CHAPTER 3

IN VITRO PLANT REGENERATION FROM COTYLEDON EXPLANTS

3.1 INTRODUCTION

Information on culture conditions favouring *in-vitro* high frequency regeneration is a pre-requisite for achieving crop improvement via somatic-hybridization, somaclonal and gametoclonal variant selection and transformation. Cotyledon explants have been found to possess a high morphogenic potential in a few grain - legumes (Mehta and Mohan Ram, 1980; Gill *et al.,* 1987a). Moreover, cotyledons can be obtained in large number by growing seeds under sterile conditions for a short period of time and at any time throughout the year. Microbial contamination of such explants is rarely a serious problem (Fazekas *et al.,* 1986). Previous studies on shoot regeneration from cotyledon explants of *Vigna radiata* have been restricted to only two cultivars, ML-5 and S-8 (Mathews and Rao, 1984; Mathews, 1987). Moreover, Mathews and Rao (1984) reported regeneration only on basal medium. Mathews (1987), however, used only MS basal medium supplemented with one cytokinin and one auxin for shoot regeneration from two day old cotyledons. Earlier workers have not studied the factors that influence plant regeneration in detail (Mathews and Rao, 1984; Mathews, 1987). In the present study, 12 cultivars have been investigated and optimal culture conditions for plant regeneration from cotyledonary explants have been worked out.

3.2 MATERIALS AND METHODS

Seeds of twelve cultivars (listed in Table 5) of *V. radiata* were obtained from the Directorate of Pulse Research, ICAR, Kalyanpur, Kanpur and Division of Genetics, IARI, New Delhi. Of these, K-851 was used for detailed studies. 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 A (Murashige and Skoog, 1962), B_5 (Gamborg *et al.*, 1968) and C (MS salts + B_5 vitamins) containing 3% sucrose and 0.7% agar (Hi-media, Bombay) in test tubes (25 mm x 150 mm). All media were adjusted to pH 5.8 before autoclaving. The seeds were allowed to germinate in 8-h dark and 16-h cool-white fluorescent light of 80 µmol m^{-2} s^{-1} at 25 $\pm$ 2°C with 60% humidity for seven days.

The cotyledons of two day old aseptic seedlings (unless mentioned otherwise) from three basal media were excised and cultured on the respective basal media supplemented with different concentrations (10^{-7} M, $5x10^{-7}$ M, 10^{-6} M, $5x10^{-6}$ M, 10^{-5} M) of BAP. Unless otherwise mentioned, cotyledons were planted with the proximal end embedded in the medium (Plate 1A). Callus isolated from explants was cut into small pieces of approximately 50 mg each (4 mm x 4 mm) and transferred on to the same or different media for plant regeneration.

To study the effect of different cytokinins on shoot / plant regeneration, cotyledons from seedlings germinated on basal medium 'C' were excised and cultured on the same basal medium supplemented with different cytokinins (KIN, 2-iP and AdS) at an equimolar concentration of $5x10^{-6}$ M. In addition, the effect of the age of donor seedling, size of the explant and genotype on regeneration from cotyledons was studied.

Well developed shoots were separated and transferred on to fresh half strength MS, MS and MS supplemented with $5x10^{-6}$ M IAA or NAA.

All cultures were maintained under same experimental conditions as for germination of seeds. For each treatment, 24 cultures were raised and each experiment was repeated at least once. Visual observations of the cultures were taken every week and the percentage of cultures showing callusing, rooting, shoot bud differentiation and number of shoots per explant were recorded after 28-d.

Plantlets with well developed roots were removed from the culture vessel and after washing their roots in the running tap water, plants were then transferred to pots containing sterile vermiculite. Glass beakers were inverted over the plants to ensure high humidity during the first few days. Subsequently, the plants were transferred to field.

3.3 RESULTS

1.3.1 Culture of cotyledon explant

Cotyledons were excised from seeds imbibed for 2-d and cultured on three basal media alone or supplemented with different concentrations of BAP (Table 1). On basal media, cotyledon explants directly produced either shoot or root at uncallused proximal end. The shoot subsequently developed roots from the base and complete plants resulted within three weeks (Plate 1B). The percentage of explants forming shoots and the number of shoots per explant varied with the basal media. The percentage of explants forming shoots was highest (36%) in B_5 medium and the number of shoots was maximum in C medium.

Effect of BAP concentration

Addition of BAP to all the three basal media induced callus formation within 7-d at the proximal end of the cotyledons, followed by shoot differentiation. These shoots showed stunted growth and lacked roots even after prolonged incubation on BAP containing medium (Plate 1C). Initially, the callus was green and compact, but later turned brown, presumbly due to production of phenolic compounds. Addition of BAP also enhanced the frequency of regeneration as well as the number of shoots per cotyledon over the control. The number of cultures showing differentiation of shoots varied with the basal media as well as with the concentration of BAP, i.e. 50% in MS medium with $5x10^{-7}$ M, 54.8% in B_5 medium with 10^{-6} M and 60% in medium C with 10^{-5} M BAP. But, the number of shoots per culture was highest on $5x10^{-6}$ M BAP in

all the basal media. The morphology of some of the regenerated shoots was not normal in all the media supplemented with BAP. Some of the abnormalities noted were (i) fasciation of shoots resulting into thick stem with free apices at tip; (ii) fusion of leaflets resulting into simple leaf and (iii) increase in number of leaflets of leaves. The green callus isolated from cotyledon culture grew profusely, but failed to differentiate shoot inception on the basal media or BAP concentrations.

Effect of different cytokinins

Since maximum number of shoots/culture was observed with $5x10^{-6}$ M BAP on all the basal media, hence this concentration was chosen to study the comparative response of four different cytokinins (See Table 2). Like BAP, all other cytokinins to basal media induced callus formation at the proximal end of cotyledon. However, further morphogenetic response of cotyledons varied with different cytokinins. BAP and 2-iP favoured only multiple shoot development from explants. KIN not only induced differentiation of shoots, but in 15% of the total explants, shoots also subsequently developed roots resulting into plantlets. AdS, in addition to the development of plantlets, also induced only root differentiation directly from 10% of the total explants. KIN induced highest frequency (35%) of differentiation, while BAP produced the highest number of regenerants/explant. The morphology of regenerated shoot was normal with KIN, 2-iP and AdS in all cultures.

Effect of age of donor seedlings

To test the optimum age of seedlings for regeneration from excised cotyledons, explants were taken various days after seed germination and cultured on medium C + BAP ($5x10^{-6}$ M). As is evident from Table 3, two day old seedlings yield most regenerative explant and those older than three days totally lacked this potentiality.

Effect of size of explant

Each cotyledon was sliced into two equal parts either longitudinally or transversely and each part was cultured on basal medium C with $5x10^{-6}$ M BAP. The regeneration response of cotyledons decreased when sliced into two equal parts either longitudinally or transversely. Regeneration, however, occurred only on those explants which possessed the petiolar tissue embedded in medium. The distal end of transversely sliced cotyledon did not show any response. The longitudinal halves showed high regeneration than the proximal transverse half (Table 4).

Effect of Genotype

To study the effect of genotype on shoot differentiation, cotyledons from two day old seedlings of 12 cultivars were cultured on basal medium C supplemented with $5x10^{-6}$ M BAP (Table 5). Of the 12 cultivars tested, nine showed the capacity of shoot regeneration. However, there was

quantitative variation in regeneration responses among the cultivars. Cultivars ML-1, ML-337 and SML-32 did not show any shoot differentiation. The responding cultivars can be grouped under three categories:

(i) Poor ML-323, PDM-11, PDM-54 and G-65; (ii) medium -- K-851, PS-7 and Pusa-baisakhi and (iii) high-Pusa-105 and Sunaina. On the whole, Pusa-105 showed the highest frequency (60%) of shoot regeneration and cv. Pusa-baisakhi produced the maximum number (8) of shoots / explant.

Rooting of shoots

Well developed shoots were excised from cotyledons and cultured on half strength-MS, MS and MS containing IAA ($5x10^{-6}$ M) formed slight callus at cut end from which roots emerged within 15-d. On MS supplemented with $5x10^{-6}$ M NAA, shoots produced only callus at cut end and later shoots senescenced. The rooted plants were transferred to pots (Plate 1D) and later established in the field with 60% success.

Free-hand sections of 28-d old cultures revealed that shoot buds differentiated from cells of original explant rather than from the callus.

3.4 DISCUSSION

Plant regeneration from cotyledons with intact cotyledonary nodes of legumes has been reported in *Cajanus cajan* (Mehta and Mohan Ram, 1980), *Glycine max* (Cheng *et al.*, 1980), *V. mungo* (Gill *et al.*, 1987a) and *V. radiata* (Mathews, 1987). However, in all these instances, regeneration of shoots has been shown from cotyledonary node, having pre-existing quiescent buds in the axil of cotyledons, cultured on media supplemented with plant growth regulators. In the present study, we have obtained plant regeneration from cotyledons (without cotyledonary node), by culturing them on basal media. The other parallel observations of shoot bud differentiation on basal media are that of *Albizzia lebbeck* (Gharyl and Maheshwari, 1980), *V. radiata* (Mathews and Rao, 1984) and *V. aconitifolia* (Gill *et al.*, 1986). The basal media used by previous workers (Mathews and Rao, 1984) for obtaining complete plant from cotyledons of *V. radiata* were different from those used in the present study. Moreover, they obtained less number of complete plants/cotyledon (2 plants/seed) than the present work (3 plants/ seeds) on the basal media.

The cytokinins (BAP and KIN) enhanced shoot bud formation in cultured cotyledons of *V. radiata*, is in accordance with previous reports on other grain-legumes (Cheng *et al.*, 1980, Gill *et al.*, 1987 a). In the present study, multiple shoots were induced directly from cotyledons on the basal medium without the intervention of a callus phase at a relatively shorter time compared to cotyledons cultured on cytokinins supplemented medium where differentiation was preceded by an intervening callus phase.

Mathews (1987) reported maximum response (80%) of explants for shoot differentiation in MS + 5 μM BAP + 20 μm IBA, but the worker has not reported the number of shoots regenerated per explant. In the present study, three different basal media supplemented with different concentrations of BAP were used. The maximum response (60%) of explants for shoot differentiation was in 'C' basal medium + 10^{-5} M BAP, whereas the maximum number of shoots (8 to 9) per explant was with $5x10^{-6}$ M BAP. This study shows that 16 to 18 plants has been obtained from single seed in 28 days.

The age, size and orientation of the explant have an important role in obtaining shoot formation in legumes (Kameya and Widhlom, 1981; Wright *et al.,* 1987). The decline in the frequency of regenerants with the age of donor seedlings beyond 2-d is perhaps due to more mobilization of cotyledon's reserve food to embryonic axis at the advance stage of germination. This observation is in accordance to Mathews and Rao (1984), but they observed decrease in cotyledonary plants after 1-d of seed imbibition. This variation may be due to the different basal medium and plant growth regulators used in the two studies. The regeneration response of cotyledons decreased when they were sliced into two equal parts either longitudinally or transversely. However, regeneration was more in longitudinal halves than proximal transverse halves. The distal transverse half of cotyledon did not show any differentiation. Thus, the longitudinal halves are more regenerative than proximal transverse half, because in longitudinal half some substances might have been contributed by intact distal half to proximal half where they have activated the epidermal cells to form more shoot buds.

Regeneration in tissue culture is a genetically controlled trait (Bhojwani *et al.,* 1984; Templeton-Somers and Collins, 1986). The results show that the frequency of shoot regeneration and number of shoots per explant vary from cultivar to cultivar. These differences are attributed to the intrinsic genetic constitution at the cultivar level. Such genotypic differences in the regeneration capacity of other grain-legumes have been reported earlier (Malmberg, 1979; Rubluo *et al.,* 1982; Rubluo and Kartha, 1985).

Formation of multiple shoots in mungbean from cotyledonary explant could be of practical application in raising hybrid seedlings of difficult crosses. There are reports in the literature that in certain legumes, cells of cotyledons become polyploid during embryogenesis (Mehta and Mohan Ram, 1980). It would be specially advantageous, if plants recovered from cotyledons of *V. radiata,* as described above, would offer a high degree of variability. Thus, the procedure of differentiating large number of plants from different cultivars of *V. radiata* on a relatively simple medium may prove useful in the isolation of somaclonal variant and in studies on plant cell transformation.

Table 1. Regenerative response of two days old cotyledons of Vigna radiata cv. K-851 on various basal media supplemented with different concentrations of BAP.

Culture period: 28 days.

BAP concentrations (M)	A (MS) Medium		B_5 Medium		C (MS salts + B_5 vitamins) Medium	
	Percent cultures form regenerating shoot buds.	Number of shoot buds per culture	Percent cultures form regenerating shoot buds	Number of shoot buds per culture	Percent cultures form regenerating shoot buds	Number of shoot buds per culture
0	16.6 ± 1.0 (6/36)	1.0 ± 0.0[a]	36.5 ± 2.5 (12/33)	1.25 ± 0.2[a]	26.6 ± 1.6 (12/45)	1.5 ± 0.1[a]
10^{-7}	30.8 ± 1.5 (12/39)	1.0 ± 0.0	26.6 ± 1.5 (12/45)	1.8 ± 0.1	46.6 ± 3.0 (21/45)	2.71 ± 0.2
$5x10^{-7}$	50.0 ± 3.5 (21/42)	2.3 ± 0.4	15.4 ± 1.0 (6/39)	2.5 ± 0.4	53.8 ± 3.0 (21/39)	2.3 ± 0.1
10^{-6}	28.6 ± 2.1 (12/42)	3.0 ± 1.3	54.5 ± 4.0 (18/33)	3.3 ± 0.7	53.3 ± 3.1 (24/45)	4.9 ± 0.9
$5x10^{-6}$	28.6 ± 1.5 (12/42)	4.5 ± 0.9	42.8 ± 3.0 (18/42)	5.5 ± 1.1	33.3 ± 2.0 (15/45)	8.2 ± 1.9
10^{-5}	40.0 ± 2.5 (18/45)	3.33 ± 0.6	27.3 ± 1.5 (9/33)	4.6 ± 1.1	60.0 ± 4.0 (27/45)	5.7 ± 0.6

a - plantlet number

Values are mean ± SE

Values in parentheses represent ratio of explants showing response.

Table 2. Effect of different cytokinins (5x10-6 M) in C medium on shoot differentiation in two days old cotyledons of Vigna radiata.

Culture medium : C basal medium
Culture period 28 days.

Media	Cultures showing differentiations (%)	% Explant forming			Average number of plants and/ or shoot per culture	Degree of callusing
		Plantlets	Shoot	Root		
BM*	30.0 ± 2.0 (12/40)	15.0 ± 1.5 (16/40)	0.0	15.0 ± 1.2 (6/40)	1.7 ± 0.3[a]	0.0
BM + BAP	33.0 ± 2.0 (12/36)	0.0	33.0 ± 2.0 (12/36)	0.0	4.7 ± 1.2[b]	+ + +
BM + KIN	35.0 ± 3.0 (14/40)	15.0 ± 1.2 (6/40)	20.0 ± 1.8 (8/40)	0.0	2.4 ± 0.2[c]	+ + +
BM + ADS	15.0 ± 1.0 (6/40)	5.0 ± 0.3 (2/40)	0.0	10.0 ± 0.9 (4/40)	2.0 ± 0.0[a]	+
BM + 2-iP	10.0 ± 0.9 (4/40)	0.0	10.0 ± 0.9 (4/40)	0.0	1.5 ± 0.0[b]	+ +

\+ Callus upto 25%
\+ + Callus 25-50%
\+ + + Callus 50-75%
\+ + + + Callus 75%

a- include only plantlet number.
b- include only shoot number.
c- include both plantlet and shoot number.

*BM = 'C' basal medium
Values are mean ± SE
Values in parentheses represent the ratio of explants showing response.

Table 3. Effect of the age of seedling on regeneration in cotyledon cultures of Vigna radiata.

Culture medium: C basal medium + 5x10-6 M BAP.
Culture period: 28 days.

Days after sowing seeds	Percent cultures regenerating	Average number of shoots per culture
1	33.3 ± 2.1 (16/48)	6.0 ± 0.8
2	41.7 ± 3.5 (20/48)	6.4 ± 0.6
3	33.3 ± 1.5 (15/45)	5.5 ± 0.6
4 to 7	0.0	0.0

Values are mean ± SE
Values in parentheses represent ratio of explants showing response.

Table 4. Effect of cotyledons size on regeneration in cotyledon culture of Vigna radiata.

Culture medium : C basal medium + 5x10-6 M BAP.
Culture period : 28 days.

Cotyledon size	Percent cultures regenerating	Average number of shoots per culture	Callus range
Complete cotyledon	33.3 ± 2.0 (15/45)	8.2 ± 1.9	+ +
Longitudinal half	12.5 ± 1.0 (6/48)	4.0 ± 0.1	+
Transverse proximal half	4.2 ± 0.5 (2/48)	4.0 ± 0.0	+ + +
Transverse distal half	0.0	0.0	0.0

+ Callus upto 25%
+ + Callus 50%
+ + + Callus 75%

Values are mean ± SE
Values in parentheses represent ratio of explants showing response.

Table 5. Regenerative response of cotyledon explants obtained from different cultivars of Vigna radiata.

Culture medium : C basal medium + 5x10-6 M BAP.
Culture period: 28 days.

Cultivar	Percent cultures regenerating	Average number of shoot buds/culture
K-851	41.7 ± 2.1 (20/48)	6.4 ± 0.6
Pusa baisakhi	30.0 ± 1.5 (12/40)	7.3 ± 0.7
PS-7	50.0 ± 3.2 (20/40)	4.8 ± 0.7
Pusa-105	60.0 ± 4.5 (24/40)	5.0 ± 0.3
ML-1	0.0 (0/36)	0.0
ML-323	8.4 ± 0.7 (3/36)	1.0 ± 0.0
ML-337	0.0 (0/36)	0.0
PDM-11	16.7 ± 1.2 (8/48)	4.5 ± 0.4
SML-32	0.0 (0/45)	0.0
G-65	8.4 ± 0.9 (4/48)	1.0 ± 0.0
PDM-54	8.4 ± 1.0 (4/48)	6.0 ± 0.6
Sunaina	58.4 ± 2.3 (28/48)	4.2 ± 0.4

Values are mean ± SE
Values in parentheses represent ratio of explants showing response.

PLATE 1 Plant regeneration from cultured cotyledons of *Vigna radiata*.

A. cotyledon explant with proximal end embedded in the medium at the time of culture;

B. A well developed plantlet from a cotyledon explant on C basal medium.

C. Production of multiple shoots from cotyledon explant cultured on C + BAP ($5x10^{-6}$M), 28-d after culture.

D. Regenerated plant growing in small pot, a week after transfer.

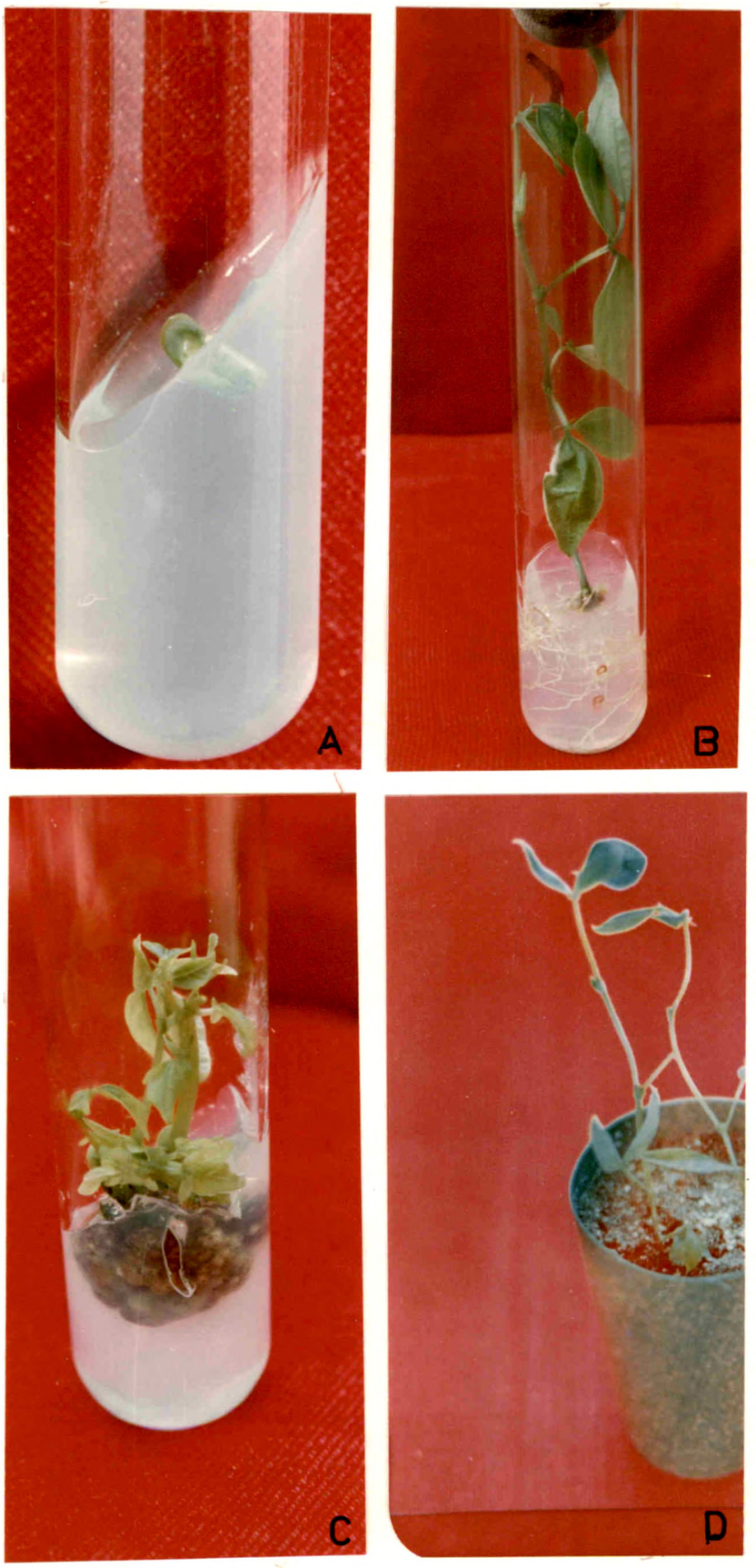

PLATE 1