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Efficient *In Vitro* Regeneration, Analysis of Molecular Fidelity and *Agrobacterium tumifaciens* - Mediated Genetic Transformation of *Grewia asiatica* L.

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Abstract

Grewia asiatica is a dietotheraphtically important fruit bearing shrub, indigenous to India. It is a rich resource of triterpinoids and flavonoids and possesses many putative health benefits. Two of the drawbacks which include short shelf life of its fruits and larger seed volume impedes its full exploitation. Seed abortion for developing seedless cultivars through biotechnological interventions is a viable option. One of the prerequisites for such a strategy is to develop an efficient plant regeneration and transformation protocols in *G. asiatica*. Against this backdrop multiple shoot induction was achieved from nodal explants with axillary buds, on culturing in Woody Plant Medium (WM) fortified with 3% (*w/v*) sucrose, 2 × 10^{-5} M Kinetin (Kn) and 1 × 10^{-5} M indole-3-butyric acid (IBA) giving rise to an average of 4.25 ± 0.71 microshoots per explant. More than 90% of the explants formed micro-shoots with mean shoot length of 10.5 ± 1.96 cm leading to whole plant regeneration. Healthy regenerated shoots showed prolific rooting of more than 95% on WM supplemented with 4.8 × 10^{-6} M indole-3-butyric acid (IBA). Following simple hardening procedures, rooted plantlets, were transferred to soil-sand (1:1; *v/v*) with about 92% success. Genetic fidelity was assessed using random amplified polymorphic DNA (RAPD). Additionally, *Agrobacterium*-mediated genetic transformation protocol was developed using *A. tumefaciens* strain GV2260 harboring binary vector p35SGUSINT containing hygromycin phosphotransferase gene (*hpt*). Transformation was verified by GUS assay and detection of the *hpt* by polymerase chain reaction. *In vitro* regeneration and ensuing molecular fidelity of regenerated plants and transformation studies are hitherto unreported for *G. asiatica*.

Keywords: *Grewia asiatica*; Woody Plant Medium; Regeneration; RAPD; *Agrobacterium*; GUS

Introduction

Grewia asiatica L. (Malvaceae), commonly known as 'Phalsa' is a multipurpose gregarious shrub, found in tropical and sub-tropical parts of South East Asia [1]. The plant is Asian in origin and is capable of growing under neglected and water scarce conditions where only a few other crops would normally survive [2,3]. It is well-known for its nutritional and therapeutic attributes [4,5]. The plant finds its use in Ayurvedic and traditional systems of medicine [6]. *G. asiatica* is a rich repository of biologically active molecules. The major chemical constituents from various plant parts are flavonoids and terpenoids including grewinol, quercetin and naringenin from flowers; taraxasterol, β-sitosterol, erythrodiol, β- amyrin, lupeol, betulin lupenone, friedelin and α-amyrin from the bark [7-9]. These constituents have been found to bestow antioxidative [10], radioprotective [11] and many other putative health benefits [12-15]. The leaves are used as cattle fodder and are applied to wounds and cuts to relieve irritation and cure painful rashes due to their antibiotic effect [16]. The root bark is used in urinary tract problems and for the treatment of rheumatism, while the stem bark is used in sugar refining [17]. The fruit is a small berry with stony seeds. It is pulpy and consumed fresh, in desserts, or processed into refreshing fruits and soft drinks [18]. The fruits are claimed to be useful for heart, blood and liver disorders [19]. Anorexia, indigestion, thirst, toxemia, stomatitis, hiccough, asthma, spermatorrhoea, fever, diarrhoea, tuberculosis and sexual troubles are some other ailments where Phalsa fruits have been reported to be effective [11,20-22].

However, short shelf-life and larger seed size of fruits limits their accessibility for commercial exploitation. Short shelf-life makes the fruits suitable only for local marketing whereas larger seeds reduce the pulp volume [23]. As compared to other cultivated fruit crops in India, so far Phalsa has not attained a place of importance largely because of high perishable nature and small size of its fruits [24,25]. However, recently one of the constituent laboratories of Council of Scientific and Industrial Research (New Dehli, India), CSIR-IIIM, Jammu has developed end-to-end technology from cultivation to product development in the form of Phalsa juice under the trade name of 'Shiwalik Berry'. The protracted seed-to-seed life cycle, woody habit, predominant propagation by seeds, asynchronous fruit set, sluggish seed germination and loss of seed viability on storage are some of the constraints which inhibit full exploitation of *G. asiatica* [26]. The dietotherapeutic and medicinal importance, demands development of commercially acceptable cultivars in *G. asiatica*. However, the conventional breeding in certain crop species is delayed due to genetic restrictions imposed by sterility, polyploidy, heterozygosity, long juvenile periods and self-incompatibility [27]. Nonetheless, these pressures have paved a way for biotechnological interventions to come into play. Tissue culture

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is one such avenue where genetic fidelity, induction of somaclonal variations and shortening of protracted life cycle can be achieved. One of the potent applications of tissue culture is micro-propagation to ensure supply of bulk and quality planting material for commercial enterprise. The purpose of micro-propagation is to develop genetically uniform, physiologically stable, developmentally normal plantlets with regained juvenility and vigor [28]. Its application in horticulture, agriculture and forestry is currently expanding world-wide. Numerous recalcitrant forest trees of economic value are still difficult to establish under *in vitro* conditions largely due to lack of morphogenetic ability, high rate of contamination and reduced rooting of the regenerated shoots [29]. Therefore, improvement in existing procedures for *in vitro* regeneration with intact genetic integrity of woody species seems to be imperative. Against this background, we have successfully developed an *in vitro* strategy for regeneration of multiple shoots from axillary buds of *G. asiatica*.

For assessing the genetic fidelity of *in vitro* regenerated plants, we employed random amplified polymorphic DNA (RAPD) technique using arbitrary decamer primers. RAPD markers are often used in genetic variation studies in tissue culture raised plants because of the smaller amount of DNA required, ease of use, low cost, reliability, not as much time consuming, and does not require prior knowledge of the nucleotide sequence of the organism under study, with no radioactive probes and no expensive restriction enzymes involved [30,31].

Further, the larger seed volume with respect to fruit pulp and short shelf-life of the fruits are two main drawbacks in Phalsa. These restrict its commercial value and as such induction of parthenocarpy or stenospermocarpy is a viable option to circumvent these shortcomings. Parthenocarpic fruits are seedless and tend to have longer shelf-life [32]. Auxin response factor (ARF) [33], inner no outer (INO) [34], deficiens (DEF) [35] and chalcone synthase (CHS) [36] are the key pathway genes which can be disrupted for induction of parthenocarpy. We have already cloned some of these genes (ARF, two isoforms of CHSs; NCBI accession nos. KX129912, KX129910, KX129911) from *G. asiatica.* For molecular interventions to disrupt the highly conserved pathway of seed formation, development of an efficient *in vitro* regeneration system with high genetic fidelity and *Agrobacterium*-mediated genetic transformation is of fundamental research application in *G. asiatica*. In this direction, an efficient and reliable method for multiple shoot induction subsequently used for genetic transformation was successfully established in *G. asiatica*.

There are no documented *in vitro* studies encompassing molecular fidelity and *Agrobacterium* mediated genetic transformation vis-à-vis *G. asiatica* or any member of this genus. The present study is the only report describing a successful method related to *in vitro* regeneration and acclimatization of *in vitro* hardened plants of *G. asiatica* under field conditions. Here, we also demonstrate the efficacy of the protocol developed to ensure genetic fidelity of ensuing regenerated plants using RAPD technique. The *Agrobacterium*-mediated transformation resulting in the introduction and transient expression of the GUS-*hpt* gene in plant tissues was also an important aspect of present investigation.

Materials and Methods

Multiple shoot induction/*in vitro* rooting and *A. tumifaciens* mediated transformation

In vitro cultures were established from seed raised field-grown plants at the Indian Institute of Integrative Medicine Jammu, India (32°.44'N 75°.55'E; 305 m in altitude). The nodal segments 2-3 cm in size containing axillary buds were originally used for the initiation of aseptic cultures of *G. asiatica*. The explants were thoroughly washed under tap water containing 1-2 drops of Tween-20 for 30-45 min and subsequently rinsed 3-4 times with distilled water. Explants were sterilized with 0.5% (*w/v*) bavistin (BASF, Mumbai, India) solution for 3-4 min followed by rinsing with sterile, double-distilled water. The sterilized explants were then transferred to 0.5% (*w/v*) streptomycin sulphate (Amresco, Ohio, USA) for 2 min and finally treated with 0.1% (*w/v*) $HgCl_2$ (HiMedia, Mumbai, India) for 2 min. The treated explants were rinsed 4-5 times with sterile, double-distilled water under a laminarhood. After blotting on a sterile filter paper, the surface-sterilized axillary buds were cultured on MS [37] basal medium, containing 3% (*w/v*) sucrose and supplemented with different concentrations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) (HiMedia, Mumbai, India) as shown in Table 1. The Medium was adjusted to pH 5.8 ± 0.1 with either 0.1 N KOH or 0.1 N HCl, and 0.7% agar was added as a gelling agent. Aliquots of 100 mL medium were dispensed in 250 mL (*v/v*) borosilicate conical flasks for explant inoculations. The medium was autoclaved at 121°C (15 psi) for 20 min. The cultures were kept under a 16-h photoperiod with a light intensity of 25-30 μE/m^2/s provided by 40-W cool white fluorescent lamps (Philips, Kolkata, India). Relative humidity (RH) of 50-60% was maintained at 23 ± 1°C. The same type of explants i.e., nodal segments were also cultured on B_5 Gamborg's medium [38] and WM [39] with different molar concentrations and combinations of plant growth regulators viz., Kn/IBA and Kn/NAA (Sigma Aldrich, St. Louis, USA) (Table 1). After 8 weeks of initial culture, the response frequency of explants was calculated as percentage of explants producing shoots per total number of explants. For rooting, elongated shoots (>4.0 cm) were transferred to WM which was supplemented with various concentrations of IBA and NAA. Rooting was also checked on WM devoid of exogenous supplements of plant growth regulators (Table 2). For *in vitro* regeneration from nodal explants, each triplicated treatment consisted of 35 explants. Subsequent transfers of 65 *in vitro* regenerated micro-shoots per treatment were substantiated for root induction. Mean and standard errors of means were computed. The statistical analysis of data was performed as per Gomez and Gomez [40]. At 5% level of significance, data were subjected to ANOVA with Duncan's multiple range test. Juvenile plant tissues (leaf petiole and internodal segments) were obtained from *in vitro* cultures of *G. asiatica* for transformation studies. Explants were cultured on WM containing 4×10^{-6} M Kn and 2.4×10^{-6} M IBA. For genetic transformation, different types of media were used for bacterial culture, pre-culture, co-cultivation, and selection for transformation (Table 3).

Hardening and transplantation

The nine week old *in vitro* rooted plants were washed free of agar and transferred to soil, sand and vermiculite mixture (1:2:1; *v/v*) in Styrofoam cups. The plants were kept under controlled environmental conditions at 27 ± 1°C under 16 h/8 h photoperiod and relative humidity RH 80-90%. Moreover, plants were watered on alternate days with 0.5 X Hoagland's solution. The hardened plants were directly transplanted in earthen pots in sand-soil mixture (1:1; *v/v*) under field conditions after three weeks.

RAPD fingerprinting

Total genomic DNA was extracted from the leaves of 13 randomly selected hardened *in vitro* raised plants after 3 months of their transfer to field conditions. *In vivo* mother plant was also subjected to DNA

Phytohormone combination	Cytokinins/Auxin molar ratios	Percentage explants producing shoots	Number of shoots/ culture	Shoot length (cm)	Internodal length (cm)	Leaves per shoot
MS-medium Kn+IBA						
2 × 10^{-6}M+5 × 10^{-7}M	4.23:1	23	Profuse	-	-	-
4 × 10^{-6}M+2 × 10^{-6}M	2.12:1	26	callusing	-	-	-
1 × 10^{-5}M+5 × 10^{-6}M	2.13:1	31	1.31^{d}	2.90^{c}	1.0^{c}	2.25^{c}
2 × 10^{-5}M+1 × 10^{-5}M	2.13:1	24	-	-	-	-
B_5-medium Kn+IBA						
2 × 10^{-6}M+5 × 10^{-7}M	4.23:1	46	Callusing	-	-	-
4 × 10^{-6}M+2 × 10^{-6}M	2.12:1	33	-	-	-	-
1 × 10^{-5}M+5 × 10^{-6}M	2.13:1	28	-	-	-	-
2 × 10^{-5}M+1 × 10^{-5}M	2.13:1	71	-	-	-	-
Woody Plant Medium Kn+IBA						
2 × 10^{-6}M+5 × 10^{-7}M	4.23:1	76	$2.50^{b,c}$	8.50^{b}	$1.25^{a,b}$	6.43^{b}
4 × 10^{-6}M+2 × 10^{-6}M	2.12:1	48	5.78^{c}	$10.9^{a,b}$	2.04^{a}	$7.33^{a,b}$
1 × 10^{-5}M+5 × 10^{-6}M	2.13:1	63	$3.40^{a,b}$	11.5^{a}	0.90^{b}	6.53^{b}
2 × 10^{-5}M+1 × 10^{-5}M	2.13:1	94	4.25^{a}	$10.5^{a,b}$	$1.50^{a,b}$	$8.00^{a,b}$
Kn+NAA						
2 × 10^{-6}M+5 × 10^{-7}M	4.6:1	59	$2.55^{b,c}$	12.40^{a}	$1.50^{a,b}$	9.70^{a}
4 × 10^{-6}M+2 × 10^{-6}M	2.12:1	86	$3.24^{a,b}$	11.27^{a}	1.30^{b}	10.20^{a}
1 × 10^{-5}M+5 × 10^{-6}M	2.13:1	68	2.00^{c}	$9.28^{a,b}$	2.00^{a}	$8.50^{a,b}$
2 × 10^{-5}M+1 × 10^{-5}M	2.46:1	55	$2.50^{b,c}$	$10.25^{a,b}$	$1.70^{a,b}$	8.75^{a}
Basal Woody medium	-	51	$10.5^{a,b}$	$3.20^{a,b}$	2.5^{a}	11.50^{a}

Table 1: A Comparative response of *G. asiatica* under different media conditions. Comparative responses of shoot proliferation and elongation from secondary axenic explants with multiple shoot buds on WM supplemented with different molar ratios of Kn/IBA and Kn/NAA, after 4 weeks of culture. Values followed by same letters are not significantly different (P ≤ 0.05) as per Duncan's multiple range test.

Phytohormone combination (molar ratios)	Rooting (%)	Mean root number per shoot
WM+IBA (2.4 × 10^{-6}M)	56	11.20 ± 0.59
WM+IBA (4.8 × 10^{-6}M)	97	17.40 ± 0.67
WM+IBA (7.2 × 10^{-6}M)	69	13.10 ± 0.58
WM+NAA (2.6 × 10^{-6}M)	47	09.70 ± 0.50
WM+NAA (5.2 × 10^{-6}M)	85	11.20 ± 0.54
WM+NAA (7.8 × 10^{-6}M)	73	10.50 ± 0.56
WM	62	06.30 ± 0.43

Table 2: Effect of IBA and NAA on *in vitro* root induction in *Grewia asiatica*. Data were recorded 3 weeks after transfer to rooting media (mean ± SE).

extraction following the modified procedure of Doyle and Doyle [41]. The DNA yield was measured by UV absorbance ratio at 260 nm/280 nm. Purity of DNA was checked on 0.7% agarose gel using an aliquot of 3 µL of the preparation. Based on amplification of mother plant through polymerase chain reaction (PCR), 5 arbitrary decamer primers were selected among the 18 primers. These primers produced easily scorable distinct amplification profiles. Master Cycler Gradient (Eppendorf, Germany) was used for DNA amplification and the PCR conditions were as follows: 5 min at 95°C; 40 cycles of : 1 min at 95°C, 1 min at 35°C, 2 min at 72°C; and as a last step 5 min at 72°C. 20 µL reaction mixture contained 1X PCR buffer (10 mM tris HCl, pH 9.0;50 mM KCl; 2.5 mM $MgCl_2$); 200 µM dNTP;200 µM random primers; 50 ng of DNA template and 0.5 U of Taq DNA polymerase (Bio Basic Inc., Canada). The separation of amplified products was performed by gel electrophoresis using 1.5% (*w/v*) agarose. All the PCR reactions were repeated at least twice to check the reproducibility.

Bacterial strain and culture

For the genetic transformation of *G. asiatica,* GV2260 an *Agrobacterium* strain harboring p35SGUSINT vector [42] was used. The *Agrobacterium* strain GV2260 was streaked on yeast mannitol agar medium containing 25 mg/L hygromycin and grown at 28°C. Prior to transformation, the primary culture was established by using a single colony of *Agrobacterium* which was inoculated on freshly prepared yeast mannitol broth (YMB) containing 25 mg/L hygromycin (Table 3). The bacteria were grown in dark in an incubator shaker with 220 rpm at 28°C for 24-48 h till OD_{600} of 0.9 ± 0.1. The cells were harvested in early stationary phase by centrifugation at 4000 rpm at 4°C for 10 min. The supernatant was discarded and the pellet was resuspended in fresh YMB medium containing 200 µM acetosyringone (HiMedia). Leaf petiole and internodal segments of 1-2 cm were pre-cultured on WM for 3 days containing 1 mg/L Kn and 0.5 mg/L IBA (Table 3). These explants were then submerged in bacterial suspension at 28°C for 30 min. Excess *Agrobacterium* were removed by blotting onto sterile filter paper. Then the explants were transferred onto co-cultivation medium, and co-cultivated in the dark for 1- 4 d at 23 ± 1°C to evaluate the effect of co-cultivation time on transformation competence. After co-cultivation, the explants were washed 3-4 times with sterile deionized water fallowed by two washes with WM containing 500 mg/L cefotaxime to remove *Agrobacterium*. Blot dried explants on a sterile filter paper were transferred to selection medium (SM; Table 3) containing 15 mg/L hygromycin for the rejuvenation of hygromycin-resistant shoots. The sub-culturing of explants were carried out after every 10 d for about 1 month on the same SM. Regenerated shoots (2.0-3.0 cm) were expunged and transferred to hygromycin-free regeneration medium (RM). Transformed and non-transformed tissues were discriminated histochemically through GUS expression [43]. Transient GUS expression of randomly selected explants was first tested 5 days after co-cultivation with GUS buffer [50 mM $NaHPO_4$, pH 7; 10 mM EDTA, pH 7; 0.5 mM $K_3Fe(CN)_6$; 0.5 mM $K_4Fe(CN)_6$; 0.1% Triton X-100; 2 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid]. Tissues for GUS expression were incubated at 37°C overnight followed by several washes with 75% ethanol. Explants after 12 days showing GUS-positive blue spots or sectors visualized under a NIKON

Stage	Medium composition	Medium	Duration
Bacterial culture	0.5 g/L K_2HPO_4+0.2 g/L $MgSO_4$+0.1 g/L NaCl+0.4 g/L yeast extract+10 g/L mannitol	YMB	1-2 days
Pre-culture	WM+4 × 10^{-6}M Kn+2.4 × 10^{-6}M IBA	RM	3 days
Co-cultivation	WM+4 × 10^{-6}M Kn+2.4 × 10^{-6}M IBA+200 µM acetosyringone	RM+Acetosyringone	3-5 days
Transformant selection	WM+4 × 10^{-6}M Kn+2.4 × 10^{-6}M IBA+15 mg/L hygromycin	SM	8-9 Weeks

Table 3: Media used for bacterial culture and transformation study of *Grewia asiatica*.

HF stereo zoom microscope (Nikon, Japan) were scored as GUS positive.

Molecular analysis of transformants

The non-transformed as control and transformed explants (hygromycin resistant shoots) were subjected to total DNA isolation using the DNeasy Plant mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The isolated DNA was analyzed for its purity using Nanodrop spectrophotometer (AstraAuriga, Cambridge, UK) and further checked on 0.8% agarose gel. The transformation was confirmed by the presence of the *hpt* transgene by using gene-specific primers (forward: 5′-GCGTGACCTATTGCATCTCC3′ and reverse: 5′-TTCTACACAGCCATCGGTCC 3′) to amplify a 700-bp region of the *hpt* gene. Master Cycler Gradient PCR was used for the DNA amplification and the PCR conditions were as follows: 94°C for 3 min; 35 cycles of 30 sec at 94°C, 35 sec at 50°C, 1 min at 72°C; and a final extension for 7 min at 72°C. The 20 µL reaction mixture contained 1X Taq buffer with NH_4SO_4, 2.5 mM $MgCl_2$, 200 µM dNTPs, and 2 µM each of the forward and reverse primers, 50-100 ng of DNA template from the control and transformants, and 0.5 U of TaqDNA polymerase. Analysis of amplified DNA fragments was performed by gel electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA) using 1.2% (*w/v*) agarose.

Results and Discussion

Effect of plant growth regulators on regeneration of *G. asiatica*

Micropropagation is the clonal perpetuation of plants under *in vitro* conditions with many advantages over conventional vegetative propagation. High multiplication frequency, molecular, cytogenetical and phenotypic stability are some of the important attributes of *in vitro* regenerated plants from organized meristems. The present study demonstrates the applicability of genetically stable micropropagated plants of *G. asiatica* and also entails development of an efficient genetic transformation protocol using *A. tumifaciens*.

The varied morphogenetic response obtained with different media supplemented with various concentrations and combinations of phytohormones is summarized in Table 1. MS medium with different phytohormonal combinations like Kn in combination with auxins IBA and NAA proved to be least effective and failed to induce micro-shoots from axillary buds of nodal explants. It also triggered profuse callusing at the cut surface of the explants (Figure 1a and 1b). Similar morphogenetic response using MS medium in *G. asiatica* has also been reported by Biswas et al. [44]. The explants also elicited poor response in B_5 minimal medium [38]. To optimize the *in vitro* response, we tested WM [39]. It proved quite efficacious to induce micro-shoots (Figure 1c and 1d). Nodal explants on WM exhibited maximum response in terms of shoot induction with phytohormone combination of 2 × 10^{-5} M Kn and 1 × 10^{-5} M IBA. Here the explants with emergent shoots averaged 94% and the mean number of shoots per explant was 4.25 ± 0.75. It also showed prolific foliage of 8.00 ± 0.96 leaves per shoot with mean shoot length of 10.5 ± 1.96 cm. There was not any significant difference in shoot induction by using Kn along with NAA (Table 1).

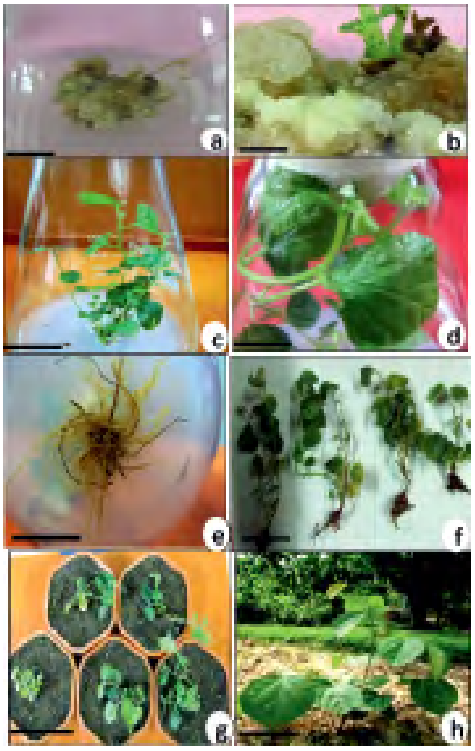

Figure 1: Plant regeneration in *Grewia asiatica* **(a-h):** Profuse callusing with sparse shoot regeneration on MS minimal organic medium supplemented with Kn 1 × 10^{-5} M and IBA 5 × 10^{-6} M (bar=8 mm) **(a, b)**. Multiple shoot induction and regeneration from nodal explants on WM containing Kn 2 × 10^{-6} M and IBA 5 × 10^{-7} M. (bar=10 mm) **(c).** Healthy regenerated shoots with well differentiated foliage and floral buds after 7 weeks of culture (bar=13 mm) **(d).** Rooted individual shoots with profuse tapering roots (bar=15 mm) **(e).** Healthy regenerated shoots with well-developed roots (bars=25 mm) **(f).** Established plants under hardening (bar=12 mm) **(g).** Field grown hardened plant (bar=31.0 cm) **(h).**

Shoots transferred to rooting medium resulted in robust rooting (Figure 1e). Optimum root induction was observed with 4.8 × 10^{-6} M IBA with rooting percentage of 97% and mean root number per shoot 17.4 ± 0.67. Further 5.2 × 10^{-6} M NAA also showed superior rooting of 85% with mean root number per shoot 11.2 ± 0.54. Moreover, this hormonal combination also tended to induce rooting in the shoot tuft of initial culture with multiple micro-shoots. IBA is by far the most commonly used auxin to obtain root initiation [45]. It is inferred that efficacious rooting by IBA is most likely due to the basis that optimum concentration of IBA might be accountable to boost the cambial growth at the base of micro-cuttings that result in differentiation of root primordia [46]. Furthermore, root inducing factors are believed to be essential for rooting, which combine with

auxin to form a complex that directs RNA to activate enzymes that cause root initiation [47]. However, 62% rooting was also observed on WM devoid of phytohormones with mean root number per shoot 6.3 ± 0.43. Induction of rooting in micro-shoots on medium without auxin supplement may be due to endogenous auxin levels along with some root inducing factors which occur naturally within the micro-shoots that may help for root primordia initiation. Root initiation started 2 week after transfer onto rooting medium. Rooting percentage with different concentrations of IBA and NAA ranged from 47 to 97%. Poor morphogenetic response and profuse callusing in *G. asiatica* on MS and B_5 media is probably due to the high nitrogen content in these media than that of WM. Micro-shoots from nodal explants arose without an intervening callus phase. Similar morphogenetic response has also been recorded in *Fraxinus profunda* [48], internodal and nodal ring regions of *Piper* sp. [49]. Following hardening procedures, 6-weeks old hardened plants (Figure 1f and 1h) were successfully transferred to field conditions, with a survival rate of about 92%.

RAPD fingerprinting

The *in vitro* raised plants, successfully established under field conditions were free of any detectable phenotypic change compared to the donor mother plant. For genotypic variability RAPD analysis was checked from *in vitro* grown plants and mother plant. Utility of RAPD as a means of molecular analysis of *in vitro* regenerated plants has been well documented [50,51]. Genetic fidelity of micro-propagated plants has immense practical utility and commercial implications [52] (Gantait et al.). With this perspective, we assessed the genetic integrity of *in vitro* regenerated plants from axillary buds in *G. asiatica*. The RAPD band pattern of 13 randomly selected *in vitro* regenerated plants was compared with the donor mother plant. Five primers (Table 4) used for amplification of the isolated DNA samples displayed same banding pattern in all the chosen plants and were virtually same as that of the mother plant. The representative profile of the 13 *in vitro* raised plants with a primer OPD-05 is shown in Figure 2. The size of polymorphic fragments varied from 200 to 2000 bp and the number of amplified fragments ranged from 5 to 18. The monomorphic banding pattern in micro-propagated plants in comparison to *in vivo* mother plant in *G. asiatica* shows that organized multiple axillary buds are least vulnerable to genetic changes and are strictly true to parent type. This establishes the suitability of protocol developed for the micropropagation of *G. asiatica*.

Agrobacterium mediated transformation

Efficient regeneration system for the generation of transgenic tissues/plants remains an important bottleneck for most of the species and cultivars. In present study, petiole of leaf was highly responsive to transformation followed by young internodes**.** Direct shoot organogenesis from the cut edges of petiolar explants was observed at the end of 4-5 weeks of culture (Figure 3a-3c). Petioles were used as the explant source for further transformation studies. Explant source has a significant impact on transformation efficiency as different tissues have different endogenous hormone levels. Endogenous hormone levels are

Primer	Sequence (5′→3′)
OPB-14	CCGCCTAGTC
OPB-19	TGTCGACA TG
OPC-11	CCCAGCTGTG
OPC-13	CCACCAGTGG
OPD-05	CATTCGAGCC

Table 4: Primers used for RAPD analysis of *Grewia asiatica*.

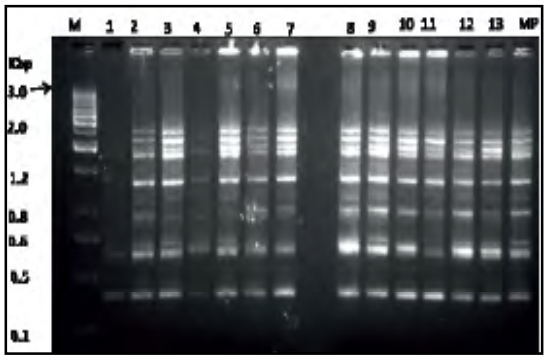

Figure 2: DNA amplification obtained with primer OPD-05: Mother plant (MP), micropropagated plants cultured on WM basal+2 × 10^{-6}M Kn+1 × 10^{-5}M IBA (lanes 1-13) and M, Molecular Weight Markers (3 kb DNA Ladder).

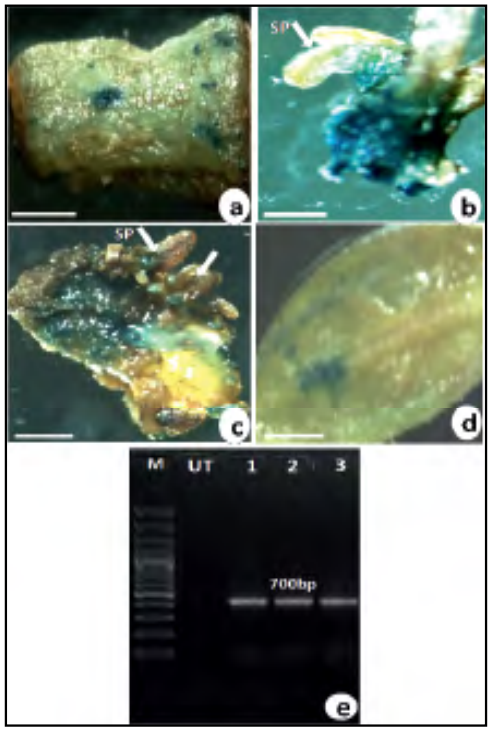

Figure 3: *Agrobacterium tumifacians*- mediated transformation of *G. asiatica* using aseptic petiole and leaf explants **(a-d)**. β-glucuronidase (GUS) expression shown by infected petiole after co-cultivation on WM **(a-c)** and leaf explants **(d)** (SP=Shoot primordia) (bar=2 mm). PCR detection of 700 bp fragment of *hpt*, M molecular weight ladder, UT untransformed control, 1-3 independent putative transgenic shoot buds **(e)**.

equally important to exogenous hormone levels which determine the rate of important processes like dedifferentiation and redifferentiation [53]. The processes of dedifferentiation and redifferentiation are required in both direct and indirect shoot organogenesis. After more than 25 years, *Agrobacterium*-mediated gene transfer is still the most used method for the transformation of fruit bearing species like apple, almond, banana, orange, grapevine and melon [54]. *Agrobacterium*-mediated transformation is highly genotype dependent for many plants and this problem is widely described in many fruit species as apricot, grapevine and others [55]. *Agrobacterium* strain GV2260 was successful for transformation of *G. asiatica* producing GUS positive explants harboring p35SGUSINT (Figure 3a and 3d).

Optimization of co-cultivation duration

The process of co-cultivation which is one of the significant steps for *Agrobacterium*-mediated transformation in plants has distinct influence on the efficiency of transformation. The temporal study of co-cultivation in *G. asiatica* from 1-6 days demonstrated that 3d was optimal with transformation efficiency of 50%. While as co-cultivation for less than 3d strikingly decreased the transformation efficiency. Nevertheless co-cultivation for more than 3d also reduced the transformation frequency which was observed due to bacterial overgrowth that in turn resulted in the explant mortality (Figure 4). Plant growth regulators also play an important role in co-cultivation medium as they enhance cell division, resulting in high regeneration frequency of transformants compared with the co-cultivation medium without plant growth regulators [56]. The optimal conditions for transformation of *G. asiatica* nodal explants utilized a pre-culture for 3d followed by co-cultivation for 3d on RM supplemented with 200 µM acetosyringone.

Selection and regeneration of transgenic shoots

The hygromycin resistant regenerants sub-cultured on SM for 7-8 weeks resulted in the poor regeneration of plants. Initially the explants showed some growth response which later on diminished. The efforts are underway to generate stably transformed plants in Phalsa. Poor transgenic plant recovery may be attributed to constraints imposed by transgenes and/or the hygromycin selection pressure that may impose undue stress on transgenic plant survival, resulting in low maturation. *Agrobacterium*-mediated transformation involves interaction between two biological systems and is affected by various physiological conditions [57,58]. Considering the large number of factors involved, it is not possible to conduct a factorial experiment and hence interaction effects, if any, cannot be assessed from present investigation. This regeneration protocol with attempted and underway transformation provides an integral framework for future genetic improvement of *G. asiatica* particularly in context to induction of parthenocarpy. This may eventually complement traditional and biotechnological approaches for the development of Phalsa as a commercial crop.

Molecular analysis of putative transgenic shoots

The hygromycin resistant *in vitro* regenerated shoots were further confirmed by PCR by amplifying *hpt* gene using gene specific primers from total genomic DNA obtained from transformed shoots. The same amplified region (700 bp) was missing from the untransformed (control) plants (Figure 3e). The transient GUS assay approach was found to be an easy and reliable way of establishing optimal conditions for transformation.

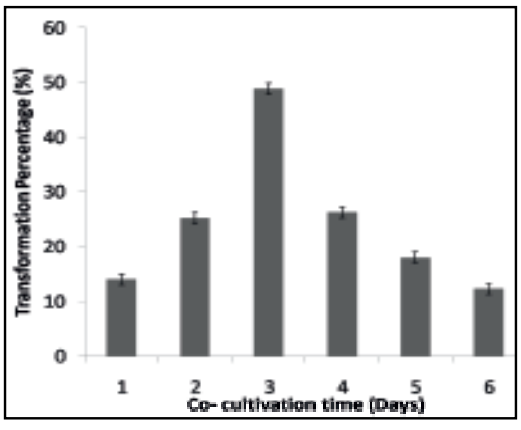

Figure 4: Effect of co-cultivation duration on transformation efficiency using *Agrobacterium tumefaciens* strain GV2260 harboring binary vector p35SGUSINT on WM.

Conclusions

In the present study, a simple and hitherto unreported *in vitro* regeneration and *Agrobacterium*-mediated genetic transformation as confirmed by GUS-transient expression analysis was established for *G. asiatica*. Although the frequency of transformation was low, the protocol is repeatable and can be further optimized for the development of transgenic plants to mobilize genes of agronomic importance into elite cultivars. Further, genetic fidelity of micro-propagated plants was assessed employing RAPD analysis. The molecular analysis revealed no genomic alterations in the plants developed through forced axillary bud induction. The effort described herein may allow the disruption of activity of key pathway genes implicated in seed formation.

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