An Efficient In Vitro Regeneration Strategy in a Recalcitrant Grain Legume

Vigna radiata (L.) Wilczek

Arpita Maity[1], Dipankar Chakraborti[2] and Rituparna Kundu Chaudhuri[1*]

[1] U.G. and P. G. Department of Botany, Bethune College, 181 Bidhan Sarani, Kolkata-6

[2] P. G. Department of Biotechnology, St. Xavier's College, 30 Park Street, Kolkata – 16

**Corresponding Author, e-mail ID: rkc2821@gmail.com*

Abstract: Cotyledonary nodes, excised from three days old seedling of *Vigna radiata* have been tested for their morphogenic potential on media containing a range of hormonal combinations including benzyladenine aminopurine (BAP), á-naphthalene acetic acid(NAA), and amino acid proline. Multiple shoots developed on cotyledonary node explants in basal medium containing BAP (1-2mg/l). Presence of both the cotyledons, either full or half, resulted in a maximum number of shoots (18) produced in Murashige and Skoog medium containing 1.5mg/l BAP and 0.01mg/l NAA, supplemented with 500mg/l proline. Mature plants showed normal phenotypes.

Key words: Legume, *Vigna*, shoot multiplication, proline, regeneration

Introduction:

Legumes, with 88% of the species examined to date are second only to the Graminiae in their importance to humans. The 670 to 750 genera and 18,000 to 19,000 species of legumes include important grain, pasture, and agroforestry species. *V. radiata*, commonly known as mung bean, a popular legume is a traditional food source of Chinese people rich in vitamins, calcium, irons, phosphorus ratio higher than crude rice. These contain 20% protein and are a good source of foliate and dietary fiber. It has good values both as food and as medicine. Taking mung bean as food regularly could help in treatment of high blood pressure, arteriosclerosis, diabetes, nephritis. Mung bean paste applied externally is reported to treat dermatitis and skin eczema. Along with various antibacterial properties mung bean shows antiviral as well as anticancerous properties. However various biotic stresses such as viral, bacterial and fungal pathogens as well as abiotic stress like drought results in limited crop performance. The seeds are recalcitrant in nature also poses a hindrance towards improved crop production (Ali and Gupta, 2012). Conventional breeding assisted for a long time to develop resistant varieties to biotic and abiotic stresses. Certain modern approaches have been developed for selection of disease resistant high yielding varieties. Molecular markers have been developed and biotechnological approaches have been undertaken towards genetic improvement of pulses that also include *V. radiata* (Gupta et al., 2014). Plant transformation is now a core research tool in plant biology and a practical tool for cultivar improvement that requires a strong regeneration strategy under *in vitro* condition.

An efficient and reproducible protocol for regeneration does not exist yet, genetic engineering has been retarded very much in this important crop species. Due to recalcitrant nature of the plant little progress in *in vitro* regeneration of this plant has been made. Gill et al., (1988), Eapen and George (1990), Acharjee et al., (2012) studied morphogenic responses of cultured cotyledons, ontogeny of somatic embryos, and study of embryonic axes to produce transgenics. Recent findings aim towards procedures which rely on proliferation of shoots around pre-existing buds of an embryo, which may provide an ideal system for transformation. Very few reports are available on genetic transformation of this species using any of the gene transfer techniques probably due to lack of an efficient and reproducible regeneration system. A very few success has been obtained with embryonal axes and cotyledonary nodes (Yadav et al., 2010, Amutha et al., 2004). Also induction of shoot oraganogenesis from cotyledonary nodes, hypocotyls and epicotyl have been reported and used for successful regeneration (Mundhara and Rashid, 2006) in this species. *In vitro* culture facilitates rapid multiplication of superior clones and is a pre-requistie for improvement of plants via genetic engineering technique. An efficient regeneration strategy has been discussed in this paper.

Material and Methods:

Seed surface sterilization and culture conditions :– Healthy seeds of *V. radiata* cultivar HUM1, were washed with autoclaved double distilled water and 0.05% 'Exalin' (detergent) solution. The seeds were sterilized for 15 min with 0.1% $HgCl_2$ and washed with sterile double distilled water. Finally, the seeds were kept immersed overnight in jam bottle containing double distilled water. Half of the seed explants with cotyledonary node/ embryonic axis were transferred to culture tubes containing 20 ml of different combinations of plant growth regulators like BAP, NAA in MS Basal medium supplemented with proline.

A set of 15 explants (3 explants/culture tube) per treatment was cultured, and each experiment was repeated three times. The pH of the culture medium was adjusted to 5.8 prior autoclaving at 1.1 kg cm^{-2} and 121ºC for 15 min. All of the cultures were incubated under a 16 h light /8-h dark photoperiod regime (artificial light supplied at 45 µE m^{-2} s^{-1}) at 22 ± 2 ºC.

Subcultures were done at 3-4 weeks intervals. The number of responding explants and shoots developed per explant was recorded from the second week. Data was recorded after every 3-4 weeks of culture.

Induction of shoots from explants :– Three days old cotyledonary nodes were cultured in MS medium supplemented with 0.5-2 mg/l BAP and 0.01 mg/l NAA. Usually, nodes were sub-cultured at an interval of two weeks in the same medium. In addition, during each subculture, one set of nodes was transferred to shoot induction medium containing BAP and NAA, supplemented with or without proline. Regeneration efficiency of 6-8 weeks old cultures was studied.

Induction of root and maintenance of complete plant :– About 4-5 cm long shoots were placed in root induction media (RIM). The medium was formulated with ½ MS salts, 3% (w/v) sucrose and supplemented either with NAA (0.5, 1, 2mg/l) or with IBA (0.5, 1, 2 mg/l). The shoots were kept for four weeks in the RIM and rooted shoots were maintained in ½ MS basal medium with 0.5% (w/v) sucrose. Frequency of root induction in auxin containing medium and number of roots developed per shoot was recorded.

Transplantation and hardening of regenerants :– Plantlets with well-developed roots were thoroughly washed in tap water and transplanted in pots containing soilrite and were covered by polythene bags and allowed to grow for two weeks under 80-90% humidity. Subsequently, bags were removed for 2-3 h every day for gradual acclimatization and finally covers were completely removed from the plantlets after one week and the plants were transferred to soil (clay:sand:compost; 1:1:1).

Analysis of experimental data :– Statistical difference between mean tabulated values were estimated ($p \leq 0.05$) using Tukey's HSD test with the Statistica Software v 5.0 (StatSoft 1995).

Results and Discussion :– Legumes, recognized to be a valuable source of protein, is unfortunately recalcitrant and poses a challenge to *in vivo* germination and *in vitro* regeneration (Hammat et al., 1986). Various earlier published reports claiming regeneration failed to show reproducibility. Among various explants tried cotyledonary nodes remain a dependable source. However in *V. radiata*, the cotyledonary node is a poor regenerator (Mundhara and Rashid, 2006). Successful regeneration from cotyledonary node in *Glycine max* was first described by Cheng et al., (1980). The number of regenerating shoots per explant in different cultivars ranged from one to a few (Mathews, 1987; Sen and Guha-Mukherjee, 1998). Tivarekar and Eapen (2001) reported regeneration with BAP and IAA. While vitrification remains a persistent problem during *in vitro* culture (Malik and Saxena, 1992) it was not observed in this study. Amutha et al. (2004) observed that inducing effect of BAP in different combinations with NAA, produced 19 shoots/explant from cotyledonary explants. Employing a low concentration of thidiazuron (1.0 µM), to avoid vitrification, induction of a few shoots was possible on *V. radiata* seedlings from cotyledonary node (Mundhara and Rashid, 2006). Noticeable multiplication in *V. radiata* was observed by Yadav et al., (2010) by using B5 media (Gamborg et al., 1968) containing 2.0 mg/l BAP and combinations of NAA, IAA, IBA. The explants yielded 27 shoots in average using double cotyledonary node explants of cultivar ML-267, *V .radiata (*Yadav et al., (2010).

In the present work, successful regeneration of shoots (7±1.1) of *V. radiata* from cotyledonary node was obtained with BAP (1.5 mg/l) and NAA (0.01 mg/l) in MS medium (Table1). An increased number of shoots (18±2.1) developed from cotyledonary node in the medium supplemented with proline (500 mg/l) (Figure 1a-f). BAP (0.5-1 mg/l) in different combinations with NAA without proline supplementation, using cotyledonary node was found to produce lesser number of shoots per explants. Some persistent problem like chlorosis of leaves associated with regular leaf falling was also observed. The problem is common for leguminous plant regeneration under *in-vitro* conditions. This work finds to overcome such problem by using different gradients of proline (200-500 mg/l). In tissue culture proline metabolism is affected by osmotic and salt stress (Pandey and Ganapathy, 1985, Duncan and Widholm, 1994). It was found from earlier reports that in Wester variety of *Brassica napus* produced multiple shoots in 100% explants with addition of 20 mM proline (O´Neil et al., 1996). Again synergistic effects of proline with enhanced level of micronutrients have been reported to increase shoot number in *Glycine max* (Kim et al., 1994). *V. radiata* is known to be relatively drought tolerant, can grow under moisture deficit condition. Additional proline contributes to such tolerance under *in vitro* condition and help in proliferation of the shoots with proper concentration and combinations of plant growth regulators. *V. radiata* plants could be regenerated by induction of roots in microshoots in basal medium (MS) with 3% sucrose supplemented with (0.5-2 mg/l) IAA and IBA (data not shown) within four weeks. The similar concentration of NAA induced callusing from the nodal regions. Regenerated plants were hardened in ½ strength MS basal medium and acclimatized under field condition.

Tables:

Table 1: Effect of plant growth regulators and proline on regeneration of shoots from cotyledonary nodal explants of *V. radiata* (6-8wks)

Set No.	Conc. Of BAP in MS media (mg/l)	Conc of NAA in MS media (mg/l)	Conc. Of Proline (mg/l)	Mean No of shoots±SE*	Remarks
1	0.5	0.01	0	4±1.1f	Chlorosis and falling of leaves observed after 6-8 weeks
2	1	0.01	0	7±1.1e	Chlorosis prevailed
3	1	0.01	200	10±0.1d	Chlorosis prevailed
4	1	0.01	500	11±0.1cd	Leaf falling and cholorosis minimized.
5	1.5	0.01	200	15±2.4b	Prominent changes observed ,as the leaves retained green colour
6	1.5	0.01	500	18±2.1a	Leaves persistent with green colour
7	2	0.01	500	10±1.1d	Persistent leaves retained green colour. Chlorosis and falling of leaves minimized

* Values represent mean ± S.E. of three experiments each with 5 replications. Data scored after six weeks of inoculation. Means within a column with the same letter are not significantly different based on Tukey's HSD test at $P \leq 0.05$.

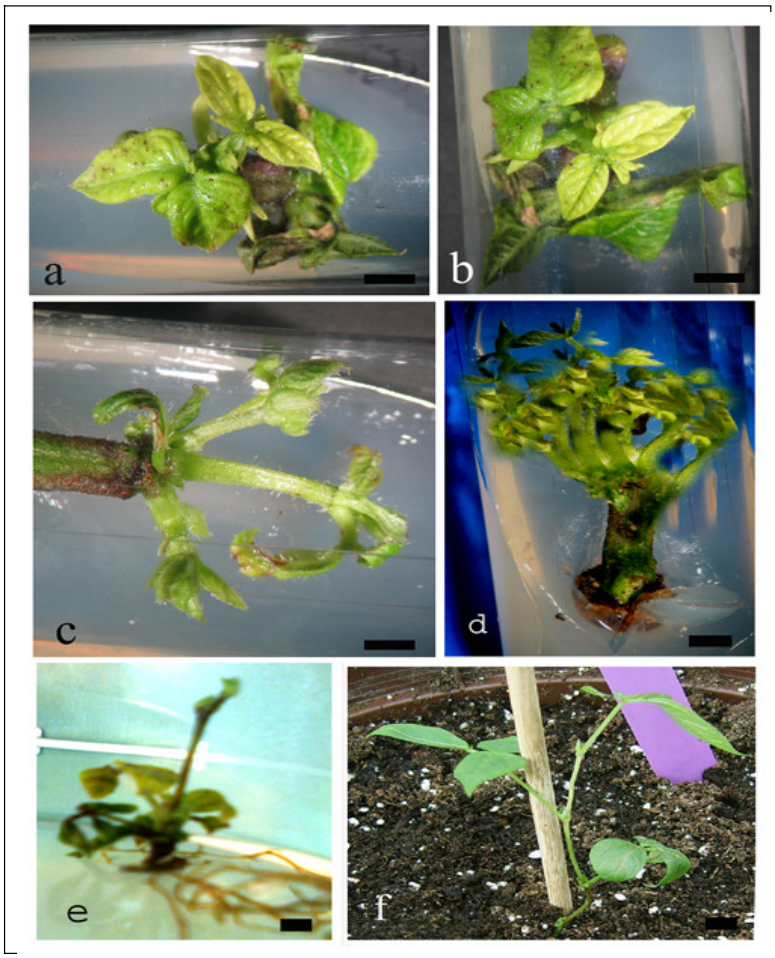

Figure 1. Regeneration of whole plant from cotyledonary explants.

a-d, different stages of shoot proliferation from explants ;

e, root induction on individual shoot;

f, plant established in soilrite. Bars represent 1 cm.

Conclusion :

The genomics and post-genomics developments in plant biology have enhanced our knowledge on the genetics and biotechnology of the various crop plants. In past recent years more emphasis has been laid on the development of transgenic plants in order to improve yield quality and disease resistance, which require a successful *in vitro* regeneration strategy. In present communication a reproducible regeneration method has been reported for *V. radiata*. BAP and NAA supplemented with proline resulted in enhanced shoot regeneration from cotyledonary node explants. Such application also found to be suitable to restrict chlorosis and regular leaf falling *in vitro* which is a common problem in legumes. This protocol for regeneration can be successfully utilized for future transgenic researches in this crop and other related species.

References:

Acharjee, S., Handique, P. J. and Sarmah, B. K . (2012) Effect of thidiazuron (TDZ) on *in vitro r*egeneration of blackgram (*Vigna mungo* L.) embryonic axes. J. Crop Sci. Biotech. 15: 311–318.

Ali, M. and Gupta, S. (2012) Carrying capacity of Indian agriculture: Pulse crops. Cur. Sci. 102: 874-881

Amutha, S., Ganapathi, A., Muruganantham, M. (2004) *In vitro* organogenesis and plant formation in *Vigna radiata* (L.) Wilczek Plant Cell Tiss and Organ Cult. 72(2): 203-207

Cheng, T, Y., Saka, H., Voqui-Dinh, T,M., (1980) Plant regeneration from cotyledonary node segments in culture. Plant Sci.Lett. 19: 91–99

Duncan, D, R. and Widholm, J, M,. (1994) Approaches for the Development of cold tolerance in Maize through regenerable callus cultures. Biotechnol. in Agriculture and Forestry, 25: 331-343

Eapen, S. and George, L. (1990) Ontogeny of somatic embryos of *Vigna aconitifolia*, *Vigna mungo* and *Vigna radiata*. *Annals of Botany* **66**: 219-226.

Gamborg, O,L., Miller, R.A., Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158

Gill, R, Eapen, S., Rao, P.S. (1988) Transformation of the grain legume*Vigna aconitifolia* Jacq Marechal by *Agrobacterium tumefaciens*, regeneration of shoots Proceedings: Plant Sciences 98.6: 495-500

Gupta, S. K., Bansal, R., Gopalakrishna, T. (2014) Development and characterization of genic SSR markers for mungbean (*Vigna radiata* (L.) Wilczek) Euphytica, 195 (2): 245-258

Hammat, N., Ghosh, T.K., Davey, M.R. (1986) Regeneration in legumes. In: Vasil IK (ed) Cell Culture and Somatic Cell Genetics of Plants, Vol. 3 (pp. 66–95). Academic Press, New York

Kim, J., Hack, E., LaMotte, C. E.(1994) Synergistic effects of proline and inorganic micronutrients and effects of individual micronutrients on soybean (*Glycine max*) shoot regeneration *in vitro J. Plant Physiol. 144:726-736*

Malik, K.A. and Saxena, P.K. (1992) Regeneration in *Phaseolus vulgaris* L. high frequency induction of direct shoot formation in intact seedlings by N6-benzylaminopurine and thidiazuron. Planta 186: 731–740

Mathews, H. (1987) Morphogenic response from in vitro cultured seedling explants of mung bean (*Vigna radiata* L.Wilczek). Plant Cell Tissue Organ Cult. 11: 233–240

Mundhara, R. and Rashid, A. (2006) Recalcitrant grain legume *Vigna radiata*, mung bean, made to regenerate on change of hormonal and cultural conditions. Plant Cell Tissue Organ Cult. 85: 265-270

Murashige, T. and Skoog, F. (1962) A revised method for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant15: 473-497

O'Neil, C.M., Arthur. A. E., Mathias. R. J., (1996) The effects of proline, thioproline and methyl glyoxal bis (Guanylhydrazone) on shoot regeneration frequencies from stem explants of *B. napus* Plant cell Rep 15: 695-698

Pandey, R. and Ganapathy, P. S. (1985) The proline enigma: NaCl-tolerant and NaCl-sensitive callus lines of *Cicer arietinum* Plant Science 40(1): 13-17

Sen, J. and Guha-Mukherjee, S. (1998) *In vitro* induction of multiple shoots and plant regeneration in *Vigna*. In Vitro Cell Dev. Biol. Plant 34: 276–280

StatSoft Inc., 1995. Statistica for Windows (Computer Program Manual). Statsoft Inc., Tulsa, OK.

Tivareker, S. and Eapen, S. (2001) High frequency plant regeneration from immature cotyledons of mung bean. Plant Cell Tiss. Org. Cult. 66: 227–230

Yadav, Y., Sreenu, K., Maheswari, P., Vanaja, M., Venkateshwarlu M. (2010) Efficient shoot regeneration from double cotyledonary node explants of green gram (*Vigna radiata* (L.) Wilczek) Indian Journal of Biotechnology 9:403-407.

Yip, W.K. and Yang, S.F. (1986) Effect of thidiazuron, a cytokinin–active urea derivative, in cytokinin-dependent ethylene production system. Plant Physiol. 80: 515–519