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IN VITRO PLANT REGENERATION OF COWPEA (*VIGNA UNGUICULATA* (L.) WALP.) USING DISTAL HALF OF COTYLEDON

RAVINDRA B. MALABADI *
Division of Plant Tissue Culture, Department of Botany, Karnatak University, Dharwad – 580003, Karnataka state, India. (E-mail : malabadi712@yahoo.com, mlbd712@rediffmail.com)
*Present address : 4816-145 Avenue, Edmonton, ALBERTA T5Y 2X8 Canada.

An *in vitro* protocol for regenerating Cowpea (*Vigna unguiculata* (L.) Walp.) cv. DFH – 1 (Deenanath-Fodder-Horsegram-1) plants was developed from distal cotyledonary segments of mature seeds. Shoot primordia were induced directly from the cotyledonary explants (distal end) when cultured on modified Murashige and Skoog (1962) (mMS) basal medium supplemented with N^6–Benzylaminopurine (BAP) (8.88 µM) and coconut water (CW) (15% v/v). The shoot primordia developed into well elongated shoots when they were subcultured on mMS basal medium fortified with 2.22 µM of N^6 - Benzylaminopurine (BAP), coconut water (CW) (15%) and adenine sulfate (AS) (75 mgl^{-1}). Elongated shoots were rooted on half strength mMS basal medium without growth regulators and were established in soil where they showed normal morphological characters.

Keywords : Cotyledon; Cowpea; Organogenesis; *Vigna unguiculata (L.)*.

Introduction

Cowpea (*Vigna unguiculata* sub sp. *unguiculata* (L.) Walp) is an important annual fodder legume of the rainfed areas and can form a component crop in the multiple cropping system. As a food, the grain is a rich source of dietary protein and staple in many countries. Fresh pods and peas, dry grain and leaves are commonly consumed in various ways. In herbal medicinal cure of kidney stone, the boiled water extract of whole grain is given to the patient. *In vitro* regeneration of cowpea has been reported from shoot and root meristem explants[1,2]. Muthukumar *et al*[3] have reported regeneration of plants from primary leaves of cowpea. Shoot regeneration *via* organogenesis was also reported from axenic cowpea hypocotyls and cotyledons of advanced breeding lines and varieties[4]. Pellegrineschi *et al*[5] have also reported successful regeneration of plants from immatue cowpea embryos. In pigeonpea (*Cajanus cajan*), plant regeneration was reported using distal cotyledonary segments of mature seeds as explants[6,7]. Among the grain legumes, *in vitro* production of multiple shoots from seeds/ explants has been achieved in mungbean[8], peanut[9], *Phaseolus vulgaris,* pea, chickpea, lentil[10] and pigeonpea[11]. Multiple shoot production was also reported from cotyledonary node explants[12].

No report is available on shoot regeneration of cowpea from distal half of cotyledon explants that lack pre-existing meristems. In this paper, we report for the first time de novo shoot organogenesis from distal half of cotyledon explants in cowpea cv. DFH-1.

Materials and Methods

Cowpea cv. Deenanath Fodder Horsegram (DFH-1) seeds procured from the Regional Fodder Research Station of the University of Agricultural Sciences, Dharwad, Karnataka state, India were surface sterilized in 70% (v/v) ethanol for 3 min and 0.1% $HgCl_2$ for 1 min. Seeds were thoroughly rinsed four to five times with sterile double distilled water and then seeds were soaked in sterile water for 15 hours in darkness at 27±2° C. In all these experiments, mMS basal medium[13] (All the macro and micro elements except potassium nitrate were reduced to half strength with the addition of 1.0 gl^{-1} L-glutamine, 0.5 gl^{-1} Casein hydrosylate) with 3% Sucrose gelled with 0.8% agar-agar (Himedia) was used. The pH of the media was adjusted to 5.8 before sterilization. The medium was dispensed in 145 mm X 25 mm glass culture tubes containing approximately 15 ml of the medium and autoclaved at 1.04 $Kg.cm^2$ for 15 min.

The morphogenetic potential of cotyledonary segments (distal end) on mMS basal medium supplemented with 8.88 µM N^6 - Benzylaminopurine and coconut water (15%) was tested. Cotyledons were split open from the presoaked seeds and the proximal meristematic ends were removed. Only the distal halves (3-3 mm^2) without any pre-existing axillary buds (Fig.1) were cultured (one explant per tube) with adaxial surface touching the medium for a period of 4 weeks. 50 explants per treatment were used and the experiment was repeated three times. The cotyledonary explants (distal end) showing shoot primordia were

Table 1. Effect of various concentrations of BAP in combination with Coconut water (15% v/v) on regeneration of shoot buds from Distal half of cotyledonary explants of cowpea (*Vigna unguiculata* L.) Walp cv. DFH-1*

Nutrient medium mMS+CW (15%) with BAP Con in µM	Total Number of Explants cultured	No. of Explants showing shoot bud regeneration	No. of shoot primordia regenerated per each explant	No. of Plants obtained per each explant
0	50	Nil	Nil	Nil
2.22	50	Nil	Nil	Nil
4.44	50	3±0.3	2±0.5	1±0.00
8.88	50	20±1.3	5±0.8	5±0.45
22.2	50	2±0.3	1±0.0	1±0.00

Data represents an average of 3 replicates.
Data scored after 6 weeks.
Experiment repeated 3 times.

Table 2. Effect of various concentrations Adenine sulfate (AS) in combination with BAP (2.22 µM) and Coconut water (15%) on shoot regeneration from shoot buds derived from distal half of cotyledonary explants of cowpea (*Vigna unguiculata* L. Walp) cultured on mMS basal medium supplemented with BAP (8.88 µM) and coconut water (15% v/v).

Nutrient medium mMS+BAP(2.22µM)+CW (15% v/v) with Adenine sulfate (mg/l)	Percentage of shoot buds showing shoot regeneration	Remarks
0	Nil	—
50	Nil	—
75	40±3.2	++++
100	20±1.7	++
150	2±0.5	+
200	Nil	——

— = Shoot buds failed to show shoot regeneration.
\+ = Very poor growth of shoots was observed.
++ = Shoot buds showed poor shoot regeneration.
++++ = Shoot buds showed shoot regeneration. Luxurient growth of leafy shoots was observed.
Data represents an average of 3 replicates.
Data scored after 6 weeks.
Experiment repeated 3 times.

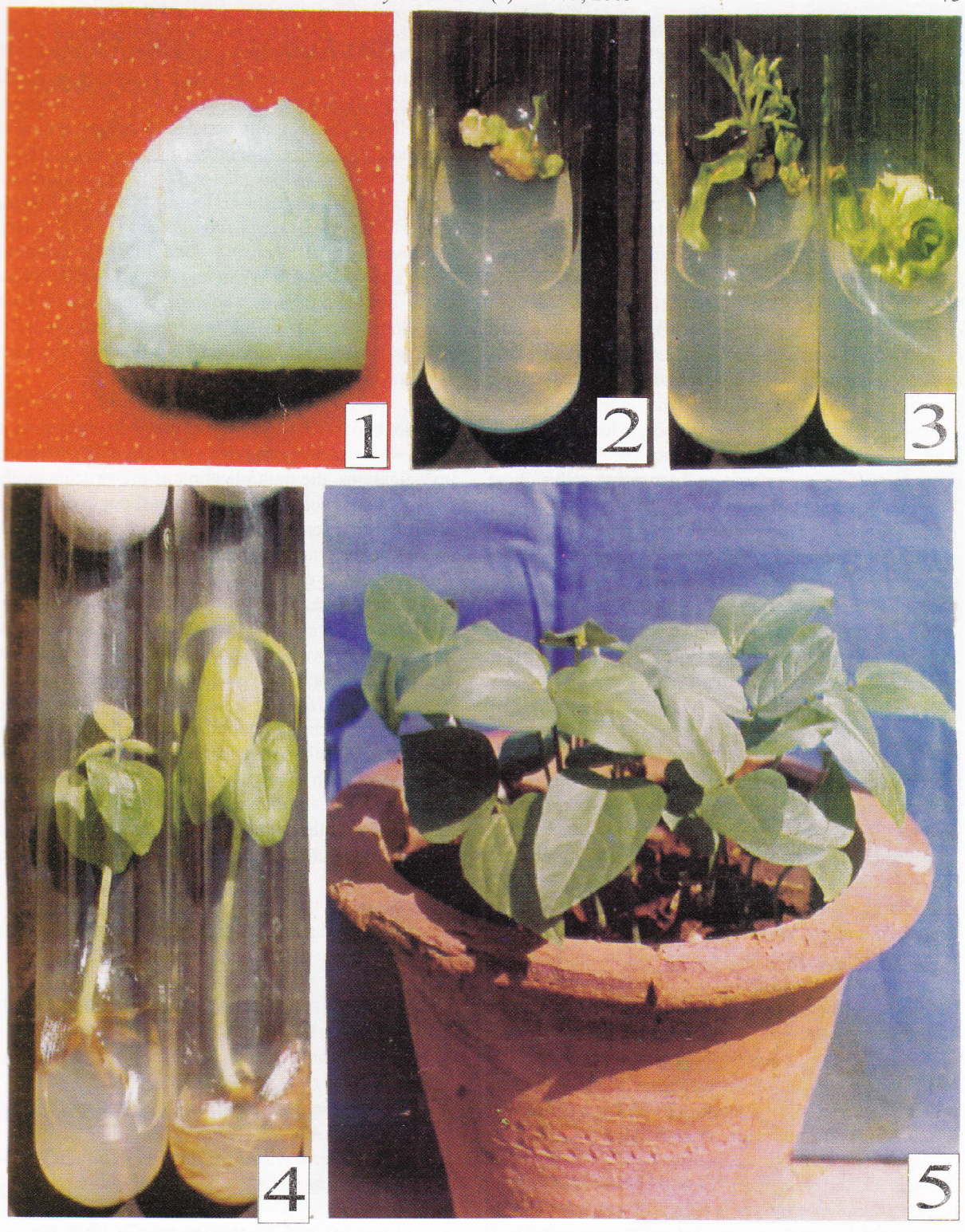

Fig.1. Plant regeneration by organogenesis from mature cotyledonary segments of Cowpea *(Vigna unguiculata* (L.) Walp. 1. Ex plant at the time of inoculation. 2. Cotyledonary segment fully covered with shoot buds after 4 weeks of culture. 3. Multiple shoots developed from cotyledonary segments. 4. Profuse *in vitro* rooting of shoots on solid medium. 5. Plantlets in pots ready for transfer to the field.

subcultured on the same medium for nearly 2 weeks. The shoot primordia developed into shoots and showed further elongation when they were subcultured on mMS basal medium containing 2.22 µM BAP, Coconut water (CW) (15%) and Adenine sulfate AS (75 mgl^{-1}). The cultures were incubated at 26 ± 2°C on a 16 hour photoperiod under cool white fluorescent light (50 µmol m^{-2} Sec^{-1}) for 2 weeks. The regenerated shoots were transferred to half-strength mMS basal medium without growth regulators for rooting. The rooted plantlets were hardened and transferred to soil.

Results and Discussion

In the present study, the distal halves of cotyledons of cowpea (*Vigna unguiculata* (L.) Walp.) cv. DFH-1 cultured on mMS basal medium supplemented with 8.88 µM BAP and coconut water (15%) swelled and turned green after 2-3 weeks in culture, producing small green dome like structures over the surface of the cotyledonary segment. After 2 weeks of culture, these structures developed into shoot buds (Fig. 1) without an intervening callus phase. The average number of shoot primordia per explant was 5. The initial shoot bud regeneration medium containing 8.88 µM BAP and coconut water (15% v/v) was modified by varying the concentrations of BAP and coconut water one at a time keeping the other one constant to obtain more shoot buds per explant. But in other combinations of BAP and coconut water, the explants turned green and failed to produce shoot buds even after 4 weeks of culture (Table 1).

Another interesting effect was the cowpea reaction to the addition of coconut water to the medium. Coconut water has a cytokinin-like-effect in cowpea or this molecule may be a precursor for endogenous cytokinin in cowpea. The earliest success in embryoid induction was achieved by using coconut water in the media. However investigations on coconut water yielded valuable information on growth promoting systems[14] and coconut water continues to be extremely useful for both somatic embryo induction and maturation[15,16]. In the present study also, cotyledonary explants (distal end) produced shoot buds only on mMS basal medium containing BAP and coconut water (Table 1). In the present study the presence of higher concentrations of BAP without coconut water and BAP with very low concentrations of coconut water, the cotyledonary explants failed to produce shoot buds which is in conformity with earlier workers[15,16]

The shoot primordia developed into shoots and showed further elongation when they were subcultured on mMS-basal medium containing 2.22 µM BAP, coconut water (15% v/v) and adenine sulfate (75 mgl^{-1}) (Table 2). The well developed shoots were transferred to half strength mMS basal medium without growth regulators for routing. The rooted plantlets were hardened and transferred to soil. Averages of 5 well developed plants were transferred to soil per each explant. Adenine in the form of Adenine sulfate can stimulate cell growth and greatly enhance shoot formation. It provides an available source of nitrogen to the cell and can generally be taken up more rapidly than inorganic nitrogen[17]. A similar concentration of coconut water (15% v/v) and adenine sulfate (75 mg/l^{-1}) has been effectively used for regeneration of plants from primary leaves of cowpea (*Vigna unguiculata* (L.)[3], Pigeonpea (*Cajanus cajan* (L.) Milsp.)[7] and a mature leguminous liana *(Bauhinia vahlii* Wight and Arnott)[18]. In the present study also shoot buds showed further development and elongated only in the presence of Adenine sulfate (75 mgl^{-1}) which also confirmed our findings with earlier workers (Fig 1).

George and Eapen[6] also observed the formation of shoot buds on the distal end of cotyledons of pigeonpea when whole cotyledons were subcultured. In the present investigations, the multiplication of pre-existing axillary buds and their possible influence on shoot bud formation was ruled out because both the proximal ends of cotyledon as well as the attached embryonal axes were eliminated. Hence, there is enough evidence to suggest that shoot bud induction was *de novo*. Totipotent cells are apparently available and are distributed all over the surface of the explants, as shown by the production of buds all along the explant. The availability of a large number of totipotent cells on the surface of a single cotyledonary segment (explant) enhances the possibility of genetic transformation by microprojectile bombardment. Bud formation is also associated with a wounding site, a prerequisite for *Agrobacterium* - mediated transformation. The present protocol fulfills the requirements for genetic transformation and hence useful for improving the crop through genetic manipulations.

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