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Augmenting a competent *in vitro* organogenesis etiquette from leaf base of country mallow, *Abutilon indicum* L. sweet: An ethno-botanically valuable medicinal plant

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ABSTRACT

An easy, efficient and highly reproducible regeneration system was established through organogenesis from leaf base mediated callus of an ethnobotanical hairy shrub *Abutilon indicum* L. sweet, which is documented to possess pharmaceutically important phytochemicals. Among various explants, leaf bases produced significant callus induction (89%) with 140 mg fresh weight on Murashige and Skoog (MS) medium supplemented with 11.31 μM 2, 4-Dichlorophenoxyacetic acid (2, 4-D). Combinations of 2, 4-D and kinetin (KIN) increased the biomass (191 mg) of embryogenic calli. Regeneration medium with a combination of 8.88 μM of 6-amino benzyl purine (BAP) and 8.06 μM of naphthalene acetic acid in MS basal media significantly influenced the initiation of shoot primordium with 91% of regeneration. Proficiently regenerated shoot apical meristems (SAMs) produced higher frequency of multiple shoot induction on 4.10 μM of zeatin. The fully regenerated mature shootlets produced high root biomass (791.67 ± 48.05 mg) on half - strength MS with 4.54 μM indole-3-butyric acid (IBA) with an excellent mat root system for efficient acclimatization. *In vitro* established plantlets were successfully acclimatized under *ex vitro* conditions with high frequency. Presence of homologous DNA banding pattern with Single Primer Amplification Reaction markers confirmed the genetic similarity. FTIR and GC-MS spectrum showed similar metabolic profiles between wild and *in vitro* regenerated plantlets. Thus the protocol is reliable for *in vitro* regeneration through leaf base derived callus which could pave a way to the large scale production of secondary metabolite through biotechnological approaches.

1. Introduction

Abutilon indicum L. a perennial sweet herb commonly called as 'country mallow', is universally found in subtropical regions of Asia, America, Australia and highly distributed in African countries including South Africa. The plant is much acclaimed for its ethnobotanical and siddha system of uses (Rao et al., 2016). Different solvent extracts of *A. indicum* are reported to show various biological activities like wound healing (Suresh et al., 2011), scavenging free radicals (Chakraborthy and Ghorpade, 2010), hepatic antioxidant (Singh and Gupta, 2008) and Ferric reducing antioxidant power (FRAP) (Ahmad and Khan, 2012). Numerous researches have reported on antibacterial properties of different solvent extracts of this plant parts against *Pseudomonas aeruginosa* (Ambarsing and Milind, 2012), *Salmonella typhi, Escherichia coli, Proteus mirabilis, Shigella* sp. and *Klebsiella pneumonia* (Abdul et al., 2010). The herb is important because of its copious pharmaceutical values. Shrikanth et al. (2014) earlier reported on pharmaceutical and *in vivo* studies with methanolic leaf extract of *A. indicum* inhibiting the

Abbreviations: PGRs, Plant growth regulators; 2, 4-D, 2,4 Dichlorophenoxyacetic acid; BAP, 6-amino benzyl purine; NAA, α Naphthaleneacetic acid; KIN, Kinetin; ZN, Zeatin; TDZ, Thidiazuron; IBA, Indole-3-butyric acid; MS, Murashige Skoog Medium; LS, Linsmaier Skoog Medium; B5, Gamborg medium; N6, Chu medium; SAM, Shoot apical meristems; SPAR, Single primer amplification reaction; ISSR, Inter simple sequence repeats; DAMD, Direct amplification of minisatellite DNA; FTIR, Fourier transform infrared spectroscopy; GC-MS, Gas chromatography Mass spectrometry; FW, Fresh weight; TC, Tissue culture; WT, wild type

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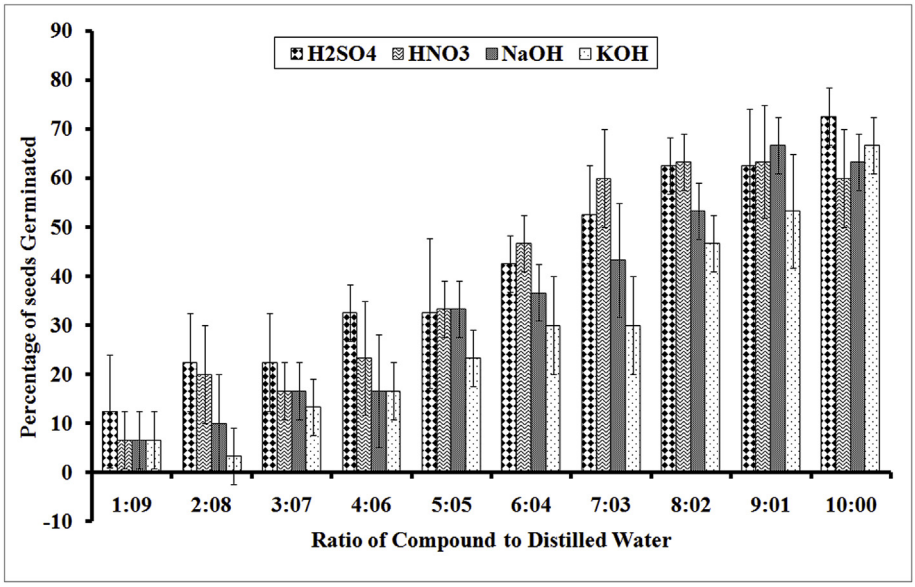

Fig. 1. Effect of different compounds on seed germination. Different ratio of compounds such as H_2SO_4, HNO_3, NaOH and NaCl with sterile distilled water used for germination efficiency testing [Compound: Water (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9)]. I Indicates standard deviation of values. * indicates significance of germination between least and the maximum ratio of the individual compounds at the level of $P < 0.5$.

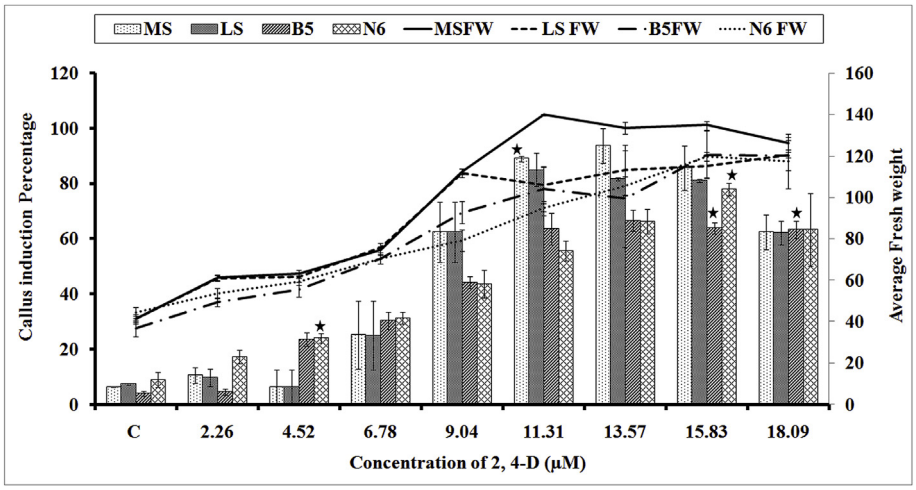

Fig. 2. Effect of synthetic hormone 2, 4-D on inducing callus. Different combination of 2, 4-D was tested on MS, LS, B5 and N6 medium for callus induction from leaf base explants. Their corresponding fresh weight was compared for significant callusing. I Indicates standard deviation of values. * indicates significance of germination between least and the maximum ratio of the individual compounds at the level of $P < 0.5$.

toxic enzymes of the *Echis carinatus* proving its anti-venom potential. Methanolic extracts of this important plant has proven anti-inflammatory (Ponnudurai et al., 2011) and anti - diarrhea activity activities (Chandrashekhar et al., 2004).

The plant has been reported for its antiproliferative (Mata et al., 2015), anti-arthritic (Bhajipale, 2012), antimicrobial (Abdul et al., 2010; Mateen et al., 2011), hepatoprotective (Porchezhian and Ansari, 2005), anti diabetic (Krisanapun et al., 2011), anti-convulsant (Bhajipale, 2012), larvicidal (Abdul Rahuman et al., 2008; Arivoli and Tennyson, 2011), wound healing (Suresh et al., 2011), anti asthmatic (Mehta and Paranjape, 2008), immunomodulatory (Dashputre and Naikwade, 2010), and anti-estrogenic activities (Mehta and Paranjape, 2008). Khanduri (2014) reported that *A. indicum* seed powder inhibits the ovarian function, changes the uterine structure and prevents implantation thus control the fertility of female albino rats. Numerous researchers have been reported different solvent extracts of *A. indicum* with their wide use in the phytoremediation, mosquito larvicidal, *in vivo* stimulation of insulin and antioxidant activity in rats against oxidative stress (Abdul Rahuman et al., 2008; Adisakwattana et al., 2009; Kaushik et al., 2011; Krisanapun et al., 2009; Seetharam et al., 2002; Varun et al., 2015).

In vitro micropropagation is one of the important tools of tissue culture for the production of large number of genetically identical plants (with or without somaclonal variation) and also to increase its principle components. Though a considerable number of reports are available for callus induction, regeneration and micropropagation (Rout et al., 2009; Ramar and Ayyadurai, 2015; Rao et al., 2016; Sudarshana et al., 2016), till date no effort has been made to evaluate the genetic fidelity of *in vitro* derived *A. indicum*. The success and efficiency of any tissue culture protocol relies on the reproducibility without any somaclonal variance. The major bioactive compounds and the functional groups present in the tissue cultured plants were analyzed using GC-MS and FTIR. Further the genetic stability between the *in vitro* regenerated and *ex vitro* maintained plants were confirmed using single primer amplification reaction (SPAR) markers such as RAPD (Shilpha et al., 2013) and ISSR (Guo et al., 2006).

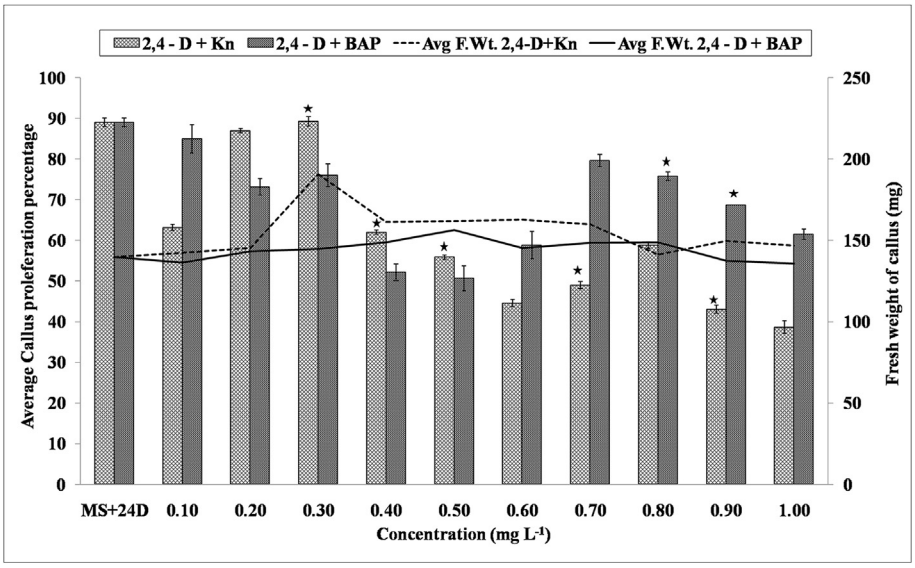

Fig. 3. Effect of combination of hormones on callus proliferation Synergistic effect of 2, 4-D with KIN and BAP were assessed for callus proliferation and fresh weight. **Note:** The concentrations mentioned here were used as mg L^{-1} and converted to µM concentration when analysis was done. For easy graphical understanding the values are depicted in mg L^{-1}. See text for more derails] I Indicates standard deviation of values. * indicates significance of germination between least and the maximum ratio of the individual compounds at the level of $P < 0.5$.

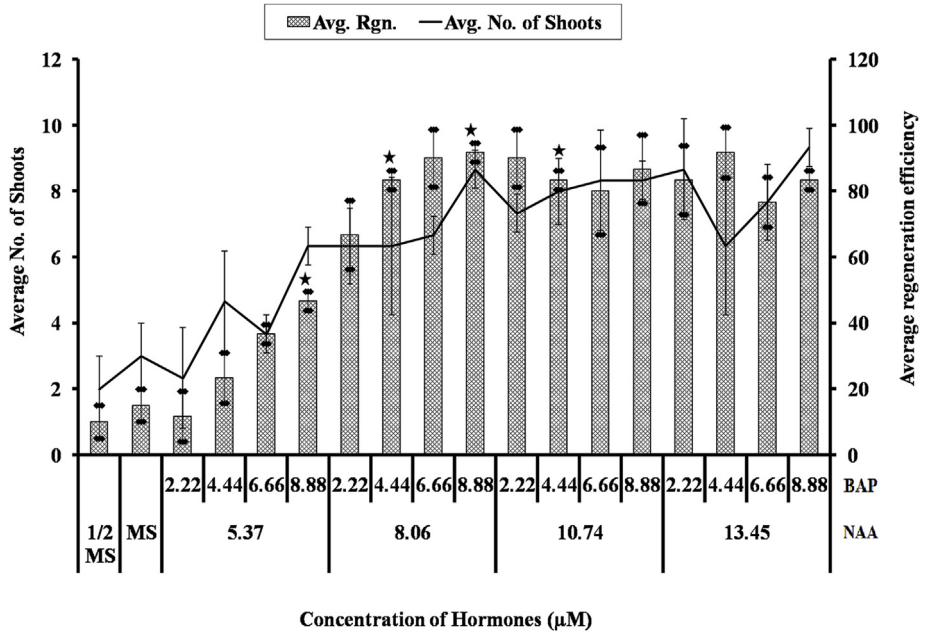

Fig. 4. Synergistic action of BAP and NAA in shoot regeneration. Combination of BAP and NAA was used to check the significant regeneration efficacy on proliferated callus I & I indicates standard deviation of values obtained for average regeneration and average no. of shoots respectively. * indicates significance of germination between least and the maximum ratio of the individual compounds at the level of $P < 0.5$.

The main aim of the present study was to establish a rapid and highly reproducible *in vitro* regeneration system from leaf base derived friable embryogenic callus of *A. indicum*. Hence, we present an appropriate protocol for *in vitro* culturing and regeneration for this ethnobotanically important plant. The results of this study will be further exploited for enhancement of secondary metabolite through biotechnological approaches for the benefit of mankind.

2. Materials and methods

2.1. Sample collection and authentication

Seeds of *A. indicum* were collected from the surrounding area of the Department of Biotechnology, Alagappa University, Karaikudi, Tamil Nadu, India. The specimen of *A. indicum* was submitted at Botanical Survey of India, Ministry of forest, environment and climate change, Southern regional centre, TNAU campus, Coimbatore - 641003. The plant specimen was identified as *A. indicum* (L.) Sweet (=*Sida indica* L.) – MALVACEAE and accession number BSI/SRC/5/23/2015/Tech.-1230.

2.2. Seed germination and selection of explant

To reduce the contamination during tissue culture (TC), seeds were surface sterilized and germinated under *in vitro* condition and the explants were collected for further studies. The tough seed coat makes it harder to germinate on autoclaved half strength MS medium (Murashige and Skoog, 1962). Therefore the seeds were treated for 30 min (150 rpm) in acids such as sulphuric acid (H_2SO_4) and Nitric acid (HNO_3) and alkali sodium hydroxide (NaOH) and potassium hydroxide (KOH) with distilled water in various ratios (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9). Followed by a wash with autoclaved

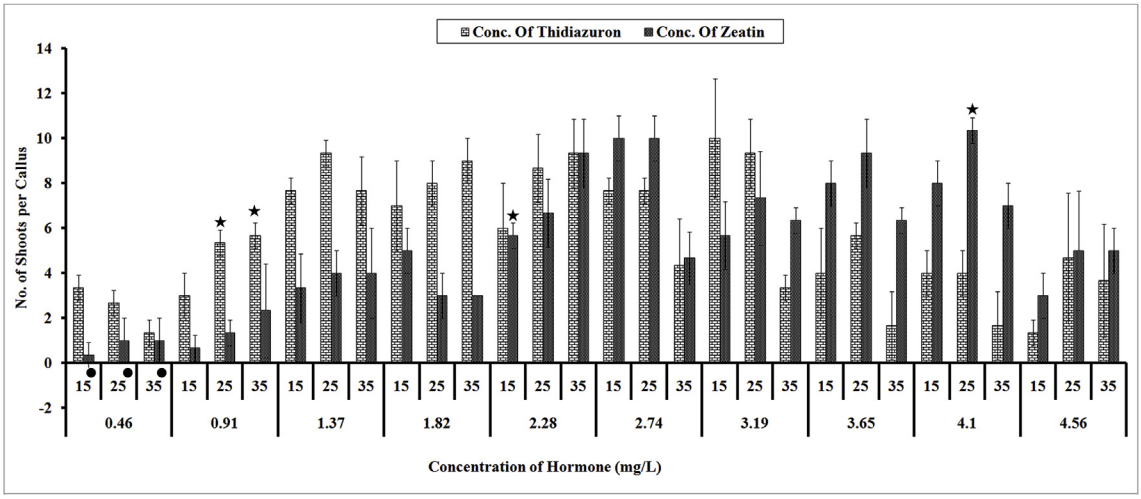

Fig. 5. Effect of Different hormones on multiple nodes induction. Relative formation of multiple nodes with Thidiazuron and Zeatin on regenerated callus derived from 15, 25 and 35 days old proliferated callus I Indicates standard deviation of values. * indicates significance of germination between least and the maximum ratio of the individual compounds at the level of $P < 0.5$.

distilled water to remove the excess acid/alkali present on the seed surface. The seeds were then surface sterilized using 70% ethanol V/V for 45 s followed by 0.1% mercuric chloride (W/V) wash for two to 3 min. Surface strilized seeds were then washed thrice vigorously with autoclaved distilled water. Then, placed on half strength MS medium, incubated in dark for 2 days (d) at 26 ± 2 °C followed by incubation under long-day light conditions with a 16 h (h) photoperiod, with an average irradiance of 50 mmol $m^{-2}s^{-1}$.

2.3. Callus induction from leaf base explants

The leaf base was excised from germinated seedlings and used as explant for callus induction. In order to assess the influence of growth hormone(s) on callus induction, different concentrations of 2,4 Dichlorophenoxyacetic acid (2,4-D) was incorporated in different media – MS, LS (Linsmaier and Skoog, 1965), B5 (Gamborg et al., 1968) and N6 (Chu, 1978). Leaf base explants were excised from *in vitro* grown *A. indicum* plantlets using sterile scalpels and inoculated on sterilized MS medium (pH:5.75) incorporated with different concentrations of 2,4-D (2.26, 4.52, 6.78, 9.04, 11.31, 13.57, 15.83 and 18.09 µM). The cultures were incubated in the dark at 26 ± 2 °C for four weeks to induce callus. The friable, nodular and creamy white embryogenic calli were screened and passaged to the callus proliferation medium [Combination of 2, 4-D (11.31 µM) with different concentrations of Kinetin (KIN) (0.46, 0.93, 1.39, 1.86, 2.32, 2.79, 3.25, 3.72, 4.18, 4.65 µM) and 6-amino benzyl purine (BAP) (0.44, 0.89, 1.33, 1.78, 2.22, 2.66, 3.11, 3.55, 4.0, 4.44 µM) discretely. **Note:** The concentrations mentioned here were used as 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg L^{-1} and converted to µM concentration when the analysis was done for two weeks to proliferate callus in the dark at 26 ± 2 °C for growth and embryo maturation. A total of 16 explants were used and the experiment was done in triplicates.

2.4. Effect of different PGRs on regeneration

For regeneration experiments, about 4–6 mm (width) of the passaged calli were transferred to MS medium supplemented with different combinations of NAA (1.0, 1.5, 2.0 and 2.5 µM), and BAP (2.22, 4.44, 6.66 and 8.88 µM). Fifty explants were used in each combination and the experiments were repeated in triplicates to obtain the significant values of regeneration frequency. All the cultures were maintained in tissue culture room at 24 ± 2 °C under a light–dark cycle of 16:8, with a light intensity of 60–70 µmol $m^{-2}s^{-1}$ for two weeks. After the appearance of shoot apical meristems (SAM), they were transferred to MS medium with different concentration(s) of thidiazuron (TDZ) and zeatin (Zn) (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mg L^{-1}), and left for incubation at 26 ± 2 °C 16/8 h day/night photoperiod for three weeks. Regenerated calli at different stages (15, 25 and 35 days old) were used to study multiple node formation.

2.5. Rooting and acclimatization

Approximately 10 cm long regenerated shoots were transferred to half strength MS medium supplemented with different concentration of Indole-3-butyric acid (IBA) (0.2, 0.4, 0.6, 0.8 and 1.0 mg L^{-1}) and kept under photoperiod for two weeks. Rooted plantlets were transferred to small plastic cups with soilrite (pH 6 ± 0.5 – Kel Tech Energies, Pvt. Ltd. Bengaluru) and incubated under photoperiod for three weeks in a versatile plant growth chamber (Sanyo) with 65% humidity at 26 ± 2 °C 16/8 h day/night. Acclimatized plantlets were then transferred to a bigger pot with vermi compost and peat soil (1:1 ratio) and kept in growth chamber with 85% humidity at 28 ± 2 °C 16 h photoperiod for three weeks.

2.6. Genetic stability analysis by SPAR markers

Genetic stability of regenerated plantlets were analyzed by directed amplification of minisatellite DNA (DAMD) and inter simple sequence repeats (ISSR) (SIGMA, USA) markers. HiPurA kit (HiMEDIA, India) was used to extract genomic DNA of soil germinated, *in vitro* germinated, *in vitro* regenerated and hardened plantlets and the quality was checked in 0.8% agarose gel. The concentration and purity of DNA were measured using spectrophotometer (Hitachi U2800, Japan) at 260/280 nm. Total genomic DNA (30 ng) was used as template for a 25 µL reaction mixture comprising of 2.5 µL of 10X buffer, 2 mM $MgCl_2$, 200 µM of dNTPs, 5 µM primers (Supplementary Table 1), 0.5 U Taq DNA polymerase (Thermo Fisher Scientific Inc., US). The PCR conditions were initial denaturation at 94 °C for 7 min, followed by 40 cycles - denaturation at 94 °C for 1 min, annealing at 55 °C (DAMD) 37 °C (ISSR) for 1 min and extension for 2 min at 72 °C, followed by a final extension for 7 min at 72 °C, using a programmable thermal cycler (Nexus gradient, Eppendorf, Germany).

2.7. GC-MS and FTIR analysis of methanol extract

Leaves of *A. indicum* were shade dried and powdered. Ten grams of

Fig. 6. Stages of *in vitro* propagation and plantlet establishment. **a)** *In vitro* germinated seeds, **b)** 21 days old calli derived from leaf base, **c)** 21 days old proliferated callus, **d)** Regenerated calli with shoot primordia from embryogenic clusters, **e)** Regenerated callus on shoot multiplication medium, **f)** Multiple shoot formation and shoot proliferation after 21 days, **g)** Roots acclimatized on MS with IBA, **h)** Mature *in vitro* derived plantlet transferred to Versatile Plant Growth Chamber (28 days old) and **i)** One month old greenhouse established plants. Scale bar: 1 cm.

Table 1
Effect of IBA on rooting.

S.No	Conc. (mg/L)	Average No. of roots	Average Main root length (cms)	Average Fresh weight (mgs)
1	MS	2.0 ± 0	1.96 ± 0.5	265.0 ± 47.89
2	1/2 MS	2.33 ± 0.58	3.4 ± 0.8	337.0 ± 110.58*
3	0.2	3.0 ± 1.0	5.33 ± 1.63	575.33 ± 54.68
4	0.4	5.67 ± 0.58	7.96 ± 0.21	791.67 ± 48.05*
5	0.6	5.67 ± 2.52	8.33 ± 1.68	724.33 ± 49.44*
6	0.8	6.0 ± 2.0	7.57 ± 1.78	651.33 ± 65.11
7	1	6.33 ± 2.52	6.06 ± 2.45	636.0 ± 54.14

-Not applicable.
Values represent means ± standard deviation; * indicates significance at the level of $P < 0.5$.

powder was weighed, dissolved in 100 mL of methanol and shaked in the dark (120 rpm for 72 h). The extract was then filtered with Whatman No. 1 filter paper, evaporated to dryness by a rotary evaporator and the final extract was dissolved in 50 mg mL^{-1}. The samples were subjected to GC-MS (Waters, US) analysis and the spectrum of wild type (WT) and *in vitro* regenerated were compared with earlier reports. For FTIR spectrophotometer analysis, 10 µL of the samples were mixed and pelleted along with potassium bromide (KBr) crystals. Subsequently the dried samples were characterized by Nicolet iS5 FTIR spectrometer (Thermo Fischer Scientific Inc., US) according to the protocol described by Razia et al. (2013) and their functional groups were recorded.

2.8. Statistical analysis

All the investigations were independently reiterated in triplicates and done in a completely randomized design. The data were subjected

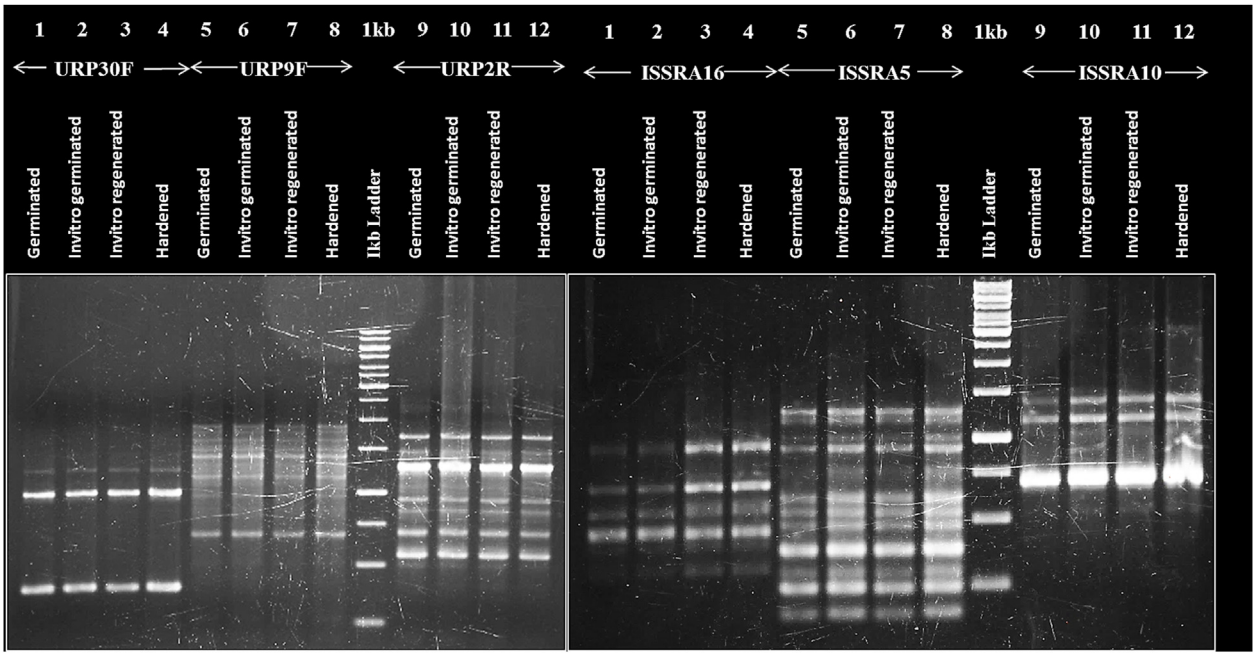

Fig. 7. DNA profile analysis by DAMD and ISSR markers for genetic variation. The genomic DNA band pattern of germinated, *in vitro* germinated *in vitro* regenerated and hardened *A. indicum*. Representative figures have been shown here.

to one way analysis of variance (ANOVA) using IBM SPSS 20.0 with significance for mean comparison at $P^{<}0.5$ level.

3. Results

3.1. Seed germination

Among the various dilutions of acid (H_2SO_4 and HNO_3) and alkali (NaOH and KOH) tested with distilled water, 10:0 ratio of H_2SO_4:H_2O gave 72% germination (Fig. 1). As the ratio of H_2SO_4 with H_2O was increased the percentage of seeds germinated also showed a steady increase. No deleterious effects of H_2SO_4 on the seeds were observed. Contrarily on surface treatment with NaOH and NaCl the seed dormancy broke and good germination was observed, but after excision of the leaf base, they failed to induce callus.

3.2. Influence of 2, 4-D, KIN and BAP in callus induction

Four weeks after incubation, the leaf base yielded good callus induction on MS medium supplemented with 11.31 µM 2, 4-D with 140 mg fresh weight (Figs. 2 and 6 b). Comparatively the calli were nodular, friable and embryogenic than those from B5 and N6 medium that produced brown and slimy callus. Leaf base mediated calli formation from seed explants were favored in the presence of 2, 4-D (11.31 µM). Subsequently proliferated on medium with a combination of 2, 4-D with BAP and KIN. 2, 4-D (11.31 µM) with KIN (1.39 µM). Two weeks after incubation significantly higher callus proliferation (89%) was observed when compared with 2, 4-D with BAP (Figs. 3 and 6 c).

3.3. Regeneration and multiple node formation

The leaf base mediated calli were used for regeneration experiments on different combinations of MS medium incorporated with PGRs. Two weeks after incubation significantly higher shoot apical meristems (SAMs) were observed on calli inoculated on MS medium incorporated with NAA and BAP (Figs. 4 and 6 d). The present research, NAA (8.06 µM) and BAP (8.88 µM) showed 91% regeneration with an average number of 8 SAMs. Further, these regenerated calluses were transferred to medium with various concentrations of PGRs like TDZ and ZN. Calluses of 15, 25 and 35 days old regenerates were used for this experiment. Among the various concentrations of TDZ, 1.36 mM induced good nodal formation wherein 25 days old regenerated calluses showed proficient and significant node formation (9.3) as compared to 15 and 35 days calluses which gave around 7 nodes but were not efficient enough to regenerate into a good shoot length. On the contrary, 25 days old regenerated calluses with ZN showed a good nodal formation (10) at 4.10 mM as compared to 8 and 7 nodes from 15 to 35 days old calluses respectively (Figs. 5 and 6 f).

3.4. Shoot branching and root adaptation

The synthetic IBA incorporated in MS medium acted as a good catalyst to enhance rooting in the regenerated callus. Half-strength MS with 0.4 mg L^{-1} IBA induced good rooting with an average of 5.67 ± 0.57 cm main root system ranging with a fresh weight of 791.67 ± 48.05 mg (Table 1). Half - strength MS media supported as a good medium for both root and shoot acclimatization. The well-developed shoots appeared green and healthy with hairy mat roots and main branch roots in the medium (Fig. 6h and i). After 2 weeks of observation the plantlets were kept on versatile growth chamber (Sanyo-MLR-351H) in commercially available Soilrite, the plants appeared very stable under 65% humidity at 26 ± 2 °C 16/8 h day/night conditions (Fig. 6h & i). The plantlets grew well in pot system when transferred after acclimatization with 85% humidity in growth chamber conditions.

3.5. Assessment of genetic stability by molecular markers

Five primers each of DAMD and ISSR were used to study the genetic stability of germinated (in soil), *in vitro* germinated, *in vitro* regenerated and hardened plantlets. A total of 256 bands (Supplementary Table 1) were obtained and no polymorphic band pattern was observed. DAMD and ISSR primers produced amplicons with a range of 200–3500 bp which further ensured that there is no genetic variation among the WT and TC derived plantlets (Fig. 7 and Supplementary Figure 1).

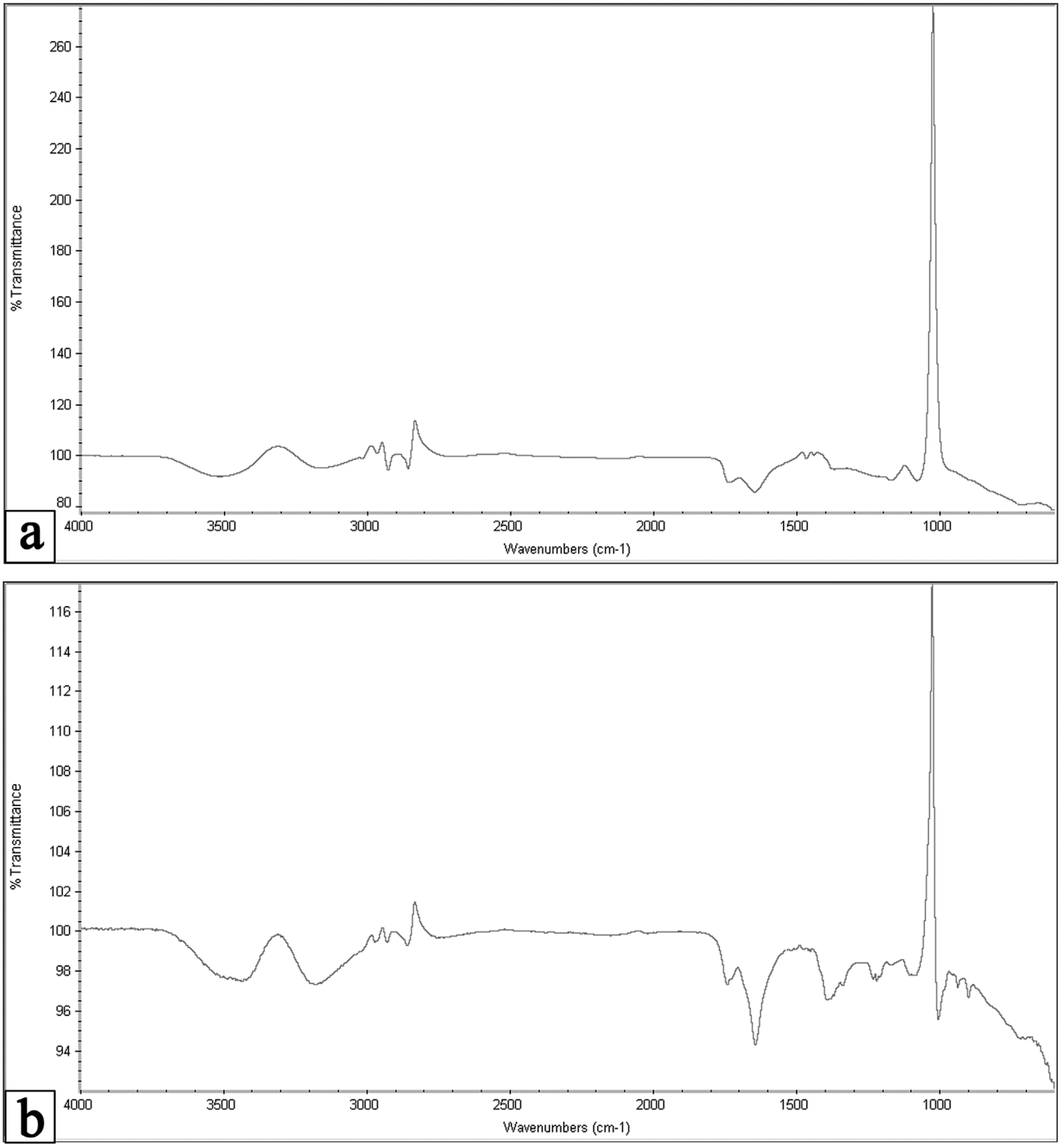

Fig. 8. GC-MS chromatogram of methanolic extract of *A. indicum*. Methanolic extract profile of **a)** hardened and **b)** direct - germinated plantlet.

3.6. FTIR and GC-MS analysis

The FTIR spectrum analysis showed no remarkable difference in the spectrum pattern between WT and the *in vitro* cultured *A. indicum*. The spectrum profile has been illustrated in Fig. 8, confirming the presence of functional groups like Alkanes, Alkenes, Ketone-6 ring, Sulphates, Sulphur halides (Supplementary Table 2).

The GC-MS spectrum of methanol extract of wild type and the *in vitro* cultured *A. indicum* were compared for their compounds present (Fig. 9). Further, the peaks were compared according to their retention times between the WT and TC raised plants. Compounds observed and their relative retention time was compared between WT and TC plants. The spectrum peaks showed 1-Dodecanol, 1-Pentadecene, 1-Hexadecene, *n*-Nonadecanol-1, 1, 2-Benzenedicarboxylic acid, Pthalic acid, isobutyl octyl ester and 1, 2-Benzenedicarboxylic acid on both WT and TC methanolic extracts indicating no change in their compound levels and the spectrum patterns (Table 2).

4. Discussion

Selection of culturing medium, suitable source for the explants and concentration of PGRs are the most important features for successful callus initiation and *in vitro* regeneration of plants (Rency et al., 2018). Seeds of *Abutilon indicum* have a poor seed germination and viability which hinders its large scale propogation and hence affects the production of important medicinal componds. Due to over exploitation for

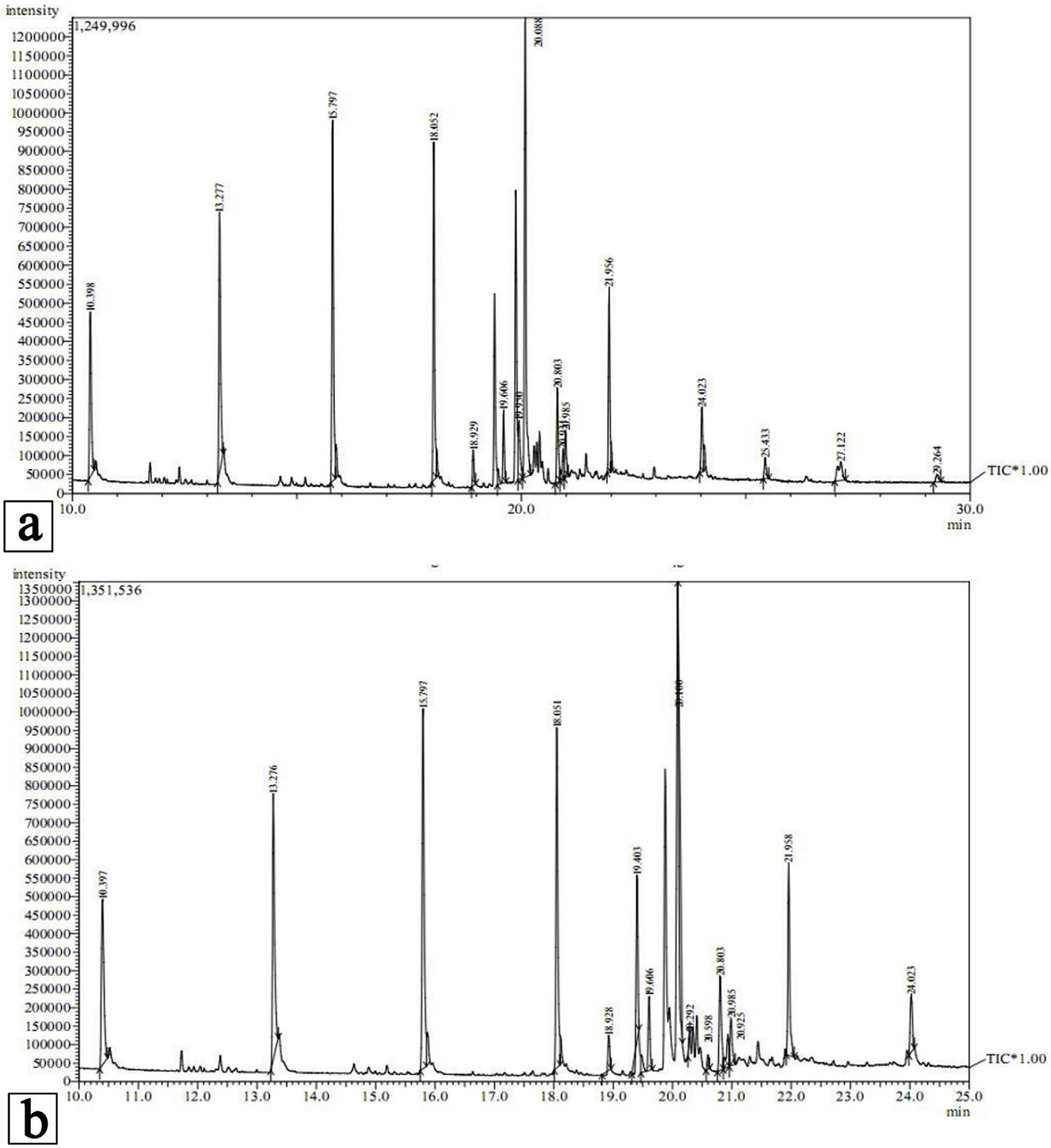

Fig. 9. FTIR spectrum of methanolic extract of *A. indicum*. Methanolic extract spectrum of **a)** hardened and **b)** direct-germinated plantlet.

its high ethnobotanic use, the natural population of this plant is restricted. Hence in the present research a series of protocols were standardized for an efficient, highly reproducible and genetically stable method of callus induction, regeneration and proper acclimatization of the ethno-botanically important weed, *A. indicum*. In this study, different explants such as seed, leaf, leaf base, cotyledonary nodes and roots to induce callus. Hitherto, there are only a couple of reports on *in vitro* organogenesis of *A. indicum*; very sparse data is available on their reproducibility and reliability of the protocols. They have used leaves (Rout et al., 2009; Ramar and Ayyadurai, 2015) and nodal (Rao et al., 2016) explants for callus mediated *in vitro* regeneration of *A. indicum*. Among various explants used, the leaf base excised from *in vitro* germinated seedlings (treated with H_2SO_4) showed most efficient callus induction (89%) on MS medium with 11.31 mM 2, 4-D. A significant increase in the fresh weight (139 mg) was observed compared to callus induced from B5, LS and N6 medium (Fig. 6. b). Our results were in agreemnet with two previous reports (Rout et al., 2009; Ramar and Ayyadurai, 2015). The protocol developed here is less time consuming

Table 2
GC-MS peaks of Methanolic extract of *A. indicum*.

S.No	Peak name		Retention time		Peak area		% Peak area	
	WT	TC	WT	TC	WT	TC	WT	TC
1	1-Dodecanol	1-Dodecanol	10.398	10.350	1209955	1278087	8.18	12.79
2	1-Pentadecene	1-Pentadecene	13.233	13.233	1747382	1782796	11.82	17.84
3	1-Hexadecene	1-Heptadecene	15.750	15.750	2317344	2413439	15.67	24.14
4	*n*-Nonadecanol-1	*n*-Nonadecanol-1	18.008	18.008	1977994	2064989	13.38	20.66
5	1,2-Benzenedicarboxylic acid	1,2-Benzenedicarboxylic acid	18.892	18.817	215614	168926	1.46	1.69
6	Pthalic acid, isobutyl octyl ester	1,2-Benzenedicarboxylic acid	19.467	19.317	492657	756305	3.33	7.57
7	1,2-Benzenedicarboxylic acid	Pthalic acid, isobutyl octyl ester	19.925	19.467	453006	534765	3.06	5.35
8	1,2-Benzenedicarboxylic acid, diundecanol	N-Hydroxymethylacetamide	20.025	20.092	3163150	−1868946	21.39	−18.70
9	Pthalic acid, butyl1 hexyl ester	3, 4-Dibrom-1, 1, 1-Triflour-2	20.758	20.258	629393	32912	4.26	0.33
10	Pthalic acid, butyl1 isohexyl ester	Pthalic acid, di (2-methylbutyl) ester	20.858	20.567	243465	67643	1.65	0.68
11	Pthalic acid, 2-methylbutyl pentyl ester	Pthalic acid, butyl hxyl ester	20.958	20.758	298929	671090	2.02	6.71
12	1-Heptacosanol	Pthalic acid, butyl tridecyl ester	21.908	20.858	998835	271113	6.76	2.71
13	Bacteriochlorophyll-*c*-stearyl	Pthalic acid, 2-methylbutyl pentyl ester	23.975	20.958	450692	331786	3.05	3.32
14	Tritriacontane	1-Heptacosanol	25.392	21.917	160194	1104867	1.08	11.05
15	Tridecanol	Nonadecene	26.983	23.983	343355	385859	2.32	3.86
16	Docosane	–	29.264	–	83690	–	0.57	–

and highly efficient for *in vitro* plant regeneration of *A. indicum*.

As the plant was found to be very recessive in breaking open its dormancy when directly sown, hence, the seeds were treated with H_2SO_4 and the leaf base derived callus showed a remarkable increase in callus mass and proliferated without hampering its embryogenic nature. The nature of callus plays a vital role in determining the efficacy of regeneration or transformation to a plantlet (Abou-Alaiwi et al., 2012). On the other hand, the callus from seeds, leaves and cotyledonary nodes were very soft and non embryogenic according to earlier reports (Ramar and Ayyadurai, 2015; Rout et al., 2009). There was a remarkable increase in the fresh weight of callus on the proliferation medium supplemented with 2, 4-D (11.31 μM) coupled with KIN (1.39 μM) which are in agreement with earlier report on proliferation and callus mass increase in medium with 2, 4-D and KIN (Sharma et al., 2010). The calli were white, creamy and embryogenic than the earlier reports (Ramar and Ayyadurai, 2015; Rout et al., 2009). The proliferated calli were efficiently regenerated on NAA (8.06 μM) and BAP (8.88 μM) with a 91% significance in regeneration and produced good shoot apical meristems (SAMs) or shoot primordia without any deleterious effects like leaf wilting or callus browning. Furthermore, parameters like age of regenerated callus and concentration of PGRs TDZ & ZN played a predominant role on the emergence of multiple internodes in the regenerated callus. Of the two PGRs, 1.36 mM TDZ was found efficient in regenerating to a whole plant as compared to that of 4.10 mM ZN, wherein the necrosis of leaves occurred, eventually leading to senescence of plant cells. Age of the regenerated callus and its internode forming capability when exposed to TDZ and ZN were pivotal in acclimatizing the plantlets to the rooting medium. Half strength MS with IBA complemented well in the lucid emergence of multiple mat roots and main root system. Chavan et al. (2011) reported the efficacy of IBA in half strength MS medium for effective root induction in endangered plant *Ceropegia attenuata* Hook. The synthetic IBA (4.54 mM) showed good mat roots than the other respective concentrations of IBA wherein the roots turned brown or wilting of leaves were observed. Thus the plants got acclimatized well in this composition of MS medium. The plants were hardened in versatile growth chambers so as to maintain a systematic control over the plant growth.

The genetic stability analyses by SPAR markers are a prerequisite so as to assess the somaclonal variation among the WT and TC plants (Krishnan et al., 2013). Changes in genetic content of *in vitro* regenerated plants are known as somaclonal variation among tissue culture raised plants (Skirvin et al., 1993). The somaclonal variation has been studied in several plant spcies using different molecular markers such as RAPD, RFLP, ISSR and AFLP (Devarumath et al., 2002; Rency et al., 2018). In this study, DAMD and ISSR markers based genetic fidelity analysis resulted in monomorphic amplicons from *in vitro* regenerated and wild plantlets, which shows true to typeness of *in vitro* regenerated plants. GC-MS and FTIR techniques by the analysis of polysaccharide was applied to evaluate the similarity between hardened and direct-germinated plantlets (Chen et al., 2015). The FTIR spectrum of methanol extract from hardened and direct-germinated plantlet showed similar spectrum and functional groups. Further the numbers of compounds were identified from the methanol extract by GC-MS spectrum analysis. Previously, Rency et al. (2018) used FT-IR analysis for the identification of functional group variations between wild and the *in vitro* regenerated plants. A total of 15 peaks were obtained of which 11 peaks shared similar or an isoform of peak name or retention time. The presence of secondary metabolites such as sitosterol, *p*-coumaric acid, caffeic acid, fumaric acid, vanillin, and *p*-hydroxybenzoic acid in *A. indicum* identified to be a ethnobotanical important medicinal plant, which is considerably exploited thereby affecting its status and habitat (Rao et al., 2016). Tissue culturing using different synthetic hormones creates physiological stress in plants and thus leads to morphological changes in plant cell and its composition (Alonso-simón et al., 2011). Hence the monomorphic bands from SPAR markers, spectrum pattern by FTIR and GC-MS validates the veracity in the efficacy and reproducibility of the TC protocol and authenticates the genetic and metabolic fingerprinting. Thus, it is apparent and this is the first report on high frequency report the high frequency somatic embryogenesis mediated by leaf base derived callus, subsequent organogenesis to shoot of *A. indicum* and its efficient regeneration demonstrating the efficacy of the protocol. These results assert the possibility to exploit *A. indicum* to enhance its secondary metabolite through genetic transformation and increase its metabolite profile.

5. Conclusions

In summary, highly efficient callus induction cum multiple shoot proliferation and plant regeneration was observed using leaf base segments from acid treated seeds of *A. indicum* plants. GC-MS and FTIR analysis shows no metabolic profile changes in *in vitro* derived and wild type *A. indicum* plants. This is the novel report for genetic fidelity analysis using DAMD and ISSR markers which reveals no somaclonal variations. Further the plant could be explored for genetic/trait improvement or secondary metabolite enhancement using transformation strategies.

Conflicts of interest

The authors declare that they have no competing interests.

Authors' contribution

SRK, MA and MR designed the study. SRK, SP, RB, PM and SJB performed the experiments. SRK and MR gave valid suggestions and inputs with regard to study. MA and MR designed the Specimen authentication and GC-MS analysis. SRK wrote the manuscript, which was further edited by SP and MR. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101125.

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