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# Plant regeneration from cell suspension cultures of *Vigna aconitifolia*

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ABSTRACT

Plant regeneration has been achieved routinely from established cell suspension culture lines of Vigna aconitifolia (moth bean), a highly drought tolerant grain legume. The cultures originated from three-week-old leaf callus. Several media including MS, $B_5$, AA, SL, PCM, SH and L-6 were tested for their effects on cell growth. Maximum growth was observed in L-6 medium containing 44.5 µM 2,4-D. After 6 to 8 weeks the suspensions were filtered through 500, 250, 125 and 60 um sieves, respectively, for four to five subcultures. An embryogenic cell line (VA-686) was obtained from the cell fraction collected below 250 µm. The VA-686 cell line is being maintained on L-6 medium with 4.5 µM 2,4-D and 2.3 µM Zeatin. Somatic embryogenesis was induced by transferring the cells to L-6 medium with 4.6 uM zeatin in which green cell clusters were produced. The somatic embryos developed from most of the cell clusters when plated on L-6 agar medium with 2.3 µM BA.

Plantlets were obtained from the embryos on L-6 medium with 10.0 µM IBA. The regenerated plants were grown to maturity in the greenhouse.

ABBREVIATIONS

BA = Benzyladenine; 2,4-D = 2,4- dichlorophenoxy-acetic acid; $GA_3$ = Gibberellic acid; IBA = Indole-3-butyric acid; IPA = Isopentenyladenine; KN = Kinetin; NAA = Napthaleneacetic acid; AA = Toriyama and Hinata, 1985; SL = Phillips and Collin, 1980.

INTRODUCTION

Morphogenically competent cell suspension cultures may be used to generate somaclonal variation with desirable traits. Amongst leguminous species, several of the forage crops, e.g. Trifolium repens, T. pretense and Medicago sativa have been established as suspension cultures from which plants were regenerated by somatic embryogenesis (Oswald et al., 1977, Phillips and Collins, 1980, McCoy and Bingham 1977). Attempts to produce similar cultures with grain legumes have generally been less successful (Gamborg et al., 1983).

Plant regeneration systems were established from immature embryo explants of soybean (Ranch et al. 1985, Ghazi et al. 1986) and pea (Kysely et al. 1987), and from callus cultures of Lathyrus sativus (Gharyal and Maheshwari, 1980), Peanut (Narasimhulu and Reddy, 1983) and pigeonpea (Kumar et al. 1983). Christianson et al, (1983) established an embryogenic cell suspension culture in soybean.

The moth bean (Vigna aconitifolia Jarq.) is a highly drought-tolerant grain legume which is grown for its seeds and as a forage crop (NRC, 1979). Plantlets have been obtained from root explants (Eapen and Gill, 1986), callus (Bhargava and Chandra, 1983, and Godbole et al, 1984) and protoplasts (Shekhawat and Galston, 1983, and Krishnamurthy et al., 1984) of Vigna aconitifolia. However, there is no report of regeneration from cell suspension cultures of any Vigna genotype. In the present paper we report on the procedures to establish cell suspension cultures from which plants can be regenerated.

MATERIALS AND METHODS

Seeds of Vigna aconitifolia, line TCVA-1 were obtained from the National Bureau of Plant Genetic Resources, ICAR, New Delhi. Seeds were surface sterilized by treatment with chlorine gas for 30 minutes as described by Gamborg et al., (1983), and germinated aseptically on hormone-free MS medium (Murashige and Skoog 1962). Leaves were excised from seven-day-old seedlings and placed on $B_5$ (Gamborg et al. 1968) agar medium with 4.5 µM 2,4-D and 0.4 uM KN, 2.0% sucrose and 0.9% agar. The pH of the medium was adjusted to 5.7. The cultures were incubated under continuous wide spectrum fluorescent light at 2000 lux and 25 ± 2°C.

Suspension Cultures

Suspension cultures were initiated in June 1986, by transferring 100 mg of three-week-old leaf callus into 250 ml Erlenmeyer flasks containing 40 ml of liquid $B_5$ medium with 4.5 µM 2,4-D and 0.4 µM KN. In all the experiments, 2.0% sucrose was used in the medium and the pH was adjusted to 5.7. The cultures were placed on a gyratory shaker at 120 rpm. Twenty-five cell suspension culture lines were initiated from the leaf callus of twenty-five different seedlings of the same genotype (TCVA-1). The cell cultures were maintained by inoculating 5 ml of suspension cultures into 40 ml of fresh medium at two-week intervals. Cells from the cultures were observed microscopically at each subculture. About six weeks after initiation, cells were subcultured into various liquid media including $B_5$, MS, AA (Toriyama and Hinata 1985), mixtures of $B_5$ and AA (25:75, 60:40, 50:50, 40:60 and 75:25), PCM (Gamborg

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et. al., 1983), SH (Schenk and Hildebrandt 1972), SL (Phillips and Collins, 1980) and L-6 medium (Table 1) each with 4.5 to 9.0 µM 2,4-D and 0.4 µM KN.

Table 1. Composition of L-6 medium used for Vigna aconitifolia suspension cultures*

| Mineral salt | mg/liter | Mineral salt | mg/liter |
|---|---|---|---|
| $NH_4NO_3$ | 1000 | $MnSO_4.H_2O$ | 22.0 |
| $KNO_3$ | 1000 | $ZnSO_4.7H_2O$ | 8.6 |
| $Ca_2(NO_3)_2.4H_2O$ | 500 | $H_3BO_3$ | 6.2 |
| $CaCl_2.2H_2O$ | 330 | KI | 0.83 |
| $KH_2PO_4$ | 170 | $NaMoO_4.2H_2O$ | 0.25 |
| $MgSO_4.7H_2O$ | 179 | $CuSO_4.5H_2O$ | 0.025 |
| NaFeEDTA | 27.85 | $CoCl_2.6H_2O$ | 0.025 |

* The medium also contained vitamins as in $B_5$ (Nicotinic acid 1 mg., Thiamine HCl 10 mg., Pyridoxine HCl 1 mg.) Myo-inositol 100 mg., Sucrose 20 gm., Casein hydrolysate 200 mg., Sorbitol 10 g.

Five ml of cell suspension from the L-6 medium with 4.5 µM 2,4-D and 0.4 µM KN, were transferred to the same medium with 44.5 µM 2,4-D. After four weeks of culture the cells were filtered through 500, 250, 125 and 60 µm stainless steel sieves for four to five consecutive subcultures, to separate embryo like structures, cell clumps and individual cells. The cell line from each fraction was then cultured separately, and the fraction passing through the 250 µm sieve was selected for further experiments. The resulting cell line (VA 686) is being maintained in L-6 medium with 4.5 µM 2,4-D and 2.3 µM zeatin, and subcultured at three-week intervals. The embryogenic capacity of the cell line is being maintained by filtering the suspensions through 250 um sieves at nine-week intervals. The procedure was repeated in September 1986, and a separate embryogenic cell line (VA-986) was obtained. Growth of these cell lines was monitored by determining the dry weights and packed cell volume (PCV) of five replicate samples. The data on PCV were collected after centrifuging the suspensions at 2000 rpm for 10 minutes in a graduated centrifuge tube.

Plant regeneration

For somatic embryo development samples of 50 mg of the cells from maintenance medium were transferred to liquid L-6 medium with 4.6 µM zeatin and cultured on a gyratory shaker under continuous light for three weeks. The green cell clumps formed in this medium were then plated on 20 X 100 mm disposable petri dishes containing 30 ml of semi-solid (0.9% agar) L-6 medium with different concentrations (2.0 to 20.0 µM)of BA, zeatin or IPA either alone or in combination with 1.0 to 5.0 µM $GA_3$. The plates were incubated under continuous wide-spectrum cool white fluorescent light at 25± 2°C. The embryos produced on L-6 medium with BA were transferred to the same medium with 10.0 µM IBA for plant regeneration.

The relative efficiency of plant regeneration in long term cultures was determined by plating the green cell clumps from liquid L-6 medium with zeatin to semi-solid L-6 medium with 2.3 µM BA at 30-days intervals. The effect of sorbitol was tested by inoculating 50 mg of cells to L-6 medium containing 0, 1.0, 2.0, 3.0 or 4.0 percent of sorbitol, respectively. The cells were cultured in sorbitol medium for four subcultures and data on PCV and dry weight were collected by harvesting the cells at the end of the fourth subculture the regenerated plantlets were transferred to a soil and vermiculite mixture (1:1) in pots and grown in the greenhouse. Chromosome numbers of the regenerated plants were verified using the root-tip squash technique of Pundir and Singh, (1986).

RESULTS AND DISCUSSION

Leaf explants cultured on $B_5$ medium produced green nodular callus within two-weeks of culture. Suspension cultures developed from the callus tissue within three weeks after transfer to the liquid medium. Possible variation in the culturability was ascertained by establishing twenty five cell lines from the leaf callus of as many different seedlings of the same genotype. There was no significant differences in the cell growth or nutritional requirements of the lines.

The suspensions produced in the $B_5$ medium consisted mostly of a mixture of highly vacuolated cells and cell aggregates, and few densely cytoplasmic cells. L-6 medium appeared to be more suitable, when compared to other media tested, in supporting cell growth and in increasing the proportion of densely cytoplasmic cells. Browning observed with other media was minimal in L-6 medium. The L-6 medium deviates somewhat from MS and other common media in the types and relative amounts of nitrogen sources, sulfates and vitamins. The reduction of inorganic nitrogen, increased calcium, presence of $B_5$ vitamins and addition of casein hydrolysate in the L-6 medium might have contributed to the observed results. The L-6 medium has also been shown to enhance somatic embryogenesis in other grain legumes including tepary bean (Phaseolus acutifolius) (Kumar et al, 1988). In preliminary experiments, casein hydrolysate was observed to be beneficial for embryo formation, while inclusion of sorbitol increased efficiency of the embryogenic callus production from embryogenic suspension (Table 2). Sorbitol was essential to retain high levels of embryogenic callus formation and subsequent plant regeneration in long-term cultures. Both sorbitol and mannitol have been reported to retain the regeneration ability in long term cultures of rice (Kavi Kishore and Reddy, 1986).

Table 2. The effect of sorbitol on callus growth and plant regeneration

| Sorbitol g/l | Dry (a) Weight (mg) | PCV (b) (ml) | Percent (c) E-callus | No. of (d) plants regenerated |
|---|---|---|---|---|
| 0 | 347 ± 11.4 | 2.02 ± .24 | 16.4 ± 1.21 | 3.2 ± .15 |
| 10 | 356 ± 6.0 | 1.86 ± 1.9 | 82.5 ± 3.4 | 14.2 ± 2.3 |
| 20 | 318 ± 8.7 | 1.76 ± .25 | 76.3 ± 1.9 | 13.8 ± 1.8 |
| 30 | 321 ± 10.2 | 1.55 ± .16 | 83.5 ± 1.9 | 12.7 ± 1.4 |
| 40 | 233 ± 6.4 | 1.38 ± .19 | 72.4 ± 2.4 | 9.4 ± 1.7 |

(a) Dry weight was determined after 14 days. The inoculum was 50 mg.
(b) ml/25 ml of culture medium after 14 days.
(c) Determined with 50 plates at each concentration.
(d) Average number of plants in 25 culture jars.

The concentration of 2,4-D had a significant effect on cell differentiation in the cultures. The cells grown in the presence of 44.5 µM 2,4-D produced large numbers of globular and torpedo-shaped structures. However, these structures failed to grow into plantlets, when transferred to L-6 agar plates with different concentrations of BA, zeatin or IPA. Although L-6 medium was superior to other media tested, the cultures consisted of a mixture of highly vacuolated cells and cells with dense cytoplasm. The latter cells divided faster and developed into clusters of 10 to 20 cells. The introduction of filtration steps resulted in a separation of the cell types and aggregates. The fraction passing through a 250 µm sieve produced a line of small cell aggregates and of isodiametric cells with dense and highly granular cytoplasm (Fig. 1).

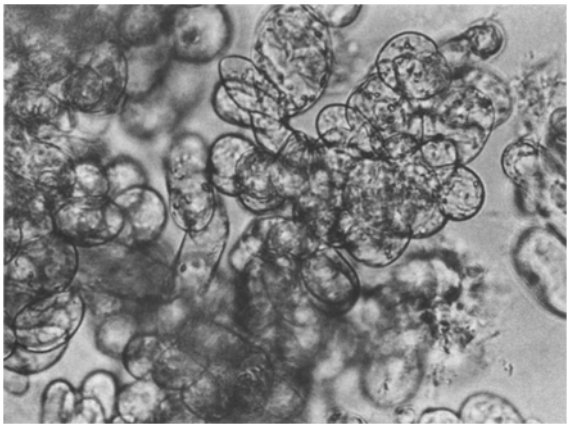

Fig.1 Cells from suspension cultures on the L-6 liquid medium with 4.5 µM 2,4-D and 2.3 µM zeatin

Such features are typical of embryogenic cells (Gamborg et al.1974). Embryogenic cell lines were recovered after four to five repeated cycles of filtration. The cell fraction between 125 and 250 µm, was the most consistent and is being maintained in L-6 medium with 4.5 µM 2,4-D and 2.3 µM zeatin. The observation that the cell line continued to retain plant regeneration capability may in fact be due to the regular filtration and selection of a particular cell fraction. The plant regeneration ability of the cell line has remained constant for more than one year (Table 3).

Table 3. Plant regeneration efficiency after prolonged culture.

| Age of (a) culture (days) | Percent E-forming (b) callus | No. of plants (c) |
|---|---|---|
| 120 | 77.5 ±. 42 | 12.3 ± 1.3 |
| 150 | 84.4 ± 3.7 | 11.6 ± .92 |
| 180 | 82.4 ± 4.2 | 13.5 ± .46 |
| 210 | 76.6 ± 4.0 | 11.6 ± .67 |
| 240 | 79.4 ± 4.86 | 12.6 ± .54 |
| 270 | 64.7 ± 7.47 | 10.5 ± .76 |
| 300 | 71.8 ± 6.9 | 11.3 ± 1.2 |
| 330 | 69.4 ± 7.86 | 11.0 ± .78 |
| 360 | 64.2 ± 9.42 | 8.7 ± .94 |
| 390 | 67.4 ± 9.78 | 10.2 ± 1.2 |

(a) The culture was initiated June 1986.
(b) Average of 25 plates.
(c) Average in 25 jars.

The growth of the cell suspensions as a function of dry weight and PCV is shown in Fig. 2. The doubling time of the VA 686 cell line is approximately three days and has remained relatively constant for more than twelve months.

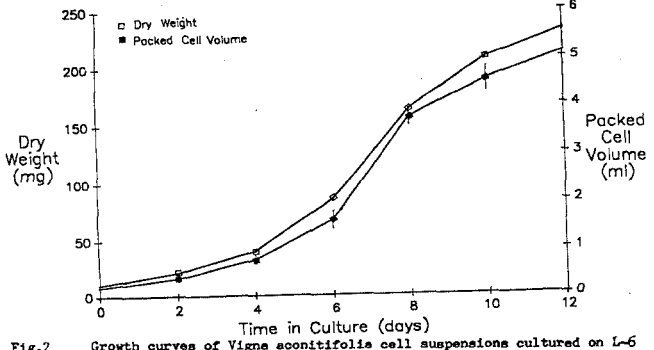


Fig.2 Growth curves of Vigna aconitifolia cell suspensions cultured on L-6 medium with 4.5 µM 2,4-D and 2.3 µM zeatin. Growth of the cell line was shown as a function of dry weights and packed cell volume with standered error bars.

For plant regeneration, the cells from maintenance medium were transferred to L-6 medium with 4.6 µM zeatin. The cells grew and produced large numbers of dark green clumps and few globular and heart-shaped embryos. The green clumps were plated on agar L-6 medium with different types and concentrations of hormones, including BA, zeatin or IPA alone or in combination with NAA and $GA_3$. BA tested at the different concentrations gave optimal response at 2.3 µM (Table 4).

Table 4. The effect of BA concentration on the efficiency on embryo formation and subsequent plant regeneration.

| BA concentration (µm) | Percent of embryo-forming plates | No. of plants regenerated/50 mg of embryo-forming callus |
|---|---|---|
| 0 | 26.0 ± 2.86 | 2.8 ± 0.5 |
| 2.2 | 86.5 ± 3.14 | 12.4 ± 2.92 |
| 4.4 | 63.2 ± 3.26 | 8.3 ± 1.73 |
| 8.8 | 29.6 ± 1.47 | 4.4 ± 6.8 |
| 17.6 | 5.6 ± 1.96 | 0 |

The number of plates per experiment was 50 and 50 mg of embryogenic cell materials was used in each plate.

Somatic embryos (Fig. 3) formed in the L-6 + BA medium were transferred to L-6 medium with 10 µM IBA. Plantlets developed within 3-4 weeks (Fig. 4).

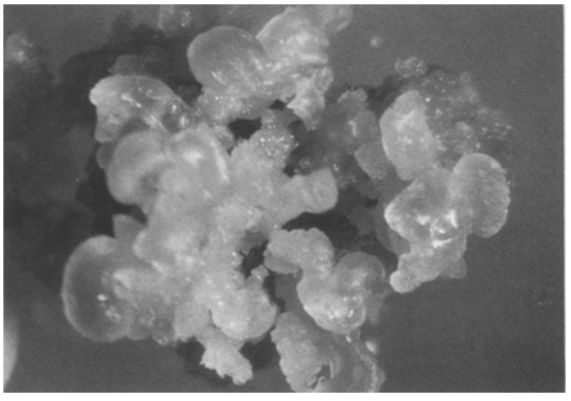

Fig.3 E-callus from suspension cultures on L-6 agar medium with 2.3 µM BA

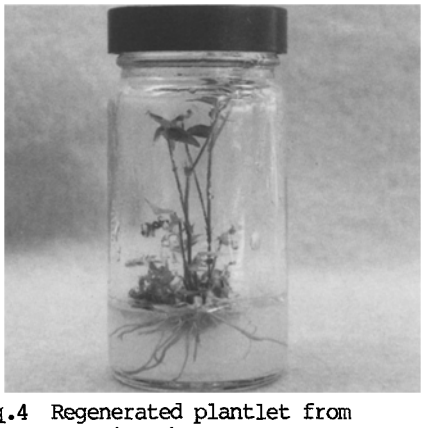

Fig.4 Regenerated plantlet from somatic embryos

Attempts to regenerate plants from cell suspension cultures in other species of Vigna were not successful (Jha and Roy, 1982; Chowdhury et al, 1983). The regenerated plants were acclimatized for two days in the greenhouse before transplanting into pots containing a soil and vermiculite mixture. More than 200 plants have been regenerated and transferred

to the greenhouse. The plants grown to maturity were visually observed to be true to type (Fig. 5). The chromosomal counts of more than 50 plants showed the normal ploidy level of 2n = 22.

Fig.5 Mature regenerated plant of Vigna aconitifolia.

Suspension culture cell lines which retain the capability for efficient plant regeneration are convenient systems for screening and selection of variants with desirable characteristics. A cell line (VA 986 ST) has been selected from cells of the VA 986 line in media with 2% NaCl and plants can be regenerated.

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