# CHAPTER 6

# REGENERATION FROM CALLUS, ANTHER AND EMBRYO

## 6.1 INTRODUCTION

*Vigna radiata* is one of the most important grain-legumes and is a rich source of dietary protein. Its qualitative and quantitative improvement is central to the needs of augmenting nutritional requirement of growing Indian population. A number of problems needing solution in this crop are amenable to solution through the use of tissue culture techniques. Grain-legumes as a class, are retractable to available culture strategies due to their limited regeneration potential (Huyghe, 1990). A very few workers have chosen mungbean as experimental material, with the result that the range of culture conditions and genotypes investigated has been very limited (Bajaj and Dhanju, 1979; Mathews, 1987). The present study describes standardization of tissue culture techniques in terms of callus initiation and development, and the effect of different combinations of growth regulators on callus growth of different seedling and reproductive explants.

## 6.2 MATERIALS AND METHODS

*Vegetative explants*

*Establishment of callus culture*

Seeds of twelve cultivars (listed in Table 3) of *Vigna radiata* were obtained from the Directorate of Pulse Research, Kalyanpur, Kanpur and Division of Genetics, IARI, New Delhi. To raise aseptic seedlings, the seeds were rinsed in 70% alcohol for 1 min. and then sterilized in 0.2% aqueous mercuric chloride solution for 10 minutes. After thoroughly washing them in sterile distilled water, the seeds were planted on basal media MS, $B_5$, C (MS salts + $B_5$ vitamins) and PC-L2, containing 3% sucrose and 0.7% agar in test tubes (25 mm x 150 mm). All media were adjusted to pH 5.8 before autoclaving. The seeds were allowed to germinate under 16-h photoperiod of cool-white fluorescent light of 80μ mol $m^{-2}$ $s^{-1}$ at 25 ± 2°C. The cotyledons from 2-d-old of seedlings and shoot-tips, epicotyls, hypocotyls,, primary leaves and root-tips from 7-d-old seedlings were excised and cultured on the respective basal media supplemented with BAP (5 x $10^{-6}$ M), NAA (2.5 x $10^{-6}$ M) and 2,4-D (2.25 x $10^{-6}$ M). The cotyledons were planted with the proximal end embedded in the medium, whereas hypocotyl, epicotyl, root tip and leaf explants were placed horizontally on the medium. The shoot tips were planted with their cut end embedded in the medium. The cultures were maintained under the same experimental conditions as for germination of seeds. For each treatment, 24 explants were cultured and each experiment was repeated twice. Visual observations of cultures were taken every week and the percentage of cultures showing callusing and fresh weight of calli were recorded.

To study the effect of genotypes on callus growth, the callus derived from primary leaf explants of 7-d-old seedlings of different genotypes was cultured on PC-L2 medium supplemented with BAP (5 x $10^{-6}$ M), NAA (2.5 x $10^{-6}$ M) and 2, 4-D (2.25 x $10^{-6}$ M).

In addition, the effect of different concentrations of BAP (2.5x$10^{-6}$, 5x$10^{-6}$, $10^{-5}$and 2.5 x $10^{-5}$ M) and NAA (5 x $10^{-7}$ and 2.5 x $10^{-7}$ M) on the growth of callus derived from different seedling explants, was also studied.

Callus (500 mg fresh weight) initiated on modified PC-L2 medium were planted on media C + BAP (5x$10^{-6}$ M). The trimming of the callus and its orientation was such that its upper surface was fairly flat and smooth. A V-shaped incision was made in the upper surface of the callus and a shoot bud, with its basal end cut wedge-shaped, was inserted in the cavity. After 28 days, the shoot buds were removed and the hard dark green callus developed at its base was subcultured on the same medium, i.e. C + BAP (5 x $10^{-6}$ M).

*Reproductive explants*

*Anther culture*

The early formed young flower buds were collected from field grown plants of mungbean cv. K-851. These buds were sterilized with 70% alcohol for one min and then with 0.2% $HgCl_2$ for 8-10 minutes. The anthers from sterilised flower buds were taken out under sterilized conditions in Laminar flow cabinet. The anthers containing uninucleate pollens were cultured on agar solidified MS basal medium supplemented with various combinations of different growth regulators (KIN, 2, 4-D, IAA) (See Table 5). The cultures were maintained under 16-h photoperiod of 80 $\mu$ mol $m^{-2}$ $s^{-1}$ at 25 $\pm$ 2°C for 28 days.

*Immature embryo culture*

The developing pods (1.5-2.0 cm in length) were excised and sterilized with 70% alcohol for one min and with 0.2% aqueous $HgCl_2$ for 8-10 minutes. The immature embryos at torpedo stage were dissected out under a binocular stereo-microscope in a Laminar flow cabinet and cultured on agar solidified MS medium supplemented with different growth regulators (listed in Table 6) under 16-h photoperiod of cool-white fluorescent light (80 $\mu$ mol $m^{-2}$ $s^{-1}$) at 25 $\pm$ 2°C for 28 days.

## 6.3 RESULTS AND DISCUSSION

*Days taken to callus for different explants*

As evident from Table 1, hypocotyl, root-tip and leaves initiated callus at the earliest, i.e. within 7-11 days followed by cotyledons which took 11-14 days. On the other hand, anthers and immature embryos had taken more time, i.e. 21-24 days for initiation of callus.

*Location of region of explant giving rise to callus and nature of callus*

The cotyledons developed green, nodular, friable to compact callus at the proximal end, while in the shoot tip and leaf explants, dark green and nodular callus developed at the cut ends. In hypocotyl, epicotyl and root tip explants, light green, friable to compact callus developed on their entire surface (Table 1).

*Selection of medium for optimal growth*

On basal medium, different seedling explants did not form callus. However, they callused with varying frequencies in all the media, i.e. MS, $B_5$, 'C' and PC-L2 containing BAP, 2, 4-D and NAA. The frequency of callusing and the growth of the callus, measured as fresh and dry weights, of all the explants except leaf, were maximum on 'C' medium. However, leaf explants showed maximum callus growth on PC-L2 medium (Table 2.). The observed differences in the callus growth on different media may be due to the differences in the concentration of inorganic nitrates and sulphates.

*Genotypic differences in callus growth*

The growth of callus derived from leaf explants of different genotypes (listed in Table 3) was compared under identical nutrient and culture conditions. ML-1, K-851 and PS-7 had high fresh and dry weights of callus, while Sunaina and G-65 had less of it (Table 3). The variation in callus growth among the genotypes being significant as also reported in *Zea mays* (Green *et al.,* 1974) and Triticales (Sharma *et al.,* 1980). On the basis of callus growth, the genotypes can be grouped under three categories:

high - ML-1, K-851, PS-7
medium - PDM-54, SML-32, ML-323 and
poor - Sunaina, G-65, M-337, Pusa-107, PDM-11.

*Influence of growth regulators on callus growth*

On basal medium, different seedling explants did not form callus. However, these explants formed callus on medium supplemented either with auxin or cytokinin alone or in combination. Preliminary experiments have shown that among auxins (2, 4-D, NAA and IAA) and cytokinins (BAP, KIN, AdS and 2-iP), NAA and BAP, respectively, were the most effective for callus growth. Combinations of auxin (NAA) and cytokinin (BAP) at different concentrations were effective in induction of callus (Table 4) as reported in *Psophocarpus tetragonolobus* (Brunel *et al.,* 1981), *Dolichos lablab* (Sounder Raj *et al.,* 1991) and other legumes.

Cotyledonary callus required high concentration of BAP and NAA for its optimal growth, whereas shoot tip and hypocotyl derived calli required low concentrations of these

growth regulators. On the other hand, leaf and epicotyl derived calli required high concentration of BAP and low concentration of NAA for their maximum growth. These results demonstrate that the growth regulators requirement for optimal callus growth varies with the explant and it may be due to the difference in their endogenous levels. Addition of 2, 4-D to BAP and NAA-containing medium had further increased the callus growth. None of these treatments was effective in inducing shoot bud differentiation from callus of different explants. However, Jain and Chopra (1988) obtained best regeneration response in *Vigna aconitifolia* callus produced on BAP ($2.5 \times 10^{-6}$ M) and NAA ($5 \times 10^{-7}$ M) supplemented medium.

Gill *et al.* (1986) reported callus formation and differentiation of shoots in *Vigna aconitifolia* on MS basal medium alone. However, in the present study, none of the explants produced callus on basal media. But they produced profuse callus on basal media supplemented with BAP ($5x10^{-6}$M), 2,4-D ($2.25x10^{-6}$M) and NAA ($2.5x10^{-6}$M).

Venketeswaren and Huhtinen (1978) in winged bean (*Psophocarpus tetragonolobus*) and Godbole and Krishnamurthy (1987) reported that plants could be regenerated from callus provided it was subcultured a number of times on basal medium. However, in the present study, the leaf derived callus exhibited no organogenetic potential when subcultured for 10 passages of one month each, on the basal medium and on the medium on which it was initiated. Thus, a different approach was followed for the regeneration of shoots from callus. The hard dark green callus developed at the base of grafted shoot bud in the leaf derived callus block, differentiated into adventitious shoots in 10% of the cultures (Plate 6). Further experiments are needed to enhance the efficiency of shoot regeneration from truely dividing callus cultures.

*Anther culture*

Anthers cultured on basal medium turned brown and undergone necrosis, while those cultured on medium supplemented with different growth regulators started proliferation in 3 weeks and developed a mass of callus in 4 weeks (Table 5.). The callus was friable and creamish - green in colour. Out of the 14 media tested, MS supplemented with KIN ($5 \times 10^{-6}$ M) + 2, 4-D ($5 \times 10^{-6}$ M) and IAA ($10^{-5}$ M) proved to be the best for inducing callus in 78% of the cultured anthers. Similar to our results, Bajaj and Singh (1980) also reported maximum induction of callus from anthers cultured on MS medium supplemented with IAA, 2, 4-D and KIN (each at $10^{-5}$ M). However, the percentage of callusing cultures was low, i.e. 28.5% compared to 78% in the present study.

*Immature embryo culture*

Most of the immature embryos underwent callusing, but some of them developed into spindler shoots (Table 6). The shoot regeneration was higher, i.e, 29% in medium containing CM than in medium containing CH, i.e. 10%. On contrary, Gosal and Bajaj (1983 b) reported higher

plant regeneration from hybrid embryos on medium containing CH than culture. These differences may be due to different genotypes used in the present study. The callus developed from immature embryo was green, compact, nodular and shiny. However, callus failed to differentiate under any hormonal / nutrient combinations.

**Table 1. Days to callus for different explants**

| Explant | Time taken for callus initiation | Location of region of explant giving rise to callus | Callus nature (morphology and colour) |
|---|---|---|---|
| Cotyledon (entire) | 11-14 days | Only at proximal end | Green, friable to compact, nodular |
| Hypocotyl | 7-10 days | Callus initiated along both the cut ends and subsequently on the surface | Light-green, friable to compact |
| Epicotyl | 7-10 days | Callus initiated along both the cut ends and subsequently on the surface | Light-green, friable to compact |
| Root tip | 7-11 days | Callus initiated along both the cut ends and subsequently on the surface. | Light-green, friable to compact |
| Leaves | 7-11 days | Along the margins | Dark green, nodular |
| Shoot tip | 7-11 days | At the base of shoot tip | Dark green, nodular |
| Anther | 21-24 days | All over the surface | Friable and creamish- green |
| Immature embryo | 21-24 days | All over the surface | Green, compact, nodular, shiny |

Table 2. Callusing response of various seedling explants of Vigna radiata cv.K-851 in different basal media each containing BAP ($5x10^{-6}$M), NAA ($2.5x10^{-6}$M) and 2,4-D ($2.25x10^{-6}$M)

| Explant | MS medium | | | $B_5$ medium | | | 'C' medium | | | PC-L2 medium | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % callusing | callus fresh weight[a] | callus dry weight[b] | % callusing | callus fresh weight[a] | callus dry weight[b] | % callusing | callus fresh weight[a] | callus dry weight[b] | % callusing | callus fresh weight[a] | callus dry weight[b] |
| Cotyledon | 100 | 2086±200 | 215±23 | 100 | 912±50 | 255±6 | 100 | 2316±100 | 260±10 | 91.6 | 2300±130 | 143±9 |
| Root tip | 100 | 970±100 | 83±7 | 100 | 354±28 | 45±2 | 100 | 1080±200 | 87±10 | 91.6 | 1001±34 | 94±3 |
| Hypocotyl | 100 | 1139±86 | 96±9 | 100 | 543±48 | 57±5 | 100 | 1450±19 | 187±15 | 100 | 1201±30 | 85±16 |
| Epicotyl | 100 | 1171±134 | 130±14 | 100 | 245±21 | 43±6 | 100 | 1426±25 | 143±2 | 100 | 1071±10 | 121±12 |
| Shoot tip | 100 | 1138±10 | 129±9 | 100 | 1272±13 | 129±17 | 100 | 1272±13 | 157±17 | 100 | 1123±19 | 140±19 |
| Leaf | 100 | 1134±13 | 106±5 | 100 | 959±16 | 84±4 | 100 | 1628±3 | 154±10 | 100 | 1806±4 | 159±8 |

a,b Fresh and dry weight (mg/culture)

Data represents an average of 24 explants ± SE

Data scored after 4 weeks of culture.

Table 3. Fresh and dry weight of 4 week old calli of leaf explant of Vigna radiata genotypes cultured on PC-L2 medium containing BAP ($5x10^{-6}M$), NAA ($2.5x10^{-6}M$) and 2,4-D ($2.25x10^{-6}M$)

| | Genotype | Callus | |
|---|---|---|---|
| | | Fresh Weight (mg/culture) | Dry weight (mg/culture) |
| 1. | ML-1 | 4187 ± 297 | 371 ± 67 |
| 2. | K-851 | 3842 ± 369 | 394 ± 15 |
| 3. | PS-7 | 3224 ± 162 | 342 ± 24 |
| 4. | PDM-54 | 2313 ± 62 | 283 ± 75 |
| 5. | SML-32 | 2194 ± 10 | 276 ± 9 |
| 6. | ML-323 | 2145 ± 153 | 238 ± 20 |
| 7. | Pusa-baisakhi | 1912 ± 452 | 234 ± 13 |
| 8. | PDM-11 | 1726 ± 167 | 214 ± 22 |
| 9. | Pusa-105 | 1670 ± 133 | 205 ± 27 |
| 10. | ML-337 | 1665 ± 391 | 179 ± 37 |
| 11. | G-65 | 1548 ± 384 | 186 ± 49 |
| 12. | Sunaina | 1529 ± 36 | 199 ± 18 |

Values represent the mean of 24 cultures ± SE.

Table 4. Fresh and dry weights of calli derived from different seedling explants of Vigna radiata cv. K-851 on 'C' medium supplemented with different combinations of growth regulators

| Growth regulators (M) | | Cotyledon | | Shoot tip | | Epicotyl | | Hypocotyl | | Leaf | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BAP | NAA | Fresh weight[a] | Dry weight[b] | Fresh weight[a] | Dry weight[b] | Fresh weight[a] | Dry weight[b] | Fresh weight[a] | Dry weight[b] | Fresh weight[a] | Dry weight[b] |
| 0 | $5x10^{-7}$ | 308 ± 50 | 48 ± 7 | 194 ± 11 | 31 ± 1 | 171 ± 25 | 39 ± 4 | 110 ± 27 | 25 ± 1 | 765 ± 0 | 86 ± 0 |
| $2.5x10^{-6}$ | $5x10^{-7}$ | 2232 ± 167 | 175 ± 11 | 2307 ± 279 | 165 ± 16 | 2388 ± 182 | 195 ± 7 | 2358 ± 126 | 181 ± 0 | 728 ± 153 | 84 ± 18 |
| $5x10^{-6}$ | $5x10^{-7}$ | 2574 ± 353 | 191 ± 19 | 2842 ± 127 | 190 ± 4 | 2133 ± 322 | 191 ± 29 | 2552 ± 171 | 203 ± 23 | 1708 ± 17 | 158 ± 16 |
| $10^{-5}$ | $5x10^{-7}$ | 2674 ± 162 | 194 ± 4 | 3330 ± 217 | 198 ± 5 | 3123 ± 104 | 222 ± 4 | 2100 ± 113 | 169 ± 0 | 1193 ± 396 | 115 ± 32 |
| $2.5x10^{-5}$ | $5x10^{-7}$ | 3316 ± 84 | 203 ± 3 | 3050 ± 622 | 190 ± 13 | 2674 ± 224 | 185 ± 7 | 1653 ± 12 | 125 ± 2 | 989 ± 15 | 73 ± 0 |
| 0 | $2.5x10^{-7}$ | 1365 ± 123 | 126 ± 10 | 642 ± 27 | 73 ± 18 | 1202 ± 19 | 140 ± 3 | 100 ± 1 | 23 ± 1 | 1184 ± 92 | 113 ± 12 |
| $2.5x10^{-6}$ | $2.5x10^{-7}$ | 3160 ± 57 | 205 ± 6 | 3741 ± 170 | 232 ± 15 | 3361 ± 199 | 231 ± 2 | 3554 ± 250 | 250 ± 2 | 1773 ± 196 | 185 ± 13 |
| $5x10^{-6}$ | $2.5x10^{-7}$ | 2834 ± 188 | 202 ± 8 | 3998 ± 138 | 238 ± 11 | 2935 ± 294 | 213 ± 4 | 3702 ± 174 | 229 ± 1 | 1660 ± 141 | 171 ± 15 |
| $10^{-5}$ | $2.5x10^{-7}$ | 2777 ± 27 | 192 ± 2 | 3384 ± 424 | 202 ± 12 | 3586 ± 84 | 245 ± 11 | 2464 ± 20 | 201 ± 0 | 2202 ± 207 | 192 ± 17 |
| $2.5x10^{-5}$ | $2.5x10^{-7}$ | 2630 ± 255 | 189 ± 20 | 2812 ± 134 | 173 ± 4 | 3071 ± 74 | 207 ± 9 | 3195 ± 375 | 217 ± 8 | 2079 ± 163 | 179 ± 12 |

a,b Fresh and dry weights (mg/culture)

Data represents an average of 24 cultures ± SE

Data scored after 4 weeks of culture.

Table 5. Callusing response of anthers of Vigna radiata cv.K-851 cultured on various media.

| | Medium | % of callusing anther |
|---|---|---|
| 1. | MS basal medium | Swelling and browning |
| 2. | MS + $2.5x10^{-6}$M KIN + $2.5x10^{-6}$M 2,4-D + $10^{-5}$M IAA | 62 |
| 3. | MS + $2.5x10^{-6}$M KIN + $5x10^{-6}$M 2,4-D + $10^{-5}$M IAA | 29 |
| 4. | MS + $2.5x10^{-6}$M KIN + $10^{-5}$M 2,4-D + $10^{-5}$M IAA | 60 |
| 5. | MS + $5x10^{-6}$M KIN + $2.5x10^{-6}$M 2,4-D + $10^{-5}$M IAA | 52 |
| 6. | MS + $5x10^{-6}$M KIN + $5x10^{-6}$M 2,4-D + $10^{-5}$M IAA | 78 |
| 7. | MS + $5x10^{-6}$M KIN + $10^{-5}$M 2,4-D + $10^{-5}$M IAA | 29 |
| 8. | MS + $10^{-5}$M KIN + $2.5x10^{-6}$M 2,4-D + $10^{-5}$M IAA | 39 |
| 9. | MS + $10^{-5}$M KIN + $5x10^{-6}$M 2,4-D + $10^{-5}$M IAA | 43 |
| 10. | MS + $10^{-5}$M KIN + $10^{-5}$M 2,4-D + $10^{-5}$M IAA | 47 |
| 11. | MS + $2.5x10^{-5}$M KIN + $2.5x10^{-6}$M 2,4-D + $10^{-5}$M IAA | 47 |
| 12. | MS + $2.5x10^{-5}$M KIN + $5x10^{-6}$M 2,4-D + $10^{-5}$M IAA | 52 |
| 13. | MS + $2.5x10^{-5}$M KIN + $10^{-5}$M 2,4-D + $10^{-5}$M IAA | 33 |
| 14. | MS + $10^{-5}$M IAA + 100 ml/l CM | 35 |

Data based on 120 anthers cultured on each medium.

Data scored after 4 weeks of culture.

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Table 6. Morphogenic response of immature embryo of Vigna radiata cv. K-851 in different media.

| | Medium | % callusing | % shoot regeneration | No. of shoots/ culture |
|---|---|---|---|---|
| 1. | MS + $5x10^{-6}$ IAA + $10^{-5}$ KIN + 7% CM | 71 | 29 | 1.0 ± 0.0 |
| 2. | MS + $4.3x10^{-5}$M NAA + $5x10^{-6}$M Thiamine + $3x10^{-4}$M Nicotinic acid | 100 | - | - |
| 3. | MS + $5x10^{-6}$M IAA + $10^{-5}$M KIN + 500 mg/l CH | 90 | 10 | 1.0 ± 1.0 |
| 4. | MS major + 4 times minor + $B_5$ vitamins + $1.3x10^{-5}$M BAP + $2x10^{-7}$M NAA + $5x10^{-6}$M Thiamine + $1.2x10^{-2}$ M Proline | No response in light, 100% callus- ing in dark | - | - |

Data based on 24 cultures.

Data scored after 4 weeks of culture.

PLATE 6 Differentiation of shoot (arrow head) from callus cultured on C + BAP ($5x10^{-6}$M).

PLATE 6