42.5°C) than *Rhizobium leguminosarum* and *R. trifolii*. Brockwell observed that up to 40°C, there was no serious mortality, but beyond this temperature the mortality rate was very high. It was reported that counts of *R. japonicum* at temperatures ranging from 28 to 35°C were appreciable, while at a temperature of 40°C the mortality rate was high. But in present investigation, survival rate of *Rhizobium* transformant was minimum at higher temperatures, i.e. above 28°C in the case of paddy husk, but in the case of sawdust and groundnut shell the count of the cells declined after two weeks. The higher temperatures affect the longevity of transformant in all carriers as shown in Figure 2.

The viability of *Rhizobium* transformant carrying reporter gene *gfp* was found to be good, since its stability was recorded up to 154 days. The *gfp* plasmid does not require any other substrate for fluorescence like other markers. Moreover being a non-conjugal plasmid, spread of the antibiotic marker encoded on it is highly restricted. It can be concluded that GFP can be used as a marker for monitoring the survival of *Rhizobium* sp. in bioinoculants. The paddy husk is a very good alternate low-cost carrier at par with conventional lignite carrier.

Peat and lignite, though good carriers, are not easily available and are expensive in India. Paddy husk, sawdust and groundnut shells are agricultural and industrial waste products. The low cost and easily available paddy husk can be used as a carrier and reporter gene *gfp* can be used as a marker for monitoring bioinoculants.


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### Plant regeneration from immature cotyledon-derived callus of *Vigna unguiculata* (L.) Walp (cowpea)


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An efficient plant regeneration protocol from immature cotyledon-derived callus was standardized for cowpea (*Vigna unguiculata* (L.) Walp). Callus cultures were initiated from immature cotyledon explants on MS + B5 medium containing IBA andKn, each at 2.0 mg l⁻¹ concentration. These cultures on transfer to a medium containing 0.1 mg l⁻¹ zeatin produced adventitious shoots. The regenerated plantlets were then transferred to root induction medium containing IBA. Histological analyses were made to confirm organogenesis from callus.

GRAIN legumes in general, and *Vigna* in particular, are recalcitrant in nature. As a consequence, genetic transformation is hard to achieve. In most cases where transgenic grain legumes have been obtained with reasonable efficiency, regeneration of shoots involved a callus phase. There have been several reports on plant regeneration through organogenesis in different explants of cowpea. However, limited success has been achieved for shoot production via callus phase in cowpea. Pellegrineschi reported regeneration of shoots from callus cultures of hypocotyl in the presence of 0.1 mg l⁻¹ zeatin. Pandey and Bansal induced organogenesis from leaf callus of cowpea in the medium containing IBA (10 µM) and Kn (10 µM). Development of reliable protocols for regeneration of grain legumes was reported in soybean, black gram, mungbean, common bean, tepary bean and pigeonpea. In the present study, a protocol for regeneration

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Figure 1. a, Regenerative nodular callus of immature cotyledon. n, nodular structure; b, Development of adventitious buds from regenerative callus; c, Regeneration of shoots from adventitious buds; d, Rooting of elongated shoots; e, Hardened plant; f, Meristematic organization of epidermal and sub-epidermal cells of cotyledon callus (bar – 900 µm); g, Meristematic zone showing shoot primordia (bar – 600 µm); and h, Histological section showing proliferation of shoots (bar – 400 µm).
of plants from immature cotyledon-derived callus of *Vigna* is reported. We demonstrate that immature cotyledon explants can be used to establish organogenic calluses and subsequent shoot regeneration from these calluses.

Plants of *Vigna unguiculata* (L.) Walp cv. Vamban 1 were raised from the experimental field at Bharathidasan University, Tiruchirappalli. The pods with extremely immature embryos were harvested 10 days after fertilization, washed in running tap water, and were soaked in Teepol (3–4 drops per 100 ml ddH2O) for 10 min. The pods were washed in ddH2O five times and treated with 70% ethanol (v/v), following which the pods were treated with 0.1% HgCl2 (w/v) for 10 min and rinsed with sterile water five times to remove the sterilant. The sterilized pods were then cut open to remove the intact seeds under aseptic conditions. The cotyledon segments dissected out from immature pods were placed on the surface of the MS-B5 medium containing MS salts11, B5 vitamins19, 30 g l−1 sucrose, 8 g l−1 agar, 160 g l−1 putrescine20, 0–2.0 mg l−1 IBA and 0–2.0 mg l−1 BAP. pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. After subculture, with a duration of 15 days, the explants were transferred to callus induction medium containing IBA and Kn (each at 2.0 mg l−1). The greenish shiny nodular calluses were transferred to regeneration medium containing zeatin (0.1–0.5 mg l−1). Calluses with regenerated shoot buds were transferred to shoot elongation medium containing BAP (0.1–0.5 mg l−1) and GA3 (0.1–0.5 mg l−1). Elongated shoots (3–5 cm) were then transferred to rooting medium containing IBA (0.1–1.0 mg l−1). The cotyledon segment cultures were kept in continuous dark for a week. For all other cultures, the culture room was maintained at 24 ± 2°C with a 16 h photoperiod of 24 μmol m−2 s−1 (white-fluorescent tubes). Rooted plantlets were transferred to plastic pots containing sterile compost for hardening. The hardened plants were transplanted to the experimental field and the survival rate was assessed after 20 days. To find out the histological events during shoot regeneration, the cotyledon-derived calluses from bud induction media were sampled at regular intervals. Samples were fixed in FAA for 24 h and dehydrated with tertiary butyl alcohol in series and sectioned with toluidine blue20 for observation. Each treatment consisted of at least 10 explants and the experiments were repeated 10 times. A complete randomized design was used in all experiments and analysis of variance and mean separations were carried out using Duncan’s Multiple Range Test (DMRT). Statistical significance was determined at the 5% level6.

Cotyledon segments cultured on IBA and Kn (each at 1.0 mg l−1) fortified with MS–B5 medium showed suppressed growth, but at the same time the explants became short, stout, stumpy and dark-green. When such cotyledon segments were vertically plated on the medium containing IBA and Kn (each at 2.0 mg l−1), the proximal ends showed good callusing response and produced greenish shiny nodular callus (Figure 1 a). Exposure of soybean

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**Table 1. Effect of growth regulators on shoot organogenesis from immature cotyledon callus**

<table>
<thead>
<tr>
<th>Growth hormone (mg l−1)</th>
<th>Mean number of shoots/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeatin (Zea) alone</td>
<td>0.1</td>
</tr>
<tr>
<td>Kn alone</td>
<td>1.0</td>
</tr>
<tr>
<td>BAP alone</td>
<td>0.5</td>
</tr>
<tr>
<td>Zea + Kn</td>
<td>0.1 + 1.0</td>
</tr>
<tr>
<td>Zea + BAP</td>
<td>0.1 + 0.5</td>
</tr>
<tr>
<td>Zea + Kn + BAP</td>
<td>0.1 + 1.0 + 1.0</td>
</tr>
<tr>
<td>Zea + IAA</td>
<td>0.1 + 0.1</td>
</tr>
<tr>
<td>Kn + IAA</td>
<td>0.1 + 0.1 + 0.1</td>
</tr>
<tr>
<td>Ka + IAA + NAA</td>
<td>0.1 + 0.2</td>
</tr>
<tr>
<td>BAP + IAA</td>
<td>0.2 + 0.1</td>
</tr>
<tr>
<td>BAP + NAA</td>
<td>0.5 + 0.05</td>
</tr>
<tr>
<td>BAP + IAA + NAA</td>
<td>0.5 + 0.1 + 0.1</td>
</tr>
</tbody>
</table>

For every treatment, hormones were tested at five different concentrations, Zea = 0.01, 0.05, 0.1, 0.2, 0.3; Kn = 0.1, 0.5, 1.0, 2.0, 3.0; BAP = 0.01, 0.1, 0.5, 1.0, 2.0, 3.0; IAA = 0.05, 0.1, 0.2, 0.3, 0.4; and NAA = 0.05, 0.1, 0.2, 0.3, 0.4 mg l−1. Where more than one hormone was used, a matrix of treatment was tested for all possible combinations of the specified hormones.

Each value represents the treatment means of 10 independent replicates with 10 explants per plate.

Means with the same alphabet in a column are not significantly different according to Duncan’s multiple range test at 5% level.

n.d., not determined.

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**Table 2. Effect of growth regulators on shoot elongation on MS–B5 medium after two weeks of culture**

<table>
<thead>
<tr>
<th>Growth hormone (mg l−1)</th>
<th>Culture response (%)</th>
<th>Mean shoot length (cm)</th>
<th>Mean number of elongated shoots/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td>0.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>0.25</td>
<td>72g</td>
<td>4.0</td>
<td>1c</td>
</tr>
<tr>
<td>0.5</td>
<td>89bc</td>
<td>4.2bc</td>
<td>2b</td>
</tr>
<tr>
<td>1.0</td>
<td>68b</td>
<td>3.0</td>
<td>1c</td>
</tr>
<tr>
<td>Kn + IAA</td>
<td>n.d.</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ka + IAA + NAA</td>
<td>0.1 + 0.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BAP + IAA</td>
<td>0.2 + 0.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BAP + NAA</td>
<td>0.5 + 0.05</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BAP + IAA + NAA</td>
<td>0.5 + 0.1 + 0.1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

For every treatment, hormones were tested at five different concentrations, Zea = 0.01, 0.05, 0.1, 0.2, 0.3; Kn = 0.1, 0.5, 1.0, 2.0, 3.0; BAP = 0.01, 0.1, 0.5, 1.0, 2.0, 3.0; IAA = 0.05, 0.1, 0.2, 0.3, 0.4; and NAA = 0.05, 0.1, 0.2, 0.3, 0.4 mg l−1. Where more than one hormone was used, a matrix of treatment was tested for all possible combinations of the specified hormones.

Each value represents the treatment means of 10 independent replicates with 10 explants per plate.

Means with the same alphabet in a column are not significantly different according to Duncan’s multiple range test at 5% level.

n.d., Not determined due to nil response.


embryonic axis to specific hormonal conditions, reprogrammed the in vitro regeneration behaviour of soybean explants\(^{22}\). After one month, the calluses obtained from immature cotyledon explants were transferred to the medium with lower level of zeatin (0.1 mg l\(^{-1}\)) for a duration of another one month (Table 1). Adventitious shoot buds were noticed on the surface of the calluses (Figure 1 b and c). Earlier, regeneration was reported from hypocotyl explants of cowpea at a lower concentration of zeatin\(^{30}\). Zeatin seems to be essential for regeneration of even cotyledonary callus as noticed in our experiments.

Certain regions of the callus when in primary culture medium showed distinct epidermal and sub-epidermal cells with dense cytoplasm and large nucleus after two weeks of inoculation. At the end of the third week, these deeply stained cells exhibited active cell divisions and subsequently organized into meristematic cell clusters containing 5 to 10 cells in each cluster (Figure 1 f). Similar results were observed in friseur fir conifers, where epidermal and sub-epidermal cells organized into meristemoids from which shoot regeneration occurred\(^{42}\). Similarly, confinement of active cell divisions after differential staining of epidermal and subadjacent layers of the in vitro cultured explants like cotyledonary leaf and petiole of legumes in response to BA, has been previously reported\(^{24,25}\). After four weeks, this culture was transferred to the medium containing a low level of zeatin. At the end of fifteenth day of subculture, differentiation of bud primordia was visible. After four weeks, completely organized shoot buds with meristematic layers and leaf primordia were obtained (Figure 1 g and h).

The regenerated shoot buds were grown in a medium containing BAP (0.5 mg l\(^{-1}\)) and GA\(_3\) (0.1 mg l\(^{-1}\)) for elongation (Table 2; Figure 1 d). All elongated shoots produced normal roots in the medium containing IBA (0.5 mg l\(^{-1}\)). Similar observations were made in tepary beans\(^{26}\) and mulberry\(^{27}\). The hardened plants after transfer to the greenhouse (Figure 1 e) showed 50% survival. The above study thus illustrated a reproducible protocol for plantlet regeneration from immature cotyledon-derived callus, thus paving the way for genetic manipulation of cowpea.


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