Organogenic plant regeneration via callus induction in chickpea 
(*Cicer arietinum* L.)—Role of genotypes, growth regulators and explants

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Organogenic plant regeneration via callus induction was studied in genotypes Pusa256 and PG186 from four explants, viz. immature embryos, immature cotyledons, mature embryo axes and mature cotyledons, on media containing different combinations and concentrations of growth regulators. Different callusing media containing varying levels of NAA (0.5 to 2 mg/l) with or without BAP (1 mg/l) were tested for callus induction response. Maximum (90%) callusing response was obtained from immature cotyledon explants on medium supplemented with 2 mg/l NAA and 1 mg/l BAP. However, only 64% of induction frequency was obtained from immature embryos on medium containing 2 mg/l NAA. After 8 weeks of induction, the calluses were transferred on different regeneration media containing varying levels of BAP (1 to 3 mg/l) with or without NAA (0.05 mg/l). Multifactorial analysis of the study revealed that calluses, induced from a medium with high callus induction frequency, did not show high shoot induction frequency. Thus, all the factors, viz. explants, genotypes and growth regulators, during callus induction play a significant role in the subsequent high frequency shoot regeneration. In general, calluses induced from embryonic explants on medium with 1.5 mg/l NAA and 1 mg/l BAP showed 17-18% shoot regeneration on a medium containing 1-2 mg/l BAP alone or in combination with 0.05 mg/l NAA.

**Keywords**: chickpea, organogenic regeneration, callus induction

**IPC Code**: Int. Cl.7 A01H4/00, 5/00

**Introduction**

Chickpea (*Cicer arietinum* L.), commonly known as gram, is one of the valuable pulse crops of Indian subcontinent with high protein and fibre-digestibility and for its ability to fix atmospheric nitrogen. The crop is susceptible to major lepidopteran pod-borers. *Helicoverpa armigera* L. alone accounts for a minimum of 30% loss in yield, creating a big gap between the yield on research plot and at farmer’s field. However, the accessible gene pool has not permitted the breeder to tailor the crop to the extent that it can tolerate various stresses and become a competitive crop. There are many reports on plant regeneration and also a few on genetic transformation of chickpea. However, the crop is relatively recalcitrant to regeneration by the commonly applied tissue culture techniques. Further, high frequency regeneration is needed for introduction of gene of interest in high yielding commercially released genotypes.

Regeneration via direct shoot induction has been reported in chickpea from various explants, viz. mature embryo axes1-3, immature embryo4, seed and cotyledonary5,6 node, hypocotyl and shoot apex7. Organogenic regeneration via callus induction approach has also been attempted using mature embryo axes8, cotyledons9, leaflets10, distal and proximal cotyledons11 and hypocotyl explants12. Higher callusing response from leaflet explants was obtained on medium supplemented with NAA13,14. However, very less frequency of *in vitro* regeneration was observed from hypocotyls, followed by shoot sections and shoot apices15. Thus, plant regeneration from callus cultures has been obtained with a very low frequency.

In view of the above, present investigation attempts to obtain shoot regeneration from callus induced from different immature and mature seed explants and identify the role of commercially available high yielding genotypes and growth regulator milieu in callusing medium on the callus induction and shoot regeneration ability.

**Materials and Methods**

**Plant Material**

Cotyledons and embryos from immature seeds and cotyledons and embryo axes from mature seeds of
genotypes Pusa256 and PG186 were used as explants. Immature pods were harvested 10-15 days after pollination and cotyledons and embryos were separated from the developing seeds after surface sterilization with 0.2% \( \text{HgCl}_2 \) solution for 8 min and then washing with several changes of sterile distilled water. Mature seeds were also surface sterilized and then washed with detergent and soaked for 24 hrs prior to explant excision. Then embryos and cotyledons were removed from the seeds. The embryo was excised and decapitated to discard root and shoot meristems, leaving only embryo axis. The cotyledon explants from both the immature and mature seeds were slightly cut at three ends leaving the attachment site of embryo intact.

**Callus Induction**

MS medium\(^{16}\), containing 0.5, 1, 1.5 and 2 mg/l naphthaleneacetic acid (NAA or N) with or without 1 mg/l benzylamino purine (BAP or B), was used for callus induction and designated as CM\(_{0.5N}\), CM\(_{0.5N1B}\), CM\(_{1N}\), CM\(_{1N1B}\), CM\(_{1.5N}\), CM\(_{1.5N1B}\), CM\(_{2N}\) and CM\(_{2N1B}\). The medium without growth regulator (CM\(_{0}\)) served as control. The data on callus induction ability was recorded after 3 weeks of explant inoculation onto the medium. After 4 weeks of inoculation, the calluses were subcultured on the same media for proliferation. The calluses were regenerated after 8 weeks of callus induction.

**Regeneration**

After 8 weeks of induction, the calluses were transferred to modified MS medium [MS basal salts (1\( \times \) macro salts and 4\( \times \) micro salts) with B\(_{5}\) vitamins\(^{17}\)] supplemented with 1, 2 and 3 mg/l BAP with or without 0.05 mg/l NAA for shoot regeneration, designated as SR\(_{1B}\), SR\(_{1B0.05N}\), SR\(_{2B}\), SR\(_{2B0.05N}\), SR\(_{3B}\) and SR\(_{3B0.05N}\). The shoot regeneration medium without growth regulators served as control (SR\(_{0}\)). The data on shoot induction ability of callus cultures was recorded after 3 weeks of transfer on shoot regeneration medium.

The cultures were kept at 20±2°C temperature in complete darkness during callus induction and 16 and 8 hrs photoperiod during shoot and root regeneration. Eight explants were cultured in one flask/petri dish with minimum of four replications per treatment. The experiments were carried out for two years and data analysis was done after transforming the values in percentage. The data was analyzed statistically for testing the significance of main and interaction effects of genotype, medium and explant. The experiments conducted for calluses, shoot and root inductions were designed in completely randomized block with three factors for callus induction and four factors for regeneration from calluses.

**Results and Discussion**

**Callus Induction**

Analysis of variance for callus induction frequency showed significant differences for individual effects of genotypes, callusing media and explants, and their two-way interactions except for genotype \( \times \) callusing media (Table 1). However, the three-way interaction between genotype \( \times \) callusing media \( \times \) explant was found insignificant.

Callus could be induced from all the explants on different callusing media with varying frequency (Table 2). Immature cotyledon explants of both the genotypes showed 90% callus induction frequency on medium supplemented with 2 mg/l NAA and 1 mg/l BAP (CM\(_{2N1B}\)), followed by mature cotyledon explants with 90% and 81% callus induction frequency in Pusa256 and PG186 genotypes, respectively on the same medium. Whereas, immature embryos and mature embryo axes showed better callus induction on medium containing 2 mg/l NAA alone. In this regard, callus induction frequency of 67% and 60% for immature embryos and 65% and 58% for mature embryo axis explants of PG186 and Pusa256 was observed. Embryonic explants showed 53-67% frequency on medium containing auxin alone with little or non-significant increase on medium containing both auxin and cytokinin (Table 2). The calluses derived from immature embryo and embryo axis explants showed 2-10% preformed shoot buds, which were discarded at an early stage so that regeneration from cultures should be \textit{de novo}. While, none of the calluses derived from cotyledon explants showed preformed shoot buds. Embryonic explants

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Callus induction frequency</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>Callusing media</td>
<td>8</td>
<td>753.5**</td>
</tr>
<tr>
<td>Explant</td>
<td>3</td>
<td>331.2**</td>
</tr>
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<td>Genotype ( \times ) callusing media</td>
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<tr>
<td>Callusing media ( \times ) explant</td>
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<td>151.7**</td>
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<td>7.4**</td>
</tr>
<tr>
<td>Genotype ( \times ) callusing media ( \times ) explant</td>
<td>24</td>
<td>0.5 ns</td>
</tr>
</tbody>
</table>

**Significant at 1% level of significance**
produced calluses tend to be friable, pale yellow and slightly loose (Fig. 1a). However, calluses from cotyledon explants were hard, non-friable and creamish-yellow. The colour intensity and compactness of calluses increased with increase in NAA concentration in the callusing media.

In case of mature and immature cotyledon explants, interaction of explants with callusing media revealed that increase in concentration of NAA (auxin) alone in the media did not show high callus induction but addition of cytokinin to the same level of auxin had a stimulatory effect on callus induction ability of both the explants (Table 2). However, embryonic explants showed insignificant differences in callus induction ability on media containing NAA alone or NAA with BAP. It implies that cotyledon explant, which is a storage tissue, has less or no endogenous cytokinin. It requires exogenous cytokinin along with auxin in the media for better and high callus induction. Whereas, tissue of embryonic explants has some endogenous cytokinin and thus exogenous addition of cytokinin during callus induction neutralizes the effect of auxin for high callus induction ability. Further, interaction of genotype and explant for callus induction

<table>
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<th>Immature cotyledon</th>
<th>Mature embryo axes</th>
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<td></td>
<td>PG186</td>
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<td>36.4</td>
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<td>39.1</td>
<td>51.5</td>
<td>36.3</td>
<td>44.9</td>
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</table>

**SE of mean**

- Genotype: 0.3
- Callusing media: 0.7
- Explant: 0.5
- Genotype × callusing media × explant: 2.0
- Callusing media × explant: 1.4

**CD at 5%**

- 0.9**
- 2.0**
- 1.3**
- 3.9**
- 1.8**
frequency showed that explants taken from genotype PG186 were better with 0.3-5% higher callus induction frequency than the explants of genotype Pusa256 (Table 2). In case of embryonic explants, genotype PG186 showed 36 to 55% callus induction frequency as compared to 36 to 50% callus induction frequency in genotype Pusa256. Immature embryos were reported to be superior for callus induction than other explants\textsuperscript{15}. However, present study showed high callusing (90%) from cotyledon explants as compared to embryonic explants particularly on medium CM\textsubscript{2N1B}. High frequency of callus induction was reported in chickpea by supplementing the media with NAA\textsuperscript{10}, while some of the workers reported high callus induction when media contained NAA along with low concentration of cytokinin\textsuperscript{9,12}. Earlier studies on leaf explants of genotype C-235 showed callusing along with rhizogenesis when cultured on medium containing auxin along with cytokinin\textsuperscript{12}. While, present study showed that all the explants induced calluses without rhizogenesis when a specific combination of growth regulators (NAA+BAP) was used.

**Shoot Regeneration**

Analysis of variance for shoot induction frequency showed significant differences for main factors, like explants, callusing media and shoot regeneration media (Table 3). Genotype factor alone and when involved with other factors showed non-significant results. Only callusing media × shoot regeneration media, callusing media × explant and shoot regeneration media × explant showed significant effects and results have been discussed from the two-way significant interactions.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Shoot induction frequency from callus cultures</th>
</tr>
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<tr>
<td>Shoot regeneration media</td>
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<td>0.25**</td>
</tr>
<tr>
<td>Explant</td>
<td>3</td>
<td>0.40**</td>
</tr>
<tr>
<td>Genotype × callusing media</td>
<td>8</td>
<td>0.12 ns</td>
</tr>
<tr>
<td>Genotype × shoot regeneration media</td>
<td>6</td>
<td>0.21 ns</td>
</tr>
<tr>
<td>Genotype × explant</td>
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<td>0.28 ns</td>
</tr>
<tr>
<td>Callusing media × shoot regeneration media</td>
<td>48</td>
<td>0.18**</td>
</tr>
<tr>
<td>Callusing media × explant</td>
<td>24</td>
<td>0.33**</td>
</tr>
<tr>
<td>Shoot regeneration media × explant</td>
<td>18</td>
<td>0.20**</td>
</tr>
<tr>
<td>Genotype × callusing media × shoot regeneration media</td>
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<td>0.16 ns</td>
</tr>
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</tr>
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<tr>
<td>Callusing media × shoot regeneration media × explant</td>
<td>144</td>
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<tr>
<td>Genotype × callusing media × shoot regeneration media × explant</td>
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</table>

\*\*Significant at 1% level of significance
Interaction of explants with callusing media on shoot induction frequency showed that embryonic explants gave significantly higher shoot induction frequency than cotyledon explants (Fig. 2). In this context, it was found that calluses induced from embryonic explants were superior with 8 to 30% shoot induction frequency over cotyledon explants, which had a maximum of 7% shoot induction frequency. Further, out of immature and mature explants, immature embryos were the best for organogenesis via callus induction. A maximum of 30-31% shoot induction frequency from embryonic explants was obtained from calluses induced on medium with 1.5 mg/l NAA and 1 mg/l BAP (Figs 1b & c). Whereas, cotyledon explants gave maximum shoot induction frequency of 6-7% from the calluses induced on a medium with 0.5 mg/l NAA and 1 mg/l BAP. Analysis reveals that with increase in concentrations of NAA from 0.5 to 1.5 mg/l with BAP (1g/l) in the callus induction media, shoot induction frequency increased from 13 to 31% for embryonic explants but it decreased by 6% at 2 mg/l NAA with BAP (Fig. 2). Whereas, shoot induction frequency for cotyledon explants decreased with increase in NAA concentration from 0.5 to 2 mg/l with or without BAP. Thus, for high shoot induction ability, callusing media with 1.5 mg/l NAA and 1 mg/l BAP was found optimal for embryonic explants (Figs 1b & c) and media with 0.5 mg/l NAA and 1 mg/l BAP for cotyledon explants (Fig. 1d). When only callus induction frequency was considered, good callusing media were 2 mg/l NAA and 2 mg/l NAA with BAP for embryonic explants and cotyledon explants, respectively. However, in context of shoot induction, these media showing high callus induction were not good because they had 10-15% less shoot induction frequency for embryonic explants and 0-8% for cotyledon explants. Thus, multi-factorial analysis reveals the importance of callusing media and explants on shoot regeneration ability.

Interactions involving shoot regeneration media and explants demonstrated high shoot induction frequency of 16-18% for embryonic explants and 3-5% for cotyledon explants on media supplemented with either 1 or 2 mg/l BAP with or without 0.05 mg/l NAA (Fig. 3). Interaction of callusing media and shoot regeneration media on shoot induction frequency showed that a maximum of 17-18% shoot induction frequency was obtained from calluses induced on media containing 1.5 mg/l NAA with 1 mg/l BAP and regenerated on media containing 1 or 2 mg/l BAP alone or in combination with 0.05 mg/l NAA (Table 4; Figs 1b & c). Callusing media devoid of cytokinin gave significantly lower shoot regeneration response irrespective of the auxin concentration in callusing media. Thus, it suggests the development of certain meristemoids in the presence of cytokinin when dedifferentiation process was taking place during callus induction in chickpea, which results in shoot induction in the prevalence of proper conditions. Further, the study emphatically shows that while working for high frequency shoot regeneration one should not only look for high callus induction ability of an explant but also
Table 4—Effect of interaction of callusing media and shoot regeneration media on shoot induction frequency of callus cultures

<table>
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<th>Callusing media</th>
<th>SR₀</th>
<th>SR₁B</th>
<th>SR₂B₀.05N</th>
<th>SR₂H</th>
<th>SR₂B₀.05N</th>
<th>SR₃H</th>
<th>SR₂B₀.05N</th>
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SE of mean

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<td>SR₂B₀.₀₅N</td>
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for the type of callus and the nutritional milieu in the media during callus induction, which is more important for its regeneration ability.

Considering significant main and interaction effects of genotypes, explants, callusing media and shoot regeneration media, it is concluded that immature embryo was the best source of explant and high shoot induction frequency was obtained on regeneration media with 2 mg/l BAP when the calluses were induced on media with 1.5 mg/l NAA and 1 mg/l BAP. So far, regeneration has not been reported from callus induced from cotyledon explants and present studies showed that a maximum of 7% shoot induction is possible on regeneration media with 2 mg/l BAP and 0.05 mg/l NAA with callus induced on media with 0.5 mg/l NAA and 1 mg/l BAP.

References