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Short Communication

High Frequency Induction of Multiple Shoots and Plant Regeneration from Cotyledonary Nodal Explant of Mung Bean [*Vigna radiata* (L) Wilczek]

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An efficient protocol for shoot bud induction and proliferation employing half cotyledonary node with intact cotyledon explants derived from two-day-old seedlings of mung bean pre-conditioned on 6- benzylaminopurine (BAP) has been achieved. Explants were cultured for four weeks each on MS B_5 + 12.5 µM BAP and MS B_5 + 5 µM BAP +0.05 µM α-naphthaleneacetic acid (NAA), respectively, as shoot bud induction and shoot elongation and proliferation media, gave the best regeneration response. The removal of the pre-existing buds from explants at 12 days in shoot bud induction medium led to enhanced regeneration response. Light microscopic observations on 14-day-old explants confirmed direct organogenesis route of regeneration. Elongated shoots (>2 cm) excised from the regenerating cultures were successfully rooted on half MS B_5 medium containing 2.46 µM indolebutyric acid (IBA). About 90% of the rooted plantlets, efficiently hardened in pots having soil and farm yard manure, flowered and produced pods with viable seeds upon reaching maturity.

***Key words*:** BAP, half cotyledonary node with intact cotyledon, mung bean, regeneration.

Mung bean (*Vigna radiata* L Wilczek) is an important grain legume and a good source of vegetable protein grown widely in Southeast Asia, Africa, South Africa and Australia. In India, it is cultivated mainly under limited and erratic rainfall conditions and on marginal and sub-marginal lands where numerous biotic and abiotic stresses limit its productivity. Conventional breeding for enhancing biotic and abiotic stress tolerance has several constraints (1). Robust *in vitro* culture methods are needed to produce high frequency regeneration for developing cultivars with desirable characters by incorporating potentially useful genes of recognized relevance. Tissue culture approaches to regenerate plants from various explants in mung bean have met with limited success owing to its recalcitrant nature (2). Thus, the potential benefit of genetic transformation for enhancing stress tolerance in mung bean has not yet been realized (3).

Most of the studies with mung bean report direct regeneration from the pre-existing meristems. Recently, mung bean has been successfully regenerated from mature cotyledons (4), cotyledonary node (5,6), cotyledonary node and epicotyl (7), petioles of primary leaves (8), cotyledon, cotyledonary node and shoot tips (9), hypocotyls, nodal segment and leaf (10) and cotyledon and hypocotyls (11). But the number of regenerating shoots remained very low and limited to a few only. Therefore, development of a simple, efficient and reproducible method for plant regeneration in mung bean is the need of hour.

Seeds of mung bean (*Vigna radiata* L Wilzeck) cultivar ML267 were obtained from Andhra Pradesh Seeds Corporation, Guntur, Andhra Pradesh, India. Healthy and uniform seeds were rinsed 2-3 times with distilled water and then washed with 70% ethanol for 1 min. After that the seeds were 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 medium containing B_5 vitamins supplemented with 8.9 µM BAP at 25±1°C under 12 h photoperiod. White cool fluorescent tubes were used to obtain 85 μmol m^{-2} s^{-1} light intensity. Cotyledonary nodes were dissected from two-day-old seedlings after removing the seed coat. A cut was made at the proximal and distal end of the seedlings selecting only 1mm of epicotyl and 2mm of hypocotyl followed by a vertical division along the

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Abbreviations: ½ CNC - half cotyledonary node with intact cotyledon MS- Murashige & Skoog; BAP-benzylzimnopurine; NAA-naphthalene acetic acid; IAA-indole acetic acid; IBA- indolebutyric acid; 2,4-D-2,4-Dichlorophenoxyacetic acid; PGRs-plant growth regulators; TDZ-thidiazuron.

growing axis leading to production of two explants each comprising of half-cotyledonary node with an intact cotyledon.

The explants were inoculated on shoot bud induction media-MS B_5 supplemented with different concentrations of BAP (0,10, 12.5, 15 and 20 µM) by inserting the abaxial surface. After 14 days of initial incubation on shoot bud induction medium, these explants were sub-cultured on to the fresh medium with same composition. After 28 days of culture, the explants with induced multiple shoot buds were transferred on to shoot proliferation media- MS B_5 supplemented with different concentrations of BAP (0, 2.5, 5 and 7.5 µM) and NAA / IAA / IBA (0.5 and 0.05 µM) with 5 µM BAP only.

In order to study the effect of age of explants on shoot regeneration, three-day-old seedlings were also used as ½ CNC explants. For evaluating the possible role of various supplements on shoot regeneration, 20 µM $AgNO_3$, 20 µM $CuSO_4$, 330 µM cysteine and 274 µM glutamine were included in shoot bud induction medium. For assessing the impact of removal of pre-existing shoot buds, 12-day-old explants were used.

The regenerated shoots attaining a length more than 2.0 cm were counted and were rooted on half strength MS B_5 medium supplemented with IBA. Hardened plants were taken to flowering, seed set and maturiy. Each experiment consisted of a minimum of 40 explants per treatment and was repeated at least three times. In all the cases the pH of the media was adjusted to 5.8 and 0.8% agar was added before autoclaving at 121 °C and 15 lbs pressure for 20 min.

For histological studies, fourteen-day-old explants with re-emerging shoot buds were fixed in methanol: acetic acid (3:1) for 48 h. The material was dehydrated through a graded ethanol series followed by embedding in paraffin (58-60°C). 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.

Half cotyledonary node with intact cotyledon explants (Fig.1a) when cultured on shoot bud induction medium containing different levels of BAP at 25±1°C under 12 h photoperiod resulted in varied response (Table 1). Meristematic growth resulted in enlargement of the explants to twice their original size within first five days of culture in the shoot bud induction medium. By 7th day itself the emergence of shoot buds initials was visible from the axillary portions (Fig.1b). Removal of the pre-existing shoot buds at 12 days resulted in sharp increase in number of axillary shoot buds. The explants cultured at 12.5 µM BAP concentration gave consistently higher shoot number

Table 1. Effect of different concentrations of various plant growth regulators on shoot bud induction and shoot proliferation

Shoot bud induction	Shoot proliferation medium MS B_5 +				Response	
medium MS B_5 + BAP (µM)	BAP(µM)	NAA(µM)	IAA(µM)	IBA(µM)	Mean shoot number	Mean shoot length (cm)
0	—	—	—	—	1.43±0.23	7.62±0.69
10	—	—	—	—	7.3±0.15	5.35±0.08
12.5	—	—	—	—	11.2±0.21	6.39±0.23
15	—	—	—	—	7.83±0.17	5.11±0.04
20	—	—	—	—	7.1±0.12	4.77±0.10
12.5	2.5	—	—	—	14.3±0.40	4.25±0.20
12.5	5	—	—	—	20.3±0.40	3.41±0.17
12.5	7.5	—	—	—	10.3±0.71	3.13±0.15
12.5	5	0.05	—	—	29.4±1.09	4.12±0.15
12.5	5	0.5	—	—	19.8±1.12	4.09±0.16
12.5	5	—	0.05	—	20.4±0.57	3.60±0.17
12.5	5	—	0.5	—	15.3±0.32	3.20±0.20
12.5	5	—	—	0.05	18.3±1.35	3.51±0.29
12.5	5	—	—	0.5	14.3±0.70	5.36±0.28

Note: Values represent mean ± SE of three replicates. All treatments gave 100 % regeneration frequency. Each observation represents a mean of 120 explants. The values are means of results from 3 individual experiments

compared to 10, 15 and 20 µM BAP upon transfer to shoot proliferation media. The explants cultured on shoot bud induction medium containing 12.5µM BAP upon transfer to multiple shoot proliferation media containing 5 µM BAP + 0.05 µM NAA gave the highest number (29.4) of shoots (Fig. 1c). It has become possible by choosing the right explant and manipulation of concentration and ratio of selected cytokinin and auxin. Vertical division in the growing axis of a germinated seedling resulted in production of double the explants. The other advantage of using explants like cotyledonary node is that it is season independent.

The average number of shoots and their length were consistently higher in two-day-old explants compared to three-day-old ones in all the shoot proliferation media tested. Addition of $AgNO_3$, $CuSO_4$, cysteine and glutamine as supplements in shoot bud induction medium at given concentrations did not improve shoot regeneration efficiency.

Half MSB_5 medium containing 2.46 µM IBA gave best rooting response within 20 days (Fig.1d). The plantlets with well-developed roots were transferred to plastic cups (7cm diameter) containing autoclaved soilrite and maintained under green house conditions for two weeks. Subsequently the rooted plantlets were transferred to 15 liters plastic buckets containing garden soil: farm yard manure (12:3). Rooted plantlets were grown under green house conditions under natural day-light. About 90% of the rooted plantlets, efficiently hardened in pots, flowered and produced pods with viable seeds upon reaching maturity (Fig. 1e).

Histological examination of vertical sections derived from 14-day-old explants from which the pre-existing buds were removed at 12 day stage indicated direct organogenesis through axillary shoot regeneration. It revealed initiation of meristemoids in the form of meristematic zones on the peripheral regions of the explants. Continuous additional meristem formation was also observed at the base of meristemoids (Fig.1f). Higher regeneration response in explants consequent to removal of the pre-existing buds indicated the possibility of the partially differentiated cells just below the excised meristem to attain a greater morphogenetic competence.

The BAP pre-conditioning in germinating mung bean seedlings stimulates formation of axillary buds in cotyledonary nodes resulting in multiple shoot

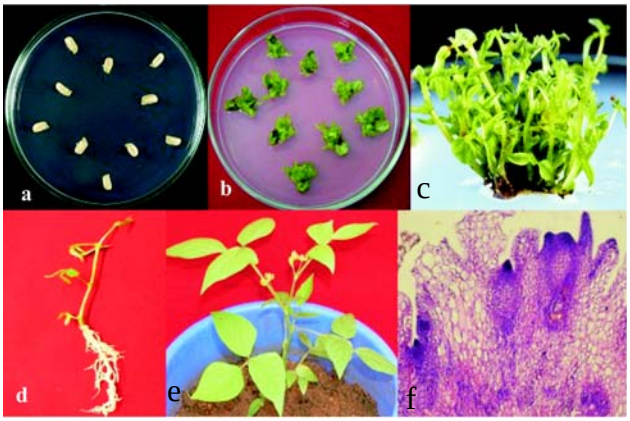

Fig.1. Regeneration in mung bean cultivar ML-267. (**a**) Cotyledonary node with intact cotyledon explant, (**b**) on shoot bud initiation medium, (**c**) shoot proliferation medium, (**d**) well rooted shoot ready for acclimatization and glass house transfer, (**e**) well established plant in green house, and (**f**) Photomicrograph of longitudinal section of the explant after removal of pre-formed shoot initials showing direct organogenesis through axillary shoot regeneration. It revealed initiation of meristemoids in the form of meristematic zones on the peripheral regions of the explants.

regeneration (12). In fact BAP has been most widely used as an effective cytokinin for multiple shoot regeneration for various legumes including *Vigna* species (13). BAP in combination with NAA/IAA produced basal callusing followed by rhizogenesis (14). *In vitro* induction of multiple shoots and plant regeneration in *Vigna mungo* and *V. radiata* have been tested for their morphogenetic potential on media containing range of phyto-hormone combinations including BA, Kinetin, TDZ and Zeatin (15).

The organogenic potential of the explant has been observed to be strongly influenced by the type, concentration and combination of plant growth regulators. Numerous reports on regeneration of shoots through organogenesis from callus and somatic embryogenesis indicate that cotyledons, primordial leaves, hypocotyls, shoot tips of mung bean are not ideal explants for *in vitro* regeneration (1,10,14). In legumes, multiple shoot induction through cotyledonary node has been widely used for regenerating shoots (16). In mung bean when mature cotyledons were used, all 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 (6,7,13,15). Whereas, with immature cotyledons, regeneration of shoot buds was obtained from all over the surface of the cotyledons (17). Regeneration from immature cotyledons of mung bean is a space, time

and labour consuming task and thus makes maintaining continuous stock of plants very tedious. 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-day-old *in vitro* cotyledonary node and hypocotyls explants from seedlings derived from mature seeds on MS medium supplemented with 0.9 µM TDZ (5).

In vitro organogenesis was successfully achieved from callus derived from cotyledon and hypocotyls derived explants of *Vigna radiata* on MS medium supplemented with NAA (1.07 µM), BA (8.88 µM) and 10% coconut water (11). Somatic embryogenesis was induced from mature cotyledons, hypocotyls, nodal segments and leaf explants using 1.809 µM 2,4-D and 3.555 µM BA (10). Somatic embryogenesis and plant regeneration from cotyledonary explants of mung bean has also been achieved using 2,4-D, NAA and TCPA singly or in combination with BAP or Kinetin (4).

Shoot multiplication can be manipulated by altering the type and concentration of hormones and timing of their presence in the shoot proliferation medium. BAP is very effective for shoot bud induction at higher concentrations but inhibited further development and growth of shoot buds. Lowering BAP concentration at later stages and inclusion of auxin (NAA) resulted in induction and proliferation of more number of shoots. Requirement of BAP for induction of multiple shoots has already been reported earlier in mung bean (1,2). IBA has been shown to have a proven role in root formation in *Vigna radiata* (11,17).

The direct *in vitro* organogenesis from explants have been shown to be a rapid multiplication method for true to elite strain (18) and is preferred for developing transgenic plants to avoid somaclonal variation. Regeneration though somatic embryogenesis may lead to abnormal plants giving rise to somaclonal variation but multiplication from the existing meristem appears to prevent that. However, in most of the studies the number of regenerating shoots remained very low and varied from one to a few only and the reported higher claims of efficient regeneration in grain legumes are either non-reproducible or show very poor results.

In conclusion, the protocol described here is very simple and efficient and can be successfully employed as an alternative protocol for genetic transformation using *Agrobacterium* mediated /biolistics approach for introduction of alien genes of interest for enhancing quality, biotic and abiotic stress tolerance in mung bean.

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