoots in cotyledonary nodes of *Milletia pinnata* L. Panigrahi (Nagar et al. 2015). Mahdi et al. (2018), found good frequency of direct shoot formation (73.8%) in cotyledon explants of *Cosmos bipinnatus* on MS media containing BAP (5 mg L^{-1}) and $AgNO_3$ (5 mg L^{-1}) with 20 mg L^{-1} ADS and highest number (5.7) of shoot per explant on 5 mg L^{-1} BAP + 5 mg $L^{-1}$$AgNO_3$ + 40 mg L^{-1} adenine supplemented MS media. However influence of ADS (45 mg L^{-1}) alone in MS media produced 79.8% multiple shoot induction in *Clitoria ternatea* L. and proved best plant growth stimulant for *in vitro* regeneration (Arockiam et al. 2018). Prashanthi et al. (2017) induced multiple shoot regeneration in the MS medium supplemented with 4 mg L^{-1} adenine sulphate and 2mg L^{-1} BAP combinations in nodal explants of *H. indicus*. Similar to our findings, maximum number (15.6) of multiple shoots were induced on MS medium supplemented with 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA + 0.4 mg L^{-1} $AgNO_3$ in *Solanum nigrum* L (Geetha et al. 2016).

Gibberillic acid is known to play prominent role in elongation of cells. In the present investigation, the combination of BAP and GA_3 with NAA/IBA was used to study the effects on multiple shoot induction and elongation in *H. indicus* (Table 2). Our findings indicated that the combination of 1 mg L^{-1} BAP + 0.1 mg L^{-1} IBA + 0.1 mg L^{-1} GA_3 was favourable in inducing shoot elongation response (82%) compared to all other combination studied with increased shoot length (6.5cm) and internodal distance (2.5 cm) in 4 weeks followed by 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} GA_3 (73.5%) (Table 2). In grapevine, Nadra et al. (2015) reported shoot formation (5.33) with elongation (2.75 cm) in the medium supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} GA_3. In contrast to our findings, Ines et al. (2015) also observed highest micro shoot multiplication rate with significantly longer shoots with more number of leaves on MS medium containing 0.5 mg L^{-1} BAP, 0.1 mg L^{-1} NAA, and 0.1 mg L^{-1} GA_3 in *Cassia absus* L. Karuppusamy and Pullaiah (2007) found that combination of 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} GA_3 was effective micropropogation.

The explants responded well to dark followed by light incubation and showed better callus induction and regeneration. In line with our observation, Mizuri et al. (2012) noticed that dark incubation period was essential for shoot regeneration for different varieties of sweet orange and grapefruit. They also identified addition of $AgNO_3$ improved shoot regeneration. Similar findings were observed in regeneration of

common bean (*Phseolous vulgaris* L.) using thin-cell-layer culture and silver nitrate (Cruz de Carvalho, 2000).

The callus cultures were genetically transformed through *Agrobacterium tumefaciens* (EHA105) harboring pCAMBIA 1301 with 35S CAMV promoter driving the expression of *GUS* gene with hygromycin as the selection marker. Validation of the transgenic callus was carried out through *GUS* histochemical assay and further confirmed through genomic DNA PCR using *GUS* gene specific primers and the percent efficiency of the transformation was 26%. In line with our study genetic transformation of *Withania somnifera* nodal explants with *A. tumefaciens* GV3101 strain showed 10.6% transformation efficiency (Mishra et al. 2016). In a tree species *Neolamarckia cadamba*, genetic transformation with *A. tumefaciens* strain GV3101 harboring pCAMBIA1305 with reporter genes *GUS* and fluorescent protein *YFP*, showed 5.4% transformation frequency (Li et al. 2019). Niazian et al. (2019) have observed 2.94% transformation efficiency in *Agrobacterium* mediated genetic transformation which was achieved in medicinal plant *Trachy spermumammi* (L.). Wissam et al. (2012) observed a transformation efficiency of 1.8% after co-cultivating leaf explants of *Centaurea montana* with *Agrobacterium tumefaciens* strain AGL1 harboring isopentenyl transferase gene with hygromycin resistance. In recent years, advances in plant genetic engineering have opened new avenues for crop improvement and various plants with novel agronomic traits have been produced. In the current study the *Agrobacterium* mediated genetic transformation was carried out using pCAMBIA1301 vector. We were successful in transforming the callus cultures which was evident through early detection of the putative transformed callus by *GUS* histochemical assay. The blue color developed upon incubation in *GUS* buffer indicated the activity of β-glucurodinase enzyme thereby conferring efficient transformation (Fig. 3). The transformed calli was further authenticated through genomic DNA PCR.

In this study, an efficient, reproducible, competent regeneration system protocol for the development of shoot culture from various explants of *H. indicus* has been established along with optimizing the *Agrobacterium* mediated genetic transformation; further this work can be extended to overexpression of genes from other plants so as to enhance the level of bioactive compounds. Genetic modifications of the plant by targeting the regulatory or biosynthetic genes offer a significant strategy to harness enhanced production of vital bioactive compounds. Elicitation of key bioactive metabolites from the same which will carve the niche for cosmetic, pharmaceutical and nutraceutical industry.

Declarations

Data Availability Statement (DAS)

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Acknowledgement:

Authors are thankful to RSST and institute for all the support and providing the infrastructure for conduction of the experiments

Author contributions:

NNR, AS designed research; NNR, AS, MR performed research, BER and MSS analysed the data; and NNR, AS wrote the paper

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Figures

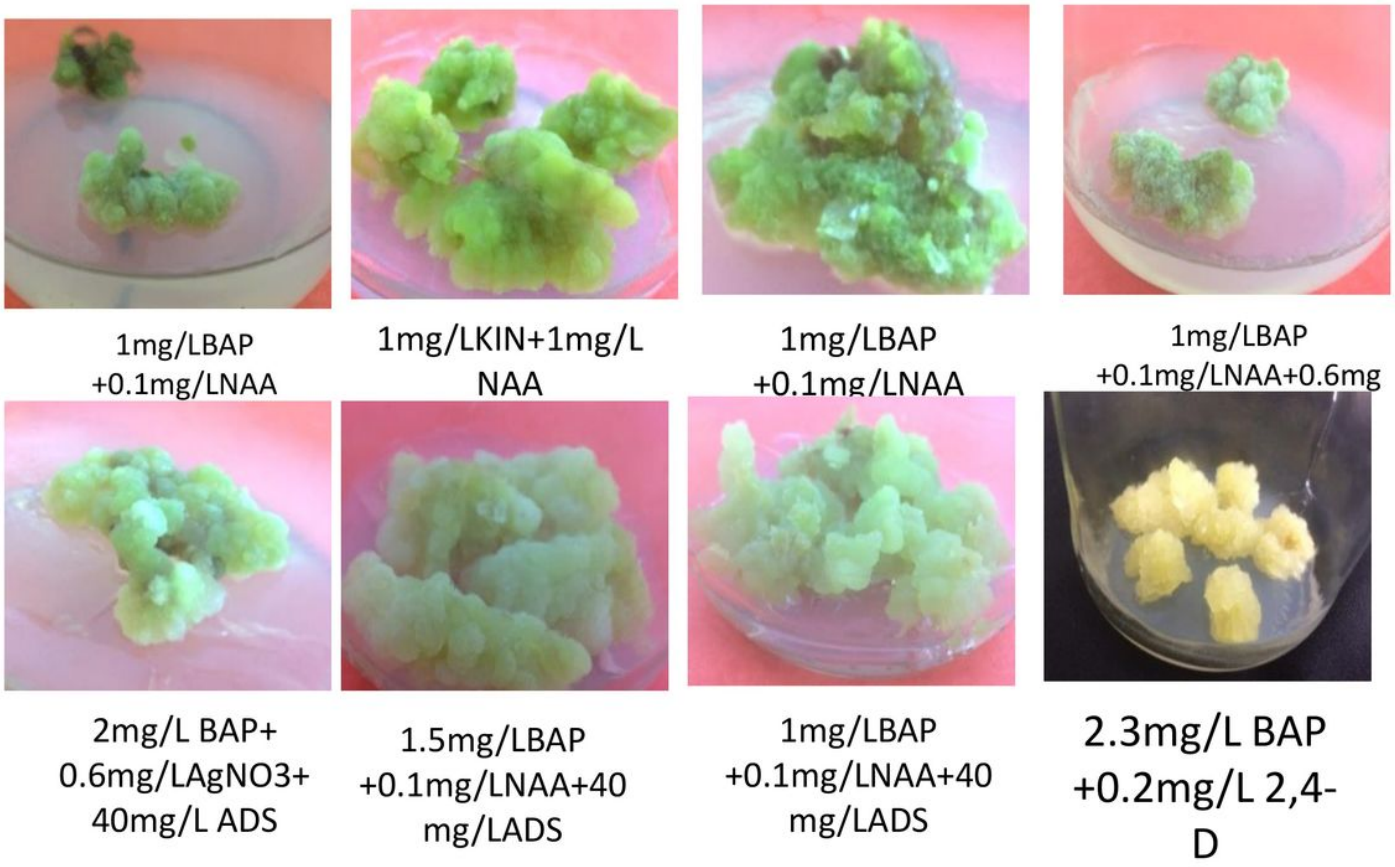

Figure 1

Different types of callus formation from leaf explants of *Hemidesmus indicus* on MS media augmented with various plant growth regulators. A) Greenish callus from 1 mg L^{-1} BAP and mg L^{-1} NAA ; B) Greenish yellow callus from mg L^{-1} KIN and mg L^{-1} NAA ; C) Greenish yellow callus from mg L^{-1} BAP +0.1 mg L^{-1} NAA +0.5 mg L^{-1} GA_3 ; D) Greenish yellow callus from 1 mg L^{-1} BAP +0.1 mg L^{-1} NAA+ 40 mg L^{-1} $AgNO_3$; E) Yellowish green callus from 2 mg L^{-1} BAP +40 mg L^{-1} $AgNO_3$ + 40 mg L^{-1} ADS ; F) Yellowish green callus from 1.5 mg L^{-1} BAP +40 mg L^{-1} $AgNO_3$ + 40 mg L^{-1} ADS ; G) Greenish yellow callus from 1 mg L^{-1} BAP +0.1 mg L^{-1} NAA+40 mg L^{-1} ADS ; H) Creamish yellow fragile callus from 2.3 mg L^{-1} BAP +0.2 mg L^{-1} 2,4-D.

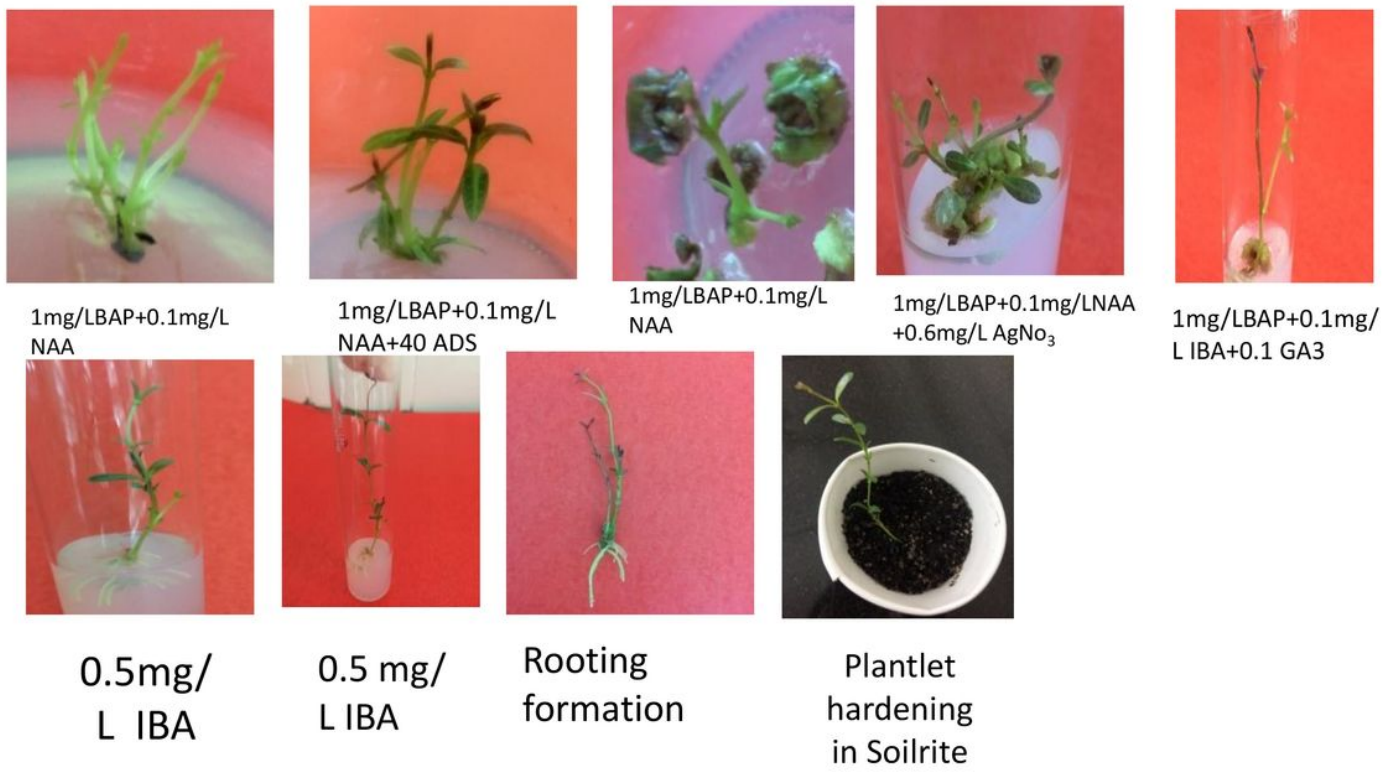

Figure 2

In vitro regeneration studies of *Hemidesmus indicus* from nodal explants. A-B) Multiple shoots induction from nodal explant and shoot regeneration from leaf explant on MS media augmented with 1mg L^{-1} BAP+0.1mg L^{-1} NAA ; C-D) Shoot morphogenesis with leaf and shoot elongation with lateral branching on MS media with 1 mg L^{-1} BAP+0.1mg L^{-1} NAA+ 40 mg L^{-1} ADS and 1mg L^{-1}BAP+0.1mg L^{-1}NAA+40 mg L^{-1} $AgNo_3$; E) Elongation with increased internodal length on 1mg L^{-1} BAP+0.1mg L^{-1} IBA+0.1 mg L^{-1} GA_3 containing MS media ; F-G) *In vitro* rooting on ½ MS media with 0.5 mg L^{-1} IBA ; H) Hardening of the plant let in sterile soilrite.

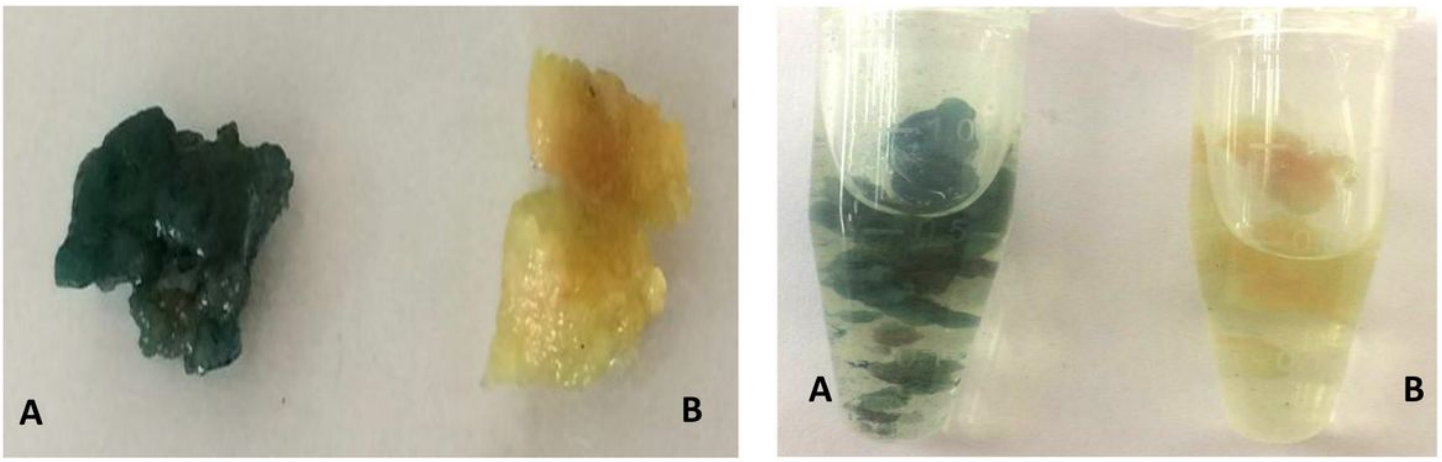

Figure 3

GUS histochemical assay in H. indicus Agrobacterium tumifaciens co-cultivated Hemidesmus indicus callus sample. In the histochemical assay, hydrolysis of X-Gluc substrate by GUS enzyme results in formation of an insoluble and coloured indigo dye, visualized as a blue precipitate at the site of enzyme activity that is easily detectable. **A.** transgenic calli and B **is** untransformed control callus

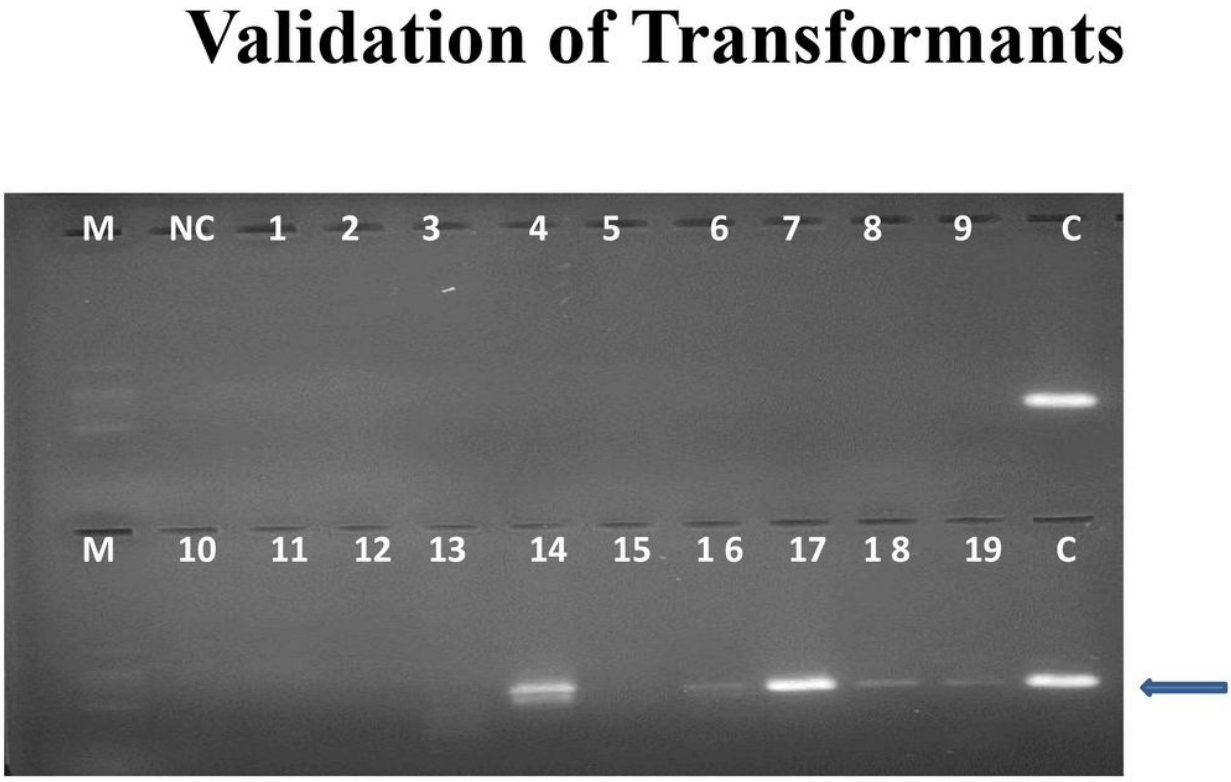

Figure 4

Genomic DNA was isolated and PCR was carried out using GUS gene specific primers. M : Molecular marker, NC is genomic DNA from untransformed cells. 1 -19: Putative transgenic calli #14,16-19 shows the presence of partial GUS gene spanning 680bps amplicon authenticating the presence of GUS transgene and the transformation percentage was approximately 26% . pCAMBIA1301 DNA as positive control. Arrow indicates the amplification of *GUS* gene (650bp).