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# Efficient shoot regeneration from double cotyledonary node explants of green gram [*Vigna radiata* (L.) Wilczek]

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An efficient shoot regeneration protocol has been developed yielding up to 27 shoots using double cotyledonary node explants of cultivar ML 267 of green gram [*Vigna radiata* (L.) Wilczek]. The explants were derived from 3-d-old seedlings germinated on Murashige and Skoog (MS) and Gamborg's medium ($B_5$) containing (2.0 mg/L) 6-benzyl aminopurine (BAP). They were initially cultured onto MS $B_5$ medium augmented with different concentrations of BAP and very low concentrations of different auxins (NAA, IAA & IBA) and cytokinin (Kn) for shoot bud induction. The explants showing multiple shoot bud initials were transferred to MS $B_5$ media containing reduced concentrations of BAP for shoot proliferation. Among the different auxins and cytokinins tested, presence of BAP+NAA in shoot bud induction and low BAP in shoot proliferation medium gave the best regeneration response. Profuse rooting was achieved in 90% of explants on ½ MS $B_5$ medium devoid of any hormones. Over 90% of the rooted plants grew well and were fertile after transfer to glass house and set seeds normally. Histological examination of 4-d-old explants confirmed direct organogenesis through axillary shoot regeneration. Protocol so developed is currently being utilized for genetic enhancement of green gram using *Agrobacterium* mediated transformation.

**Keywords:** BAP, double cotyledonary node, green gram, regeneration, *Vigna radiata*

## Introduction

Legumes associate with nitrogen fixing bacteria and play a central role in low input production systems, particularly on small-scale farms under rainfed situations. All the legume-growing regions are characterized by unpredictable weather, limited and erratic rainfall and nutrient poor soil and suffer from a host of biotic and agricultural constraints. Drought is single most important abiotic stress and terminal drought stress, which occurs during pod filling phase of the crop, is common and major yield reducer for crops growing with current rainfall. Despite tremendous increase in cereal production, the pulse production has been stagnating over the years. The per capita availability of pulses in diet has drastically declined from 60.7 g in 1951 to 32 g in 2000. The Recommended daily allowance for a vegetarian person as per ICMR and WHO guidelines are 47 and 80 g, respectively.

Green gram [*Vigna radiata* (L.) Wilczek] is an important grain legume grown mainly in arid and semi-arid situations across the country during kharif season and contributes nearly 15% of the total pulse production. The cultivation of this crop is gaining popularity by virtue of its early maturity, outstanding nutritional values and easy digestibility. Absence of sufficient and satisfactory level of genetic variability within the germplasm has been the major hurdle in its improvement through conventional breeding[1]. The immense potential of biotechnological tools for improving against biotic and abiotic stresses can be realized by supplementing the breeding programmes through introduction of alien genes of recognized relevance into elite germplasm of green gram. Legumes in general are recalcitrant to tissue culture and are highly genotype specific[2-3]. Owing to their recalcitrant nature in culture, progress in transgenic development in legume crops has been very slow[4-6].

An efficient regeneration and transformation protocol will be the key to success of genetic transformation. Cotyledonary nodes, shoot apices, leaflets and embryo axis are the most commonly used explants to regenerate legume crops. Regeneration of shoots through organogenesis from callus and somatic embryogenesis indicated that cotyledons, primordial leaves, hypocotyls, shoot tips of green gram are not ideal explants for *in vitro* regeneration[7-8]. Direct *in vitro* organogenesis from explants could be a better

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and rapid multiplication method and recently a reasonable number of researchers have reported successful regeneration from various explants[9-18]. But in most of these reports, the number of shoots regenerated remained very low and limited to a few only besides the protocol being cumbersome and not easily reproducible. The present study describes the development of simple and efficient shoot regeneration protocol from double cotyledonary node explants derived from 3-d-old seedlings.

## Materials and Methods

Seeds of mung bean [*Vigna radiata* (L.) Wilzeck] cultivar ML267 were obtained from Andhra Pradesh Seeds Corporation, Guntur, Andhra Pradesh. Healthy and uniform seeds were rinsed 2-3 times with distilled water and then washed with 70% ethanol for 1 min. The seeds were then surface sterilized with 0.1%(w/v) mercuric chloride for 8 min followed by four to five times repeated thorough washings with sterile distilled water to remove any residual disinfectant. These surface sterilized seeds were aseptically germinated on MS $B_5$ medium supplemented with 2.0 mg/L BAP at 25±1°C under light-dark (12-12 h) photoperiod. White cool fluorescent tubes were used to obtain 85 µmol $m^{-2}s^{-1}$ light intensity. Double cotyledonary nodes were dissected from 3-d-old seedlings after removing the seed coat. A cut was made at the proximal and distal ends of the seedlings selecting only 4 mm of epicotyl and 4 mm of hypocotyl. Cotyledons were also cut from apical tops retaining basal halves with explants.

The explants were inoculated on MS medium containing $B_5$ vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar and a combination of various plant growth regulators with BAP viz., NAA, Kn, IBA and IAA. The various BAP concentrations tested were 4.43, 8.87, 11.09, 13.31 and 17.75 µ*M*. Other plant growth regulators tested in combination with 11.09 µ*M* BAP included NAA (0.1, 0.5 and 1.0 µ*M*) and Kn, IBA, IAA (1.0 µ*M*). Explants were cultured for 30 d with sub-culturing at 15 d interval on the above mentioned medium. Subsequently, the explants showing multiple bud initials were transferred onto lowered concentrations of BAP for next 30 d with one sub-culture at 15 d interval. All these cultures were maintained in light-dark (12-12 h) photoperiod at 25±2ºC. Elongated individual shoots so separated were transferred onto half strength MS $B_5$ medium for rooting. Plantlets with well-developed roots were transferred in plastic cups (7 cm diam) containing soil:sand (1:1) and covered with a transparent polythene sheet. They were watered every alternate day and subsequently these plantlets were transferred into plastic buckets (14 l capacity) containing farm soil, sand and farm yard manure (10:4:4) after two wks. These plants were taken to flowering, seed set and maturity. All the cultures were maintained on the respective multiple shoot proliferation media for 30 d with one sub-culture at 15 d interval. After 60 d of total culture in shoot bud induction and multiple shoot proliferation media, the number of shoots attaining a length higher than 2.0 cm were counted and harvested for rooting. Each experiment consisted of a minimum of 40 explants per treatment and was repeated at least three times. Observations were recorded on number and length of the regenerated shoots. In all the cases the *p*H of the media was adjusted to 5.8 and the gelled with 0.8% agar before autoclaving at 121°C and 15 lbs pressure for 20 min. All the cultures were incubated at 25±1°C under 12 h photoperiod. Various supplements viz., $AgNO_3$, $CuSO_4$, proline, glutamine and cysteine were added to regeneration medium to study their effect on shoot proliferation.

For histological studies, 4-d-old explants from shoot bud initiation medium with emerging shoot buds were fixed in methanol:acetic acid (3:1) solution for 48 h. The material was dehydrated through a graded ethanol series followed by embedding in paraffin (58-60ºC). Serial sections of 6 microns thickness were cut on a rotary microtome. These sections were then affixed to slides, de-waxed and stained with hematoxylin and eosin and viewed under Olympus CX31 microscope.

## Results and Discussion

An efficient shoot regeneration protocol yielding up to 27 shoots has been achieved using double cotyledonary node explants of variety ML 267 of green gram. Explants (Fig. 1a) were derived from 3-d-old seedlings germinated on MS $B_5$ medium pre-conditioned with 2 mg/L BAP under light-dark (12-12 h) cycle. They were first cultured on MS $B_5$ shoot bud induction medium (Fig. 1b) supplemented with 11.09 µ*M* BAP in combination with different concentrations of NAA (0.1, 0.5 and 1.0 µ*M*) (Table 1). Besides NAA, other plant growth regulators evaluated included Kn, IBA, IAA for their possible role in shoot bud induction and proliferation (Table 1). Different concentrations of BAP (4.43, 8.87, 11.09, 13.31, 17.75 µ*M*) were assessed in combination with 0.1 µ*M* NAA (Table 1).

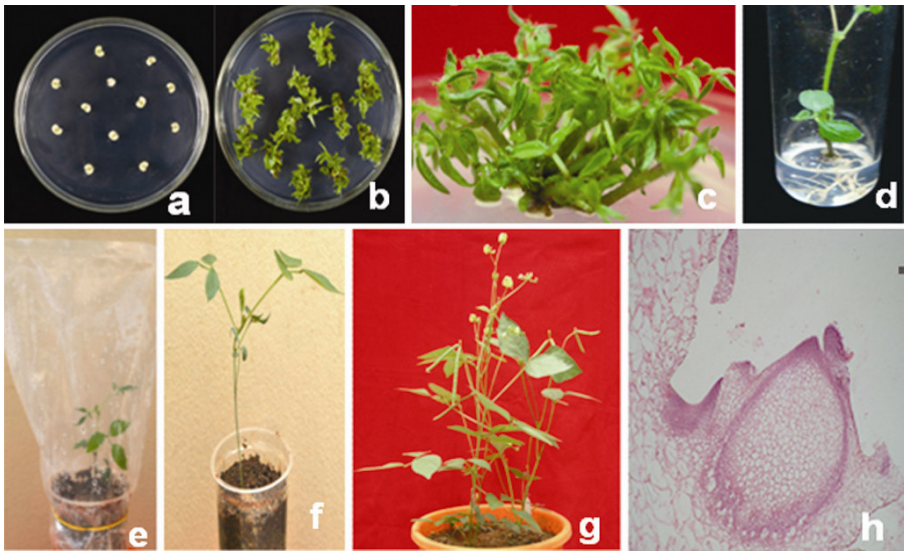


Fig. 1—Regeneration in mung bean (*Vigna radiata* L. Wilczek) cultivar ML-267; a. Double cotyledonary node explant; b. Explants on shoot bud initiation medium; c. On shoot proliferation medium; d. On ½ MS $B_5$ rooting medium rooted shoot ready for acclimatization and glass house transfer; e & f.. Primary hardening of a rooted shoot; g. Well established plant in green house; and h. Photomicrograph of longitudinal section of the explant showing direct organogenesis through axillary shoot regeneration.

Table 1—Regeneration response of DCN explants cultured on $MSB_5$ medium containing different concentrations of BAP supplemented with low concentrations of various auxins and cytokinins

| Plant growth regulator (µ*M*) | Mean shoot number | Mean shoot length (cm) |
|---|---|---|
| 00 | 2.0±0.0 | 7.1±0.1 |
| 4.43 BAP+0.1 NAA | 13.2±0.3 | 3.1±0.4 |
| 8.87 BAP+0.1 NAA | 19.1±0.6 | 2.4±0.1 |
| 11.09 BAP | 11.8±0.5 | 2.5±0.2 |
| 11.09 BAP+0.1 NAA | 26.7±0.2 | 2.6±0.2 |
| 11.09 BAP+0.5 NAA | 19.9±0.2 | 2.6±0.2 |
| 11.09 BAP+1.0 NAA | 14.1±0.8 | 2.6±0.1 |
| 11.09 BAP+0.1 Kn | 15.3±0.5 | 2.3±0.2 |
| 11.09 BAP+0.1 IAA | 16.9±0.6 | 2.5±0.1 |
| 11.09 BAP+0.1 IBA | 15.0±0.7 | 2.5±0.1 |
| 13.31 BAP+0.1 NAA | 18.2±0.5 | 2.3±0.2 |
| 17.75 BAP+0.1 NAA | 15.1±0.1 | 2.5±0.3 |

Subsequently, these explants were transferred to MS $B_5$ shoot proliferation medium containing only half the concentration of BAP used in shoot induction medium. The regeneration response varied with different PGR combinations tested and BAP (11.09 µ*M*) and NAA (0.1 µ*M*) yielded highest number of shoots (26.50; Fig. 1c). It has become possible by choosing the right explant and optimal manipulation of cultural conditions by aptly altering concentration of the selected plant growth regulators. The regenerants could be obtained rapidly and in a more reproducible manner round the year independent of the season. Plain MS $B_5$ and ½ MS $B_5$ media were tested for their response to rooting. Shoots more than 2.0 cm were isolated and efficiently rooted on ½ plain MS medium (Fig. 1d). Primary hardening was achieved in plastic cups containing soil:sand (1:1) which were covered with a transparent polythene sheet to minimize transpiration losses for a minimum of 10 d (Fig. 1e & f). They were watered every alternate day. The rooted shoots were successfully hardened in pots having soil and farm yard manure. These plantlets flowered and produced pods with viable seeds upon reaching maturity (Fig. 1g).

Earlier a high frequency shoot and plant regeneration protocol was established using explants derived from half cotyledonary node with intact cotyledon (unpublished data). The number of regenerated shoots obtained with ½ CNC was very close to those derived with DCN explant during the present investigation. With ½ CNC, it was established that a 2-d-old germinated seedling made a better explant and removal of pre-existing buds improved the regeneration response. Procedure described above for DCN explants is simpler compared to the one developed earlier for ½ CNC explants.

The effect of adding various supplements viz., $AgNO_3$, $CuSO_4$, proline, glutamine and cysteine in the shoot regeneration medium with respect to shoot induction and proliferation was also studied. None of the supplements tried enhanced the multiple shoot induction and proliferation.

Histological examination of 4-d-old double cotyledonary node explants was carried out to examine the nature and route of shoot regeneration. Vertical sections derived from these explants indicated initiation of meristemoids in the form of meristematic zones on the peripheral regions of the explants and continuous additional meristem formation was observed at the base of meristemoids. It confirmed direct organogenesis through axillary shoot regeneration. Figure 1h shows peripheral meristematic tissue getting differentiating to shoot primordia.

The direct *in vitro* organogenesis from explants had been reported to be a rapid multiplication method for true to elite strain and is preferred for developing transgenic plants to avoid somaclonal variation. In legumes, multiple shoot induction through cotyledonary node has been widely used for regenerating shoots[19]. In mung bean when mature cotyledons were used, all the regenerated shoots were obtained from the proximal side i.e., from the pre-existing buds or through the proliferation of shoots around pre-existing buds[7,16-17,20-24]. Whereas, with immature cotyledons, regeneration of shoot buds was obtained from all over the surface of cotyledons[25]. The main advantage of using explants like cotyledonary node for *in vitro* regeneration is their access to uniform explant sources all through the year. Prolific shoot regeneration has been achieved in mung bean from 3-d-old *in vitro* cotyledonary node and hypocotyl explants from seedlings derived from mature seeds on MS medium supplemented with 0.9 μ*M* TDZ[15].

The BAP pre-conditioning in germinating mung bean seedlings stimulates formation of axillary buds in cotyledonary nodes resulting in multiple shoot regeneration[26]. In fact BAP has been most widely used as an effective cytokinin for multiple shoot regeneration for various legumes including *Vigna* species. BAP in the regeneration medium was found to enhance regeneration frequency in mung bean[7,22] and chick pea[27]. IBA has been shown to have a proven role in root formation in *V. radiata*[11, 25].

BAP in combination with NAA/IAA produced basal callusing followed by rhizogenesis[28]. *In vitro* induction of multiple shoots and plant regeneration in *V. mungo* and *V. radiata* have been tested for their morphogenetic potential on media containing range of phyto-hormone combinations including BA, Kn, TDZ and Zeatin[23]. The organogenic potential of the explant has been observed to be strongly influenced by the type, concentration and combination of plant growth regulators[1,29-30]. Meristematic growth in the nodal regions of the cotyledons resulted in enlargement of the explants to twice their original size within first 5 d of culture in the shoot bud induction medium. By 7$^{th}$ d itself the emergence of shoot buds initials was visible from the axillary portion. Results indicate that an optimal level of BAP along with very low concentration of NAA in the shoot bud induction medium and subsequently culturing explants in reduced concentrations of BAP for shoot proliferation gave the best results. It indicates that the particular ratio of cytokinin and auxin is very important for the induction and proliferation of shoots.

BA was found to enhance regeneration frequency as reported by Gulati and Jaiwal[31]and Chandra and Pal[22]. In other *Vigna* species, namely *V. unguiculata*[30] and *V. aconitifolia*[32] Kn (1.0 μ*M*)/2,4-D (0.8 μ*M*) were used to initiate organogenic calluses from hypocotyl and cotyledon explants. In fact BA is the most widely used and effective cytokinin for various legumes including *Vigna* species[7,33-34]. Thus, by manipulating the concentrations of BAP, NAA and IAA, in the culture medium, it has been demonstrated that *Vigna* cotyledons could produce robust and green plantlets. *In vitro* organogenesis was successfully achieved from callus derived from cotyledon and hypocotyl-derived explants of *V. radiata* on MS medium supplemented with NAA (1.07 μ*M*), BA (8.88 μ*M*) and 10 % coconut water[11].

The protocol described here offers a relatively simple explant to prepare, which is season independent. The proliferation results in individual isolated shoots. The selection of DCN explant offers an option of using either side of the meristematic region of the cotyledonary node for transformation. Since the procedure does not involve removal of shoot buds as observed earlier, minimizing the chance of production of phenolics mainly due to the injury caused to the explant. In conclusion, the procedure developed could be successfully employed as an alternative protocol for genetic transformation using *Agrobacterium* mediated/biolistics approach making it suitable for introduction of alien genes of interest for enhancing biotic and abiotic stress tolerance and quality in green gram. The protocol is currently being used for genetic transformation of green gram with *annexin 1 bj* gene for enhancing abiotic stress tolerance.

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