Regeneration and transformation studies in *Terminalia chebula* Retz.

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This study presents in vitro regeneration of *Terminalia chebula* Retz. to obtain complete plantlets from juvenile explants (hypocotyl and cotyledon). Dried seeds were inoculated on MS basal medium after surface sterilization with Bavistin (0.2%) alone and followed by HgCl2 (0.1%), resulted in maximum (75%) germination. Hypocotyl showed 90% and cotyledon 75% callus induction on MS basal medium containing 1.0 mg/l 2, 4-D after 30 days of inoculation. Shoot regeneration was recorded only from cotyledonary callus on shoot induction medium comprising 1.5 mg/l BAP with 0.10 mg/l NAA with maximum 36.67% shoot regeneration. Maximum (43.75%) rooting was reported in ½ strength MS medium with 0.5% activated charcoal. Transgenic callus was produced through *Agrobacterium tumefaciens* mediated genetic transformation carrying gus and npt-II gene from cotyledonary explants. Co-cultivation (72 h) preceded by pre-conditioning (72 h) was found best for callus induction. Successful integration of gus gene was reported.

**Keywords:** *Agrobacterium tumefaciens*, Hypocotyl, Cotyledon, Regeneration, *Terminalia chebula*

**Introduction**

*Terminalia chebula* Retz. (Family, *Combretaceae*), commonly known as Harad, is an indigenous, multipurpose and deciduous tree of great economical importance1. It occurs in Northern tropical wet evergreen forests, tropical seasonal swamp forests, Southern and Northern tropical deciduous forests2. Fruit and dried flesh surrounding seeds are the most important product. Seeds contain tannin (30-32%), which varies with season of collection and locality3. *T. chebula* is always listed first in the Ayurvedic metiria medica4. Fruits have antiamoebic properties5 and are used in fevers, cough, asthma, urinary diseases, piles, worms and rheumatism, and scorpion-sting6. *Triphala* (*Belleric myrobalans, Embelic myrobalans* and *Chebulic myrobalans*) is an important Ayurvedic formulation used in the treatment of liver and kidney dysfunctions2. Natural regeneration of *T. chebula* from seeds in situ and ex situ is extremely low8 and is a slow growing tree compared to other species of *Terminalia*9. *In vitro* propagation has also been developed10,11. Direct sowing of seeds results in queer, inadequate germination and low survival of seedlings, all of which contribute to high production cost of seedling stock. Micropropagation of *T. chebula* has already been reported12 from shoot buds of mature tree. Reports on induction of callus are also there.

This study presents indirect regeneration in *T. chebula* Retz. using juvenile explants [hypocotyl (Hy) and cotyledon (Co)].

**Experimental Section**

Seed kernels obtained after excising hard seed coat were surface sterilized with sodium bavistin (0.2%) solution for different time durations (0-15 min) followed by washings with autoclaved distilled water, by 0.1% HgCl2 and then again 5-6 washings with autoclaved distilled water under aseptic conditions. Surface sterilized seed kernels were inoculated on MS basal medium (full strength) for germination. *In vitro* grown seedlings (15-20 days old) were used as a source of explant (Co & Hy) to carry out regeneration studies. Callus induction (CI) was achieved on Murashige and Skoog® (MS)13 basal medium containing agar (0.8%), sucrose (3%) and supplemented with different concentrations of 2,4-D (2,4- dichlorophenoxy acetic acid). Type, growth and colour of callus were observed. All constituents of media were procured from SISCO Research Laboratories Pvt Ltd, Mumbai, while 2, 4-D was procured from Merck Chemicals Pvt Ltd. Subculturing of callus was done on same medium for two times at an interval of 4 weeks for proliferation. All
cultures were kept in culture room at 26 ± 2°C under 16 h photoperiod at 20 µmol m⁻² s⁻¹. Regenerative callus was transferred to shoot induction medium [MS basal medium + different concentrations of BAP (6-benzyloaminopurine) alone and in combination with NAA (α-naphthalene acetic acid)]. Shoot regeneration%, number and length of shoots were noted. Micro shoots (1.5-2.0 cm long) were taken after 6 weeks from shoot induction medium and transferred on a different media for root induction. Shoot induction%, average number of shoots and average shoot length were recorded for each subcultured shoot. Similar observations were recorded in case of rooting.

In vitro rooting of micro shoots is done by dipping them in pre-autoclaved IBA solution for 2 h under dark aseptic conditions and thereafter subculturing in half-strength MS medium supplemented with different concentrations of activated charcoal. In present study, different concentrations of activated charcoal (0.1-1.0%) in ½ strength MS medium were tested for in vitro rooting (Fig. 1). Agrobacterium tumefaciens strain LBA 4404 was used for genetic transformation experiment. In co-cultivation experiment, explants were inoculated on MS medium supplemented with 2,4-D for different time durations and pre-conditioning was done by culturing explants on same medium devoid of Agrobacterium. All experiments were laid out in a completely randomized design (CRD)¹⁵. Significance level for F-test was 5%.

Table 1Δ Effect of different duration of 0.2% bavistin (alone) and 0.2% bavistin in combination with 0.1% HgCl₂ (5 min) on surface sterilization of seed kernels (Figures in parentheses are arc sine transformed)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration min</th>
<th>0.2% Bavistin</th>
<th>0.2% Bavistin and 0.1% HgCl₂ 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₁</td>
<td>6</td>
<td>46.67 (43.09)</td>
<td>60.00 (50.79)*</td>
</tr>
<tr>
<td>B₂</td>
<td>8</td>
<td>63.33 (52.78)</td>
<td>68.33 (55.77)</td>
</tr>
<tr>
<td>B₃</td>
<td>10</td>
<td>73.33 (58.93)</td>
<td>75.00 (60.08)</td>
</tr>
<tr>
<td>B₄</td>
<td>12</td>
<td>68.33 (55.77)</td>
<td>70.00 (56.84)</td>
</tr>
<tr>
<td>B₅</td>
<td>15</td>
<td>53.33 (46.91)</td>
<td>63.33 (52.74)</td>
</tr>
<tr>
<td>CD₀.₀₅</td>
<td>6.64</td>
<td>4.79</td>
<td></td>
</tr>
<tr>
<td>S.E.</td>
<td>2.98</td>
<td>2.15</td>
<td></td>
</tr>
</tbody>
</table>

Results and Discussion

In vitro Regeneration in T. chebula Retz.

To achieve surface sterilization of seed kernels, 0.2% bavistin alone for 10 min resulted in 73.33% uncontaminated surviving cultures. However, 0.1% HgCl₂ dipping for 5 min resulted in higher number (75%) of uncontaminated surviving cultures after 15 days of incubation (Table 1). After treatment with each surface sterilant, seed kernels were washed 5-6 times with autoclaved distilled water. Surface sterilized seed kernels were established on solid MS basal medium under 16 h photoperiod. Germination of Anogeissus pendula Edgew. viable seeds on MS medium has also been reported¹⁶. High rate of germination frequency in T. chebula embryos has been obtained on MS medium supplemented with 0.5 mg dm⁻³ gibberellic acid (GA). In Morus alba, zygotic embryos inoculated on MS basal medium supplemented with sucrose (30 g/l) showed 97.0% germination and formed seedling¹⁷.

Indirect Shoot Regeneration from Cotyledon (Co) and Hypocotyl (Hy)

Auxin is the primary hormone used to produce callus. Often 2,4-D is used to produce callus. In some species, high concentration of auxin and low concentration of cytokinin in medium promotes abundant cell proliferation with callus formation¹⁸. Callus is produced on explants in vitro as a result of wounding and in response of hormones endogenous or exogenously¹⁹. In present study, Co and Hy explants, which were excised from 3 weeks old in vitro raised seedling, were subjected to different concentrations of 2,4-D supplemented in MS medium for CI. Among Co and Hy explants (Table 2), Hy that showed 90% CI in treatment C₃ is the best explant;
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highest CI recorded in Co was 75% for the same treatment C3. Similarly, Hy explants were found better for callusing in Morus alba and M. indica. Better CI and proliferation from Co explants than leaf explants of Punica granatum L. cv. Ganesh is also reported. However, in present study, response of CI reduced for both explants with further increase in concentration of 2,4-D (Table 2). CI% was more in Hy explants as compared to Co part. Best recognized treatment for CI was 1.0 mg/l 2,4-D in MS medium (Table 2, Fig. 2).

Shoot Bud Induction and Development

Small callus pieces (0.5-0.8 cm²) from Co and Hy explants were cultured on MS medium with different concentrations of BAP alone (1.0-2.0 mg/l) and in combination with NAA (0.05-0.15 mg/l) for in vitro shoot induction. Co derived callus exhibited a high potential for shoot differentiation while Hy derived callus did not show any differentiation. Maximum shoot bud differentiation in Co derived callus in pomegranate on MS medium supplemented with 1.50 mg/l BAP + 0.5 mg/l NAA is also reported. BAP alone had no effect on shoot regeneration (Table 3) till 6 weeks. Highest shoot regeneration (36.67%) in case of Co was found in treatment D8 containing 1.50 mg/l BAP + 0.10 mg/l NAA, which was superior to all other combinations. It showed maximum number (3) of shoots per callus clump with maximum average shoot length (1.56 cm). Lowest shoot regeneration (1.67%) was observed in D4 containing 1.00 mg/l BAP + 0.05 mg/l NAA with least number of shoots (0.33) and shoot length (0.53 cm).

Unlike Co derived calli, the calli obtained from Hy explants did not respond at all to in vitro shoot bud induction and turned brown on prolonged culture (Fig. 3). In another study, best shoot multiplication response was obtained from nodal explants of T. arjuna on modified MS medium containing 4.44 μM BA (benzyladenine) and 0.5 μM NAA. Shoot induction from calluses is reported highest (98%) in Co derived callus cultured on MS medium supplemented with 0.5 μM NAA + 5.0 μM BA. In vitro shoot proliferation from nodal segments in T. chebula
on WPM supplemented with 1.50 mg/l BAP + 0.05 mg/l NAA was also observed\textsuperscript{24}. However, in present study, best medium for shoot bud induction and proliferation is MS medium supplemented with 1.5 mg/l BAP + 0.10 mg/l NAA (Fig. 4). D\textsubscript{8} treatment containing 1.50 mg/l BAP + 0.10 mg/l NAA was found to be best for \textit{in vitro} shoot

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BAP mg/l</th>
<th>NAA mg/l</th>
<th>Shoot regeneration%</th>
<th>Number of Shoots</th>
<th>Shoot length cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>1.0</td>
<td>0.00</td>
<td>0.00 (0.00)\textsuperscript{*}</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D2</td>
<td>1.5</td>
<td>0.00</td>
<td>0.00 (0.00)</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D3</td>
<td>2.0</td>
<td>0.00</td>
<td>0.00 (0.00)</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D4</td>
<td>1.0</td>
<td>0.05</td>
<td>1.67 (4.30)</td>
<td>0.33</td>
<td>0.53</td>
</tr>
<tr>
<td>D5</td>
<td>1.0</td>
<td>0.10</td>
<td>3.33 (8.61)</td>
<td>0.67</td>
<td>0.87</td>
</tr>
<tr>
<td>D6</td>
<td>1.0</td>
<td>0.15</td>
<td>8.33 (13.74)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>D7</td>
<td>1.5</td>
<td>0.05</td>
<td>23.33 (28.86)</td>
<td>2.00</td>
<td>1.17</td>
</tr>
<tr>
<td>D8</td>
<td>1.5</td>
<td>0.10</td>
<td>36.67 (37.26)</td>
<td>3.00</td>
<td>1.56</td>
</tr>
<tr>
<td>D9</td>
<td>1.5</td>
<td>0.15</td>
<td>21.67 (27.71)</td>
<td>2.00</td>
<td>1.33</td>
</tr>
<tr>
<td>D10</td>
<td>2.0</td>
<td>0.05</td>
<td>15.00 (22.60)</td>
<td>1.57</td>
<td>1.16</td>
</tr>
<tr>
<td>D11</td>
<td>2.0</td>
<td>0.10</td>
<td>13.33 (21.34)</td>
<td>1.33</td>
<td>1.13</td>
</tr>
<tr>
<td>D12</td>
<td>2.0</td>
<td>0.15</td>
<td>10.00 (18.44)</td>
<td>1.00</td>
<td>0.93</td>
</tr>
<tr>
<td>CD\textsubscript{0.05}</td>
<td>2.72</td>
<td></td>
<td>4.02</td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>5.61</td>
<td>8.30</td>
<td></td>
<td>0.35</td>
</tr>
</tbody>
</table>

Fig. 3\textsuperscript{6} Callus induction from hypocotyl explant (Browning of callus on shoot regeneration medium after 6 weeks of incubation; on MS medium supplemented with 1.0 mg/l 2,4-D )
regeneration from Co derived calli and statistically significant.

**In vitro Root Induction**

Microshoots (length, 1.5-2.0 cm) were isolated, excised and transferred to root regeneration medium for root induction to get complete plantlets. Root induction initiated within 15-20 days in culture, and within 6 weeks well developed root system was obtained (Fig. 5). Rooting in *M. alba* on MS medium supplemented with 0.05% activated charcoal is also reported\(^{25}\). However, in present study, microshoots of *T. chebula* obtained by indirect regeneration system were rooted on ½ strength MS medium + 0.5% activated charcoal after IBA (2 mg/ml) treatment. Out of 4 treatments, maximum (43.75%) rooting was obtained with 2.2 number of roots per shoot and root length 2.15 cm in treatment E\(_3\) containing ½ strength MS medium + 0.5% activated charcoal (Fig. 5). But on further increasing concentration of activated charcoal in ½ strength MS medium, rooting decreased (37.50%) with 1.56 number of roots per shoot and root length of 1.77 cm. In ½ strength MS medium without activated charcoal, microshoots did not respond to rooting at all (Table 4).

**Genetic Transformation of *T. chebula* Retz.**

Of several recombined strains of *A. tumefaciens*, LBA 4404 has proved to be most successful\(^{26-28}\). *Escherichia coli* β-glucuronidase was developed as a reporter system for transformation of plants to overcome difficulties faced in using other reporter genes\(^{29}\). An efficient technique for introducing cloned genes into plant cells using *Agrobacterium* was also standardised\(^{30-32}\). Woody species tend to be difficult and often inefficiently transformed due to lack of proper regeneration system\(^{33}\). *A. tumefaciens* strain LBA 4404 carrying β-glucuronidase and neomycin phosphotransferase-II marker genes were used for genetic transformation.
studies to develop putative transgenic callus in *T. chebula* Retz. using Co (Fig. 2). In present study, genetic transformation in *T. chebula* was carried out up to callus phase.

**Effect of Pre-conditioning and Co-cultivation Duration on Transformation Frequency**

A pre-conditioning time of 72 h followed by co-cultivation for 72 h resulted in highest transformation frequency for CI through Co explants. During pre-conditioning, explants undergo a physiological and developmental shift to enter morphogenic competency. After T-DNA insertion, recipient cells have already entered regeneration pathway\(^{34-37}\). Co-cultivation experiments included inoculation of Co explants on MS medium supplemented with 1.0 mg/l 2,4-D for 24, 48 and 72 h. Maximum transformation rate (7.77) was recorded in treatment F9 with 2.33 explants showing CI when 72 h of pre-conditioning was followed by 72 h of co-cultivation (Table 5). However, in F6 treatment, pre-conditioning for 48 h followed by 72 h of co-cultivation resulted in decreased transformation rate (4.44) with 1.33 explants showing CI. Low response was recorded when 48 h of co-cultivation was preceded by different durations (24, 48 & 72 h) of pre-conditioning, while no response was recorded when 24 h of co-cultivation was preceded by different durations (24, 48 & 72 h) of pre-conditioning. Co-cultivation for 2-3 days is standard for most of the transformation protocols, as longer periods have

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**Table 5**

Effect of pre-conditioning and co-cultivation of explants on transformation frequency in MS medium (Figures in parentheses are square root +1 transformed)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-conditioning h</th>
<th>Co-cultivation h</th>
<th>Number of explants</th>
<th>Explants producing callus</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>24</td>
<td>24</td>
<td>30</td>
<td>0.00</td>
<td>0.00 (1.00)</td>
</tr>
<tr>
<td>F2</td>
<td>24</td>
<td>48</td>
<td>30</td>
<td>0.00</td>
<td>0.00 (1.00)</td>
</tr>
<tr>
<td>F3</td>
<td>24</td>
<td>72</td>
<td>30</td>
<td>1.00</td>
<td>3.33 (195)</td>
</tr>
<tr>
<td>F4</td>
<td>48</td>
<td>24</td>
<td>30</td>
<td>0.00</td>
<td>0.00 (1.00)</td>
</tr>
<tr>
<td>F5</td>
<td>48</td>
<td>48</td>
<td>30</td>
<td>0.33</td>
<td>1.11 (1.36)</td>
</tr>
<tr>
<td>F6</td>
<td>48</td>
<td>72</td>
<td>30</td>
<td>1.33</td>
<td>4.44 (2.31)</td>
</tr>
<tr>
<td>F7</td>
<td>72</td>
<td>24</td>
<td>30</td>
<td>0.00</td>
<td>0.00 (1.00)</td>
</tr>
<tr>
<td>F8</td>
<td>72</td>
<td>48</td>
<td>30</td>
<td>0.66</td>
<td>2.22 (1.72)</td>
</tr>
<tr>
<td>F9</td>
<td>72</td>
<td>72</td>
<td>30</td>
<td>2.33</td>
<td>7.77 (2.95)</td>
</tr>
</tbody>
</table>

C.D. 0.05 2.1
S.E. 0.41 0.36

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Fig. 6- a

Genetic transformation and callus induction from cotyledon explant

a) Co-cultivation of cotyledon explants on MS medium + 1.0 mg/l 2, 4-D (for 72 hrs)

b) Putative transformed callus on selective medium after 4 weeks of culturing

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Fig. 6- b
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frequently resulted in *Agrobacterium* overgrowth\(^38\). Maximum transformation frequency with 72 h of co-cultivation in almond has also been reported\(^39\).

**Selection of Putative Transgenic Callus**

To observe growth and regeneration on selective medium, co-cultivated explants were transferred to selective medium containing antibiotics (30 mg/l kanamycin + 500 mg/l cefotaxime) alongwith 2,4-D for CI. A selective advantage was given to transformed cells through introduction of *npt-II* gene conferring resistance against kanamycin, which allows transformed cells to grow where non-transformed cells were unable to grow. The *npt-II* gene encodes enzyme neomycin phosphotransferase-II, which inactivates sugar containing antibiotics by phosphorylation\(^40\). Co-cultivated as well as control Co explants were transferred to selective CI medium. Out of 30 explants, maximum (2.33%) explants showed CI on selective medium after 6 weeks (Table 5, Fig. 6). Phosphorylation of kanamycin by *npt-II* enzyme prevents its action and therefore, only transformed bacteria and plant tissue can grow effectively on kanamycin containing media. To eliminate *A. tumefaciens* from plant cultures, another antibiotic cefotaxime is used because it is a broad spectrum antibiotic for bacterial inhibition\(^40\). Control and some of co-cultivated explants became completely necrotic within a week of culture on selective medium. After 3 weeks on CI medium comprising of 1.0 mg/l 2,4-D supplemented with 500 mg/l cefotaxime and 30 mg/l kanamycin, callus was formed on cut surface of Co explant (Fig. 6b). Data was recorded after 6 weeks on selective medium and a transformation frequency of 7.77 was observed. However, inoculated control explants did not survive at all on selective medium. Control showed higher CI% on non-selective medium as compared to the growth of *Agrobacterium* inoculated explants on selective medium. This may be consequence of antibiotic stress.

**Confirmation of Gene Integration**

Polymerase chain reaction was carried out to confirm transfer of *gus* gene from *A. tumefaciens* into genome
of cells (callus) of *T. chebula* Retz. Total genomic DNA was isolated from randomly selected 3 callus samples by already standardised method. Gene specific primers (Table 6) were used to amplify a 0.7 kb fragment of *gus* gene by PCR. Stable integration of transgene in citrus plants was confirmed by PCR analysis in similar studies. Integration of transgene (*gus* gene) into citrus genome was confirmed by polymerase chain reaction. PCR product was visualized after electrophoresis in 1.5% agarose gel (Fig. 7). Lane 1 representing DNA size marker of 100 bp, lane 2 containing sterile water and lane 3 containing reaction mixture were taken as negative controls, lane 4 containing plasmid pBl121 was taken as positive control, whereas lane 5 contains DNA of none transformed control C and lanes 6-8 contain DNA of transformed samples T1, T2 and T3 (Fig. 7). Lane 4 containing plasmid pBl121 showed the band of amplified *gus* gene, while lane 2, 3 and 5 did not show any such band. Out of 3 putative transgenic callus samples in lanes 6-8, only lane 6 and 8 showed amplification of band of integrated gene that is at par with band of positive control, thus showing confirmation of *gus* (Fig. 7). Similarly, transgenic nature of transformants was demonstrated by PCR analysis in *Jatropha curcas*.

Conclusions

This study reports for the first time *A. tumefaciens* mediated genetic transformation of *T. chebula* up to callus phase using *gus* gene from Co explant on selective medium containing 30 mg/l kanamycin and 500 mg/l cefotaxime. Major gap still exists for providing superior planting material with stably inherited traits associated with many important industries. Also, because of deviation from allopathic towards traditional Ayurvedic treatment, demand for *Terminalia* will always rise. Therefore, present research provides a platform for mass propagation of *T. chebula* and further carrying out genetic transformation studies using agronomically important traits in this species.

References