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ARTICLE

Agrobacterium tumefaciens – Mediated transformation of *Woodfordia fruticosa* (L.) Kurz

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Southern blotting

Abstract In the present study, *a protocol for Agrobacterium tumefaciens*-mediated transformation has been optimized for *Woodfordia fruticosa* (L.) Kurz. Precultured axenic leaf segments were co-cultivated with *A. tumefaciens* strain LBA4404 harboring the binary plasmid pCAMBIA1301 with β-glucuronidase (*uidA*) containing intron as the reporter gene and hygromycin phosphotransferase (*hpt*) as a selectable marker gene. After 3 days of co-cultivation, leaf segments were cultured on MS medium containing Thidiazuron (TDZ 4.54 μM) and Indole-3-acetic acid IAA (1.14 μM) + 20 mg/l hygromycin + 200 mg/l cefotaxime ($PTSM_1$) for 4 weeks (includes a single subculture onto the same medium at a 2 week interval). They were subsequently cultured for 3 weeks on MS medium containing Thidiazuron (TDZ 4.54 μM) and Indole-3-acetic acid IAA (1.14 μM) + 25 mg/l hygromycin + 100 mg/l cefotaxime ($PTSM_2$) medium for further development and shoot elongation. The hygromycin resistant shoots were rooted on a rooting medium (PTRM) containing half strength MS medium + 4.90 μM IBA + 25 mg/l hygromycin. A highest transformation efficiency of 44.5% with a mean number of 2.6 transgenic shoots per explant was achieved. Successful transformation was confirmed by the histochemical GUS activity of the regenerated shoots, PCR and RT-PCR analysis using respective primers. Southern blot analysis revealed that the *hpt* gene integrated

Abbreviations: AS, Acetosyringone; GUS, β-glucuronidase (*uid A*) gene; *hpt*, hygromycin phosphotransferase gene; IAA, Indole-3-acetic acid; MS, Murashige & Skoog (1962); OD_{600}, Optical Density at 600 nm; PCR, Polymerase Chain Reaction; RT-PCR, Reverse transcription Polymerase Chain Reaction; TDZ, Thidiazuron

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into the genome of transgenic *W. fruticosa*. Establishment of genetic transformation protocol may facilitate the improvement of this medicinal plant in terms of enhancement of secondary metabolites.

1. Introduction

Woodfordia fruticosa (L.) Kurz, an important threatened woody medicinal shrub, belongs to the family Lythraceae. It is distributed in the tropical and subtropical regions of Indian forests, and also in a majority of the countries Viz. Sri Lanka, Pakistan, China, Malaysia, Indonesia, Japan and as well as Tropical Africa [12]. *W. fruticosa* proved to be a rich storehouse of pharmaceutically active compounds used in treating various diseases [21]. There is a great demand for woodfordia flowers both in domestic and international market [20]. Many marketed drugs comprise flowers, fruits, leaves and tender twigs of this plant [1,5,8,19]. The compound woodfordin C (an inhibitor of DNA topoisomerase II) exhibits antitumor activity [17,25]. Woodfordin I is shown to induce apoptosis in human chronic myelogenous leukemia (CML) K562 cells [15].

Recently, a number of speculating medicinal values of *W. fruticosa* L. have been validated by scientific research [2,3,21,24]. Despite of the threatened status [26]; very little attention was paid toward its clonal propagation [14]. However, recently we have developed successful plant regeneration using nodal segments and leaf segments of *W. fruticosa* L. [4,13]. It is essential to establish an efficient transformation system for this valuable medicinal plant for the enhancement of secondary metabolites (For example, woodfordin C, Woodfordin I).

In this study, we developed an efficient *Agrobacterium*-mediated genetic transformation method by using leaf segments of *W. fruticosa* L. We have shown that *hpt* gene was successfully integrated into the genome and expressed in the regenerated plants. To our knowledge, this is the first report, of developing transgenic *W. fruticosa* plants via *A. tumefaciens*-mediated transformation.

2. Material and methods

2.1. Plant material

The leaves were excised from the *in vitro* raised plantlets and cut into segments of 1.0 cm^2 size (axenic leaf segments, hereafter these explants called as leaf segments) and used as the experimental material in the present genetic transformation studies. MS [18] media formulation used for entire transformation methodology is shown in the Table 1. The pH of all the media was adjusted to 5.8 prior to the addition of agar and autoclaved at 121 °C for 20 min. All the media contained 2% w/v sucrose and 0.8% agar. All cultures were incubated at 25 ± 2 °C under a 16/8 h light/dark regime with a photosynthetic photon flux density of 35 $mmol^{-2}\,s^{-1}$ that was supplied by fluorescent tubes (Philips, India). The Acetosyringone (AS) and antibiotics (hygromycin, cefotaxime) were filter-sterilized after they were dissolved in distilled autoclaved water, and added to the autoclaved medium prior to solidification.

2.2. Agrobacterium tumefaciens strain and culture conditions

Transformation studies were carried out using *Agrobacterium tumefaciens* strain LBA4404 [23] harboring a binary vector pCAMBIA1301 (CAMBIA, Australia). The T-DNA of pCAMBIA1301 contains an intron-interrupted ß-glucuronidase (*uidA*) and hygromycin phospho transferase (*hpt*) genes under the control of 35S cauliflower mosaic virus (CaMV) promoter (Fig. 2a). Single colony of *A. tumefaciens* was inoculated into fresh 3 ml of LB liquid medium supplemented with rifampicin 25 mg/l, Kanamycin 50 mg/l and chloramphenicol 75 mg/l and grown at 200 rpm at 28 °C for 20–22 h in an incubator shaker. The 3 ml culture was added to 50 ml of LB liquid medium supplemented with the same concentrations of antibiotics and cultured for 24 h until an OD_{600} reached between 0.4 and 0.6. Bacterial cells were collected using centrifugation at 6000 rpm for 10 min at 4 °C temperature and then resuspended in liquid MS medium supplemented with 100 μM AS.

2.3. Inoculation, co-cultivation

Leaf segments were pre-cultured on MS fortified with TDZ (4.54 μM) and IAA (1.14 μM) (MSPGM-Preculture/Regeneration medium) for 1, 2, 3 or 4 d were inoculated in the bacterial suspension (inoculum) for about 15 min with

Table 1 Composition of different media used for transformation study of *W. fruticosa*.

Stage	Medium composition	Abbreviation of medium	Duration (d/w)
Preculture	MS medium fortified with TDZ (4.54 μM) and IAA (1.14 μM)	MSPGM	2 d
Co-cultivation	MS medium fortified withTDZ (4.54 μM) and IAA (1.14 μM) + 100 μM AS	CCM	3 d
Hygromycin resistant shoot formation	MS medium fortified with TDZ (4.54 μM), IAA (1.14 μM) + 20 mg/l hygromycin + 200 mg/l cefotaxime	$PTSM_1$	2 + 2 w
Hygromycin resistant shoot elongation	MS medium fortified with TDZ (4.54 μM), IAA (1.14 μM) + 25 mg/l hygromycin + 100 mg/l cefotaxime	$PTSM_2$	3 w
Hygromycin resistant shoots Rooting medium	Half-strength MS salts fortified with 4.90 μM IBA and 25 mg/l hygromycin	PTRM	2 w

d = days; w = weeks.

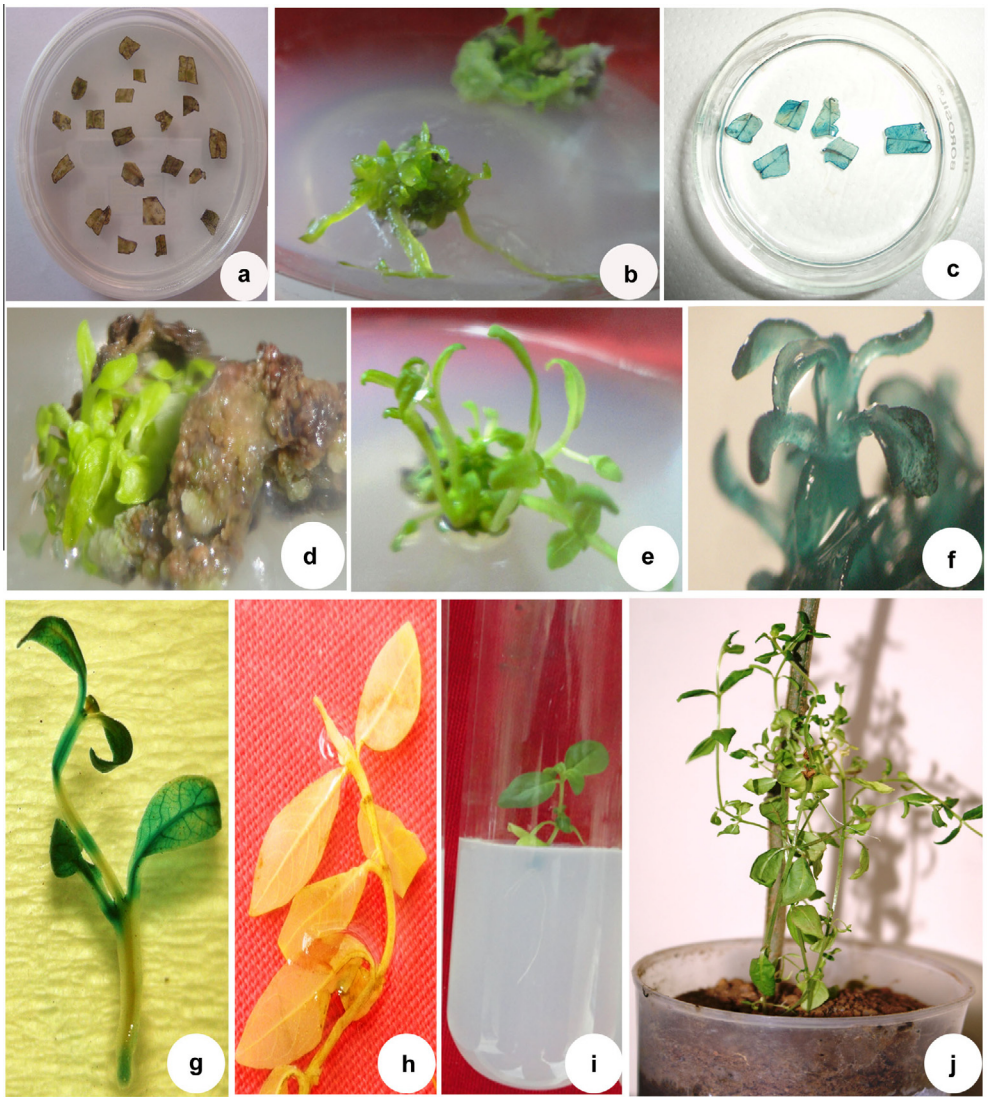

Figure 1 *A. tumefaciens* mediated genetic transformation and production of transgenic (hygromycin resistant) *Woodfordia fruticosa* (L.) Kurz. Using leaf segments as explants. (a) No shoot bud induction from the control leaf segments on selection medium supplemented with Thidiazuron (TDZ 4.54 μM) and Indole-3-acetic acid IAA (1.14 μM) + 25 mg/l hygromycin ($PTSM_1$). (b) Induction of multiple shoots from the control leaf segments on MSPGM (MS medium with 4.54 μM TDZ + 1.14 μM IAA). (c) Co-cultivated leaf segments showing Transient GUS expression after 3d of co-cultivation. (d) Regeneration of shoots from the cutting ends of leaf segments cultured on the $PTSM_1$ medium. (e) Elongation of shoots on $PTSM_2$ medium. (f) Hygromycin resistant shoot buds expressing blue coloration due to *GUS* gene expression observed under a stereo microscope. (g) Hygromycin resistant elongated shoot showing blue coloration. (h) No blue color was observed in control/wild type (WT) plant shoots. (i) Well-rooted transgenic plantlet on PTRM medium. (j) Transformants (Hygromycin resistant plantlets) in the plastic pots after greenhouse acclimation.

gentle shaking, and blotted dry with sterilized filter paper to remove excess bacterial suspension. Then they were co-cultivated on MS medium fortified with TDZ (4.54 μM) and IAA (1.14 μM) (CCM-Co-Cultivation medium) for 1, 2, 3 or 4 d under dark conditions at 25 ± 2 °C. The co-cultivation medium was also fortified with 0, 50, 100 and 150 μM AS, to determine the optimum concentration of AS for transformation.

2.4. Hygromycin sensitivity test of the leaf segments and statistical analysis

In order to use the effective concentration of selection agents, the uninfected (control) leaf segments were cultured on regeneration medium [MSPGM – MS medium containing TDZ (4.54 μM) and IAA (1.14 μM)], with different concentrations of hygromycin (0, 5, 10, 15, 20 or 25 mg/l) or cefotaxime (100, 200, 300 or 400 mg/l), respectively, while the leaf segments cultured on hygromycin-free regeneration medium (MSPGM) were regarded as control. In each 9 cm Petri dish 20 leaf segments were cultured for transformation studies. The experiments were repeated at least three times and three replicates kept per treatment (180 leaf segments/experiment). The data were subjected to ANOVA (analysis of variance, $P < 0.05$). Further, the differences in means were contrasted using Duncan's [7] new Multiple Range test.

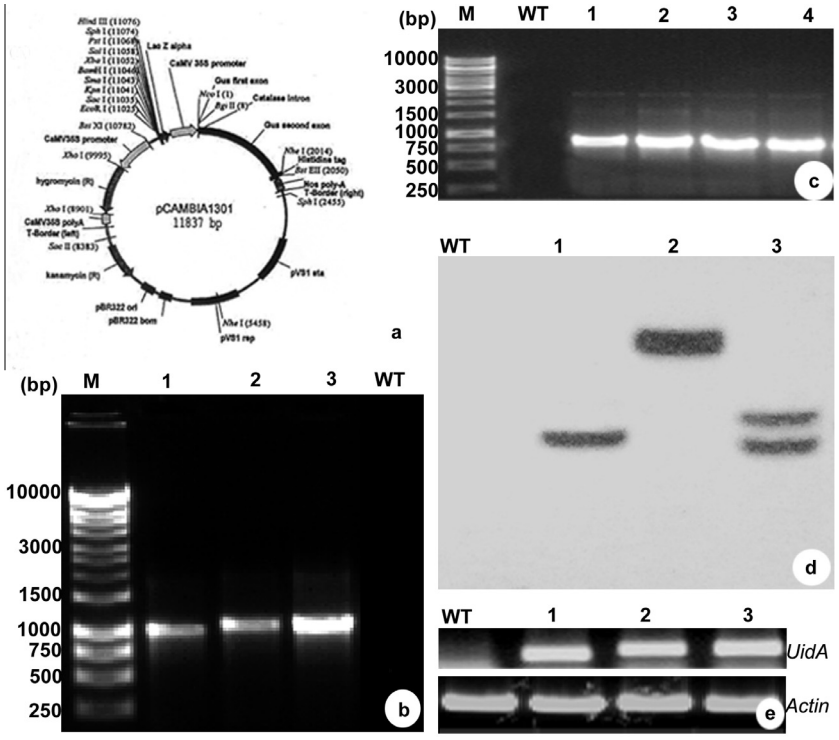

Figure 2 Molecular confirmation of hygromycin resistant *W. fruticosa* (L.) plantlets (a) Vector map used in *A. tumefaciens*-mediated transformation of *W. fruticosa* (b) PCR amplification of the 1 kb *hpt* gene of different lines (1–3) of the transformants, control plant (WT) (**c**) PCR amplification of a 900 bp fragment of *uidA* of different lines (1–4) of the transformants, no corresponding band from control plant (WT) (**d**) Southern hybridization of transgenic lines (1–3) using probe, specific to *hpt* gene (**e**) RT-PCR analysis for the expression of *GUS* gene (upper panel) of different transgenic lines (1–3), control plant (WT) and actin gene as loading control (lower panel).

2.5. Selection of transformants

Following co-cultivation, to prevent *A. tumefaciens* overgrowth the leaf segments were initially rinsed with sterile distilled water and then with liquid PGR-free MS medium containing 200 mg/l cefotaxime, blotted dry and cultured for 2 weeks on to MS medium supplemented with TDZ (4.54 μM), IAA (1.14 μM) + 20 mg/l hygromycin, as selective agent, +200 mg/l cefotaxime as a bactericide ($PTSM_1$-Putatively transformed shooting medium; Table 1). Hygromycin (20 mg/l) resistant leaf segments with shoot primordial aggregates/buds were sub-cultured for an additional 2 weeks on the $PTSM_1$ media, and subsequently cultured on $PTSM_2$ medium for 3 weeks, which is a similar medium like $PTSM_1$ but with increased hygromycin (25 mg/l) and reduced (100 mg/l) concentration of cefotaxime (Table 1). Following 7 (2 + 2 + 3) weeks, hygromycin-resistant, healthy, elongated shoots (2–3 cm) were excised and cultured for 2 weeks on to rooting medium composed of half-strength MS salts fortified with 4.90 μM IBA and 25 mg/l hygromycin (PTRM-Putatively transformed rooting medium; Table 1). Putatively transformed plantlets were taken out from the culture tubes, washed gently under running tap water to remove adhering medium and transferred to plastic pots containing a mixture of vermiculite and perlite (1:1). Plants were covered with transparent polyethylene bags to retain humidity for a week and transferred to the greenhouse. After a week, the plastic covering was removed and the plantlets were maintained in the greenhouse in plastic pots containing normal garden soil.

2.6. Histochemical GUS assay

To confirm the transformation events, histochemical analysis of *GUS* activity was carried out in 3d co-cultivated leaf segments and regenerated hygromycin-resistant shoots. Co-cultivated leaf segments/hygromycin-resistant shoots were incubated in X-glucuronide staining solution at 37 °C overnight. The stained tissues were rinsed several times with 75% ethanol to bleach chlorophyll [11]. Leaf segments stained with indigogenic dye were scored, and stable *GUS* expression was tested in regenerated hygromycin-resistant shoots. The transformation efficiency was calculated by percent of *GUS*-positive co-cultivated leaf segments showing shoot regeneration on selection medium (Table 4).

2.7. Molecular confirmation of putatively transformed plants

Genomic DNA was isolated from the regenerated hygromycin-resistant (putatively transformed) and control plant leaves by cetyl trimethylammonium bromide (C-TAB) method described by Doyle and Doyle [6]. The 90 bp fragment specific to *uidA* gene and 1 kb *hpt* gene fragment were amplified using the following primer pairs: *uidA*: Forward-5′-CGACGGCCTGTG GGCATTTCA-3′ and Reverse-5′-TGGTCGTGCACCAT CAGCAC-3′; *hpt*: Forward-5′-TAGAAAAAGCCTGAACT CACCG-3′ and Reverse-5′-TATTTCTTTGCCCTCGGACG-3′. PCR reactions were carried out in 20 μl reaction mixture containing 0.5 units of Ex Taq polymerase and 1× Taq buffer

(Takara, Dalian, China), 0.2 mM of each dNTP, 0.5 µM of each primer, and 50 ng of template DNA. The PCR cycling conditions for *uidA* included initial-denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 40 s, annealing at 58 °C for 40 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR conditions for *hpt* gene detection were set as initial-denaturation at 94 °C for 5 min, 30 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 1 min, extension at 72 °C for 40 s and final extension at 72 °C for 15 min.

To know the expression of transgenes by RT-PCR (reverse transcription-PCR), total RNA was isolated from *in vitro* regenerated transgenic plant lines following the established standard protocol [27] and treated with DNase I (Takara, Dalian, China) to remove DNA traces. Total RNA (2 µg) was used as a template for synthesis of first-strand cDNA with oligo $(dT)_{18}$ (First strand cDNA synthesis kit, Invitrogen, India). PCR of the *uidA* gene was carried out according to the conditions described above. The house keeping gene *actin* was used as an internal control to check the expression levels of transgenes. Amplified PCR products were electrophoresed on 1.0% (w/v) agarose gel stained with ethidium bromide, visualized and photographed under gel documentation system (Bio-Rad; Gel docXR +).

Stable integration of *hpt* gene in host genome was determined by Southern blot hybridization. Randomly three independent transgenic lines and control plant were selected, from which 20 µg of total DNA digested with *Eco*RI (Fermentas, USA) and separated on 0.9% agarose gel, blotted on positively charged nylon membrane (Hybond-N, Amersham Life Sciences) and hybridized with DIG-labeled probe specific to the 500 bp of *hpt* gene. Labeling, hybridization and chemiluminescent detection were performed according to the manufacturer's instructions (Roche Applied Science).

3. Results and discussion

3.1. Optimization of preculture, co-cultivation period & hygromycin sensitivity

The combination of appropriate plant growth regulators and optimization of different factors (preculture, co-cultivation, elongation, development to final transgenic plantlet formation) affecting transformation protocol were crucial for the development of transgenic plants. Initially in the present study, the concentration of hygromycin (0, 5, 10, 15, 20 or 25 mg/l) that suppressed shoot bud formation of leaf segments was determined. The control leaf segments exhibited sensitivity by showing necrosis and browning after 3 weeks of culture on MSPGM medium containing 25 mg/l hygromycin (Fig. 1a), (Fig. 3). While, such medium was devoid of hygromycin selection, a maximum of 95% of regeneration efficiency was observed after 4 weeks of culture (Fig. 1b). 89% of shoot regeneration was inhibited at 20 mg/l hygromycin selection pressure and almost all the leaf segments showed necrosis by the end of the second week (Fig. 3). So, during the first round of selection on medium ($PTSM_1$) 20 mg/l of hygromycin was used, whereas on the subsequent selection medium ($PTSM_2$) and rooting medium (PTRM), hygromycin was gradually increased from 20 to 25 mg/l with reducing usage of cefotaxime from 200 to 100 mg/l. With hygromycin at an increased 25 mg/l concentration the shoot regeneration was completely inhibited. Obviously, the inclusion of 25 mg/l hygromycin during the final rounds of selection ($PTSM_2$ or PTRM) for hygromycin resistant regenerates facilitated us to eliminate transgenic escapes.

From the results it is also noticed that different days of preculture period dramatically influenced the transformation efficiency. Preculture of 2 d was found to be optimal for improvement of *W. fruticosa* transformation (Table 2). Preculture period shorter or longer than 3 d reduced the transformation efficiency. Various concentrations of Cefotaxime were also determined to find an appropriate dose, which could give rise to maximum shoot bud survival during the entire selection process. It was observed that the selection medium should at least contain 200 mg/l cefotaxime to suppress the overgrowth of *A. tumefaciens*. While, at higher concentration (250 mg/l) leaf segments necrosis was observed in *W. fruticosa*.

Co-cultivation for a period of 3 d (Table 2) supplemented with 100 µM AS has resulted in optimum transformation efficiency of 44%. Among the different concentrations of AS tested, 100 µM was found to be the optimized concentration for maximum transformation efficiency (Table 3). This

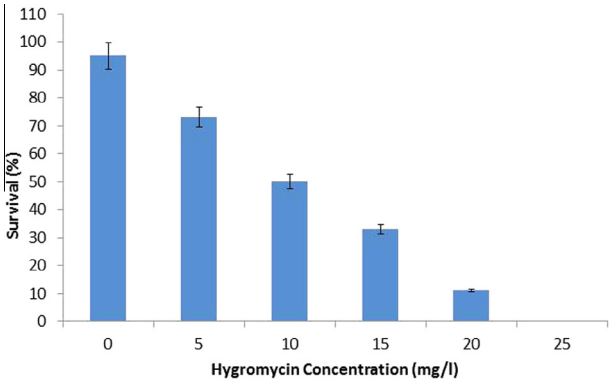

Figure 3 Survival rate of uninfected leaf segments cultured on different concentration of hygromycin (0, 5, 10, 15, 20 or 25 mg/l).

Table 2 Influence of Preculture and Co-cultivation on the transformation efficiency of *W. fruticosa*.

Parameters in days	No. of responding explants/petriplate	Transformation efficiency (%)[t]
Preculture period[*]		
0	2.20 ± 0.07	11.0[a]
1	5.90 ± 0.43	29.5[c]
2	8.95 ± 1.30	44.7[d]
3	6.70 ± 0.61	33.5[b]
Co-cultivation period[**]		
1	6.20 ± 0.35	31.0[b]
2	6.90 ± 0.12	34.5[b]
3	8.96 ± 0.07	44.8[c]
4	4.4 0 ± 0.45	22.0[a]

[t] Means with different letters were significantly different ($p < 0.05$).
[*] Preculture on MSPGM medium.
[**] Co-cultivation on CCM medium + AS.

efficiency of transformation (Table 4) was calculated by percent of co-cultivated leaf segments showing shoot regeneration on $PTSM_{1,2}$ medium. Our results are inconsistent with earlier reports in regard to optimization of different parameters co-cultivation [10,16], optimum AS concentration [22], preculture [9] required for standardized for *A. tumefaciens* – mediated transformation system.

3.2. Regeneration of hygromycin resistant plantlets

Hygromycin resistant shoots regenerated directly (without intervening callus phase) from the infected leaf segments. While, the uninfected leaf segments cultured on the selection medium ($PTSM_1$ or $PTSM_2$) showed browning and senescence (Fig. 1a and b). On average 50–60% transformation efficiency was evidenced (transient GUS expression) as expressed by the 3 d co-cultivated leaf segments (Fig. 1c). After 2 + 2 weeks (include single subculture onto the same medium at 2 week interval) of culture on $PTSM_1$ medium the leaf segments showed shoot bud formation (Fig. 1d). In the follow up the cultures on $PTSM_2$ which were retained for 3 weeks produced healthy and maximum number of hygromycin resistant elongated shoots/leaf segments (Fig. 1e). Leaf segments with elongated shoots on $PTSM_2$ medium exhibited a dark blue color after histochemical GUS assay (Fig. 1f). The elongated shoots dissected for rooting displayed dark blue color after histochemical GUS assay, showing their transgenic nature (Fig. 1g). On $PTSM_2$, the explants with the shoot bud formation declined (due to 25 mg/l hygromycin) as a result elongating shoots buds/clumps started to decline from 60% to 44%, while, the explants cultured on hygromycin-free media (control) showed 95% survival after 3 weeks culture. The elongated hygromycin-resistant shoots those transferred onto the rooting medium (PTRM) after 12–14 d survived to rooting (Fig. 1i). Based on the percent of co-cultivated leaf segments producing shoots on hygromycin medium, the average transformation efficiency of 44.5% was achieved (Table 4). A mean number of 2.6 transgenic plantlets per explants were recorded. After 12–14 d on PTRM medium, complete transgenic plantlets ready for green house transfer were produced. The plantlets survived with a 90% transplant success in the greenhouse (Fig. 1j). The transformants produced, exhibited similar morphology to those of wild plants.

Table 3 Effect of Acetosyringone (AS) concentration in co-cultivation medium during transformation studies in *W. fruticosa*.

AS concentration (μM)[a]	No. of responding explants/Petri plate	Transformation efficiency (%)[t]
0	2.16 ± 0.07	10.8
50	4.90 ± 0.12	24.5
100	8.99 ± 0.13	44.9
150	2.90 ± 0.10	14.5

[a] Acetosyringone was added to MS medium fortified with TDZ (4.54 μM) and IAA (1.14 μM).
[t] Percent of transformation efficiency; Means with different letters were significantly different ($p < 0.05$).

3.3. Analysis of transformants

Stable *GUS* expression was observed (with typical dark-blue) by transgenic plant lines/leaves after the GUS assay. On the other hand, leaves from the control plants, developed no color after GUS assay (Fig. 1h). To confirm the integration of T-DNA, molecular analysis (PCR and Southern blot) was carried using genomic DNA isolated from hygromycin resistant plant lines (*GUS*-positive lines) keeping a control/wild type (WT) plant. Specific primers were employed in PCR analysis to verify the presence of transgenes (*uidA* and *hpt*). Respective sizes of (900 bp for *uidA* (Fig. 2c) and 1 kb (Fig. 2b) for *hpt*) fragments were amplified from genomic DNA of all the transgenic plants, whereas corresponding bands were not detected in the control/wild type plants. RT-PCR was carried out to confirm the expression of *uidA* in the transgenic plants. cDNA synthesized from total RNA isolated from independent transgenic lines, as well as untransformed plants were subjected to PCR as mentioned above. As expected, 900 bp of fragment of *uidA* was observed in transgenic plants (Fig. 2e). While control/wild type plants showed no amplification. *Actin* gene was used as a control in the RT-PCR analysis of the transgenic plants (Fig. 2e). Southern blot analysis was performed to confirm the stable integration of *hpt gene* in PCR positive plants. Genomic DNA of PCR positive and non-transformed plants was digested with *Eco*RI and probed with probes specific to *hpt* gene. The results confirmed the integration of *hpt* gene into the *W. fruticosa* transgenic plants (Fig. 2d). No hybridization signal was detected in control/ wild type (WT) plant (Fig. 2d).

Table 4 Percent transformation efficiency by *A. tumefaciens* strain LBA4404 (p CMBIA 1301) in *W. fruticosa*.

Experiment[z]	No. of responding explants under hygromycin selection pressure	No. of putative transgenic plantlets/explant	No. of GUS positive transgenic plantlets[y]	Transformation efficiency (%)[t]
1	15.50	2.24	34.72 ± 0.22	38.75[a]
2	19.92	2.80	55.77 ± 0.09	49.80[c]
3	17.98	2.77	49.80 ± 0.51	44.95[b]
Mean	17.80	2.60	23.1 ± 0.29	44.50[b]

Means with different letters were significantly different ($p < 0.05$);
[z] Plantlets regenerated on medium containing 25 mg/l hygromycin.
[y] Plantlets exhibiting GUS gene expression at the fully matured stage.
[t] Percent of transformation efficiency.

4. Conclusion and future remark

In conclusion, the present study describes a simple, efficient, stable and reproducible *A. tumefaciens* – mediated gene delivery system for *W. fruticosa* using axenic leaf segments. We anticipate the present study paves future studies toward its metabolic pathway engineering for higher content of pharmaceutical compounds.

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References

[1] B.S. Ahuja, Medicinal Plants of Saharanpur, first ed., Dhrampal Vidhyalankar Publisher, Gurukul Kangri Vishawvidyalaya, Hardwar, India, 1965, pp. 10–11.
[2] Y. Baravalia, Y. Vaghasiya, S. Chanda, Asian Pac. J. Trop. Med. 4 (2011) 342–346.
[3] P.D. Bhondave, P.P. Devarshi, K.R. Mahadik, A.M. Harsulkar, J. Ethnopharmacol. 151 (2014) 183–190, http://dx.doi.org/10.1016/j.jep.2013.10.025.
[4] M. Bulle, S. Kota, R. Deepa, M. Aileni, V.R. Kokkirala, G. Kranthi Kumar, S. Abbagani, J. Herbs Spices Med. Plants 18 (2012) 178–190.
[5] R.N. Chopra, S.L. Nayar, I.C. Chopra, Glossary of Indian Medicinal Plants, CSIR, Delhi, India, 1956.
[6] J.J. Doyle, J.L. Doyle, Focus 12 (1990) 13–15.
[7] D.B. Duncan, Biometrics 11 (1955) 1–42.
[8] U.C. Dutt, The Materia Medica of the Hindus, Adi Ayurveda Machine Press, Kolkata, India, 1922.
[9] Z. Hu, Y.R. Wu, W. Li, H. Gao, In Vitro Cell Dev. Biol. Plant 42 (2006) 461–466.
[10] D.J. James, S. Uratsu, J. Cheng, P. Negri, P. Viss, A.M. Dandekar, Plant Cell Rep. 12 (1993) 559–563.
[11] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, EMBO J. 6 (1987) 3901–3907.
[12] K.R. Kirtikar, B.D. Basu, Indian medicinal plants. Parts 1–3, L. M. Basu, Allahabad, India, 1935.
[13] V. Kokkirala, K. Srinivas, Y. Rajesh, B. Mallesham, A. Mahender, G. Kranthi kumar, A. Sadanandam, Med. Aromatic Plant Sci. Biotechnol. Global Sci. 6 (2012) 50–53.
[14] P.N. Krishnan, S. Seeni, Plant Cell Rep. 14 (1994) 55–58.
[15] M.J. Liu, Z. Wang, H.X. Li, R.C. Wu, Y.Z. Liu, Q.Y. Wu, Toxicol. Appl. Pharmacol. 194 (2004) 141–155.
[16] A. Mahender, A. Sadanandam, P. Zhang, Plant Biotechnol. Rep. 5 (2011) 147–156.
[17] A. Motegi Kuramochi, H. Kuramochi, F. Kobayashi, H. Ekimoto, K. Takahashi, S. Kadota, Y. Takamori, T. Kikuchi, Biochem. Pharmacol. 44 (1992) 1961–1965.
[18] T. Murashige, F. Skoog, Physiol. Plant. 15 (1962) 473–479.
[19] K.M. Nadkarni, Indian Materia Medica, third ed., Mumbai, India, Popular Book Depot, 1954, vol. 2, pp. 489.
[20] P. Oudhia, 2003. Interaction with the herb collectors of Gandai Region, Chhattisgarh, MP, India. www.botanical.com.
[21] K.D. Pratap, G. Suchandra, Ch. Annalakshmi, P. Nilendu, B. Sukdeb, P.S. Niranjan, A. Basudeb, J. Ethnopharmacol. 110 (2007) 189–199.
[22] N. Shimoda, A. Toyoda-Yamamoto, J. Nagamine, S. Usami, M. Katayama, Y. Sakagami, Y. Michida, Proc. Natl. Acad. Sci. USA 87 (1990) 6684–6688.
[23] L. Vander Fits, E.A. Deakin, J.H. Hoge, J. Memelink, Plant Mol. Biol. 43 (2000) 495–502.
[24] N. Verma, G. Amresh, P.K. Sahu, C.V. Rao, A.P. Singh, Indian J. Exp. Biol. 50 (2012) 351.
[25] C.C. Wang, L.G. Chen, L.L. Yang, Cancer Lett. 140 (1999) 195–200.
[26] Z.S. Wochok, Biol. Conserv. 20 (1981) 83–89.
[27] J. Xu, A. Mahender, A. Sadanandam, P. Zhang, Phytochem. Anal. 21 (2010) 395–398.