# Standardization of Callus Induction and Plant Regeneration from Leaf Explants of Black Gram (*Vigna mungo* var. *silvestris)*

**Priya Srivastava, Anjana Pandey***

Nanotechnology and Molecular Biology Laboratory, Centre for Biotechnology,University of Allahabad, Allahabad, 211002, U.P, India.

**Abstract**

The present study optimized the regeneration protocol by using leaf explant in *Vigna mungo* (L) *silvestris* organogenesis. Primary immature leaf segment were inoculated on MS medium supplemented with 2, 4-dichlorophenoxyacetic acid (0.5 µM to 72.5 µM), naphthaleneacetic acid (0.5 µM to 72.0 µM). Callus initiation was observed in all media evaluated and the highest cell proliferation was obtained from explants cultivated in the presence of 2, 4-dichlorophenoxyacetic acid. Shoot induction was obtained from callus induced on 6.0 µM 2, 4-D at 6 weeks after transferring the callus and then regenerated plants were hardened and acclimated in greenhouse conditions. A medium supplemented with 6.0 µM 2, 4-D, 3.0% sucrose, was effective to achieve a high frequency of callus induction, maturation, and further development.

**Key Words***: Vigna mungo,* callus induction, plant regeneration, 2, 4-D, naphthalene acetic acid.

## INTRODUCTION

Black gram (*Vigna mungo*) is a tropical leguminous plant which belongs to genus *Vigna* and under subgenus Ceratotropis. Subgenera Ceratotropis includes 8 cultigens which have been used for their seed pod or forage production in Asian countries, these are- black gram (*Vigna mungo* (L.), mung bean (*Vigna radiata* (L.), azuki bean (*Vigna angularis* (Willd) etc. Black gram forms one of the important constituents in the dietary practices of the local communities. It is highly prized pulse, very rich in phosphoric acid and its genetic diversity is found in India **(**1). Natural distribution of *V. mungo* var. *silvestris* ranges from India to Myanmar **(**2). It is believed that black gram was domesticated in Northern South Asia from *V. mungo* var. *silvestris* that commonly grows there (3**).** It is a rich protein food which contains about 26 percent protein, almost three times that of cereals.

***Corresponding Author**
Centre for Biotechnology
University of Allahabad
Allahabad, 211002
U.P., India
Email: apandey70@rediffmail.com

Legumes are one of the most important groups of the crop plants and have been the subject of effort to improve desirable traits including their in vitro culture response. Since legumes are notoriously recalcitrant to regenerate from tissue culture, much effort has been devoted for developing and optimizing efficient in vitro regeneration system to facilitate a variety of technologies. The ability to regenerate plants from cultured cells, tissues or organs constitute the basis of producing transgenic crop. The crop improvement was done by breeding methods in early days. However, breeding is difficult due to the fact that *Vigna mungo* is self-pollinating crop and the genetic variation among the Black gram varieties is narrow. The regeneration system used to generate genetically modified plants from cotyledonary nodes (4). Callus regeneration is advantageous over direct regeneration for transformation since effective selection of transgenic cells can be achieved. The present study was to standardize the optimum protocol for callus induction and plant regeneration from leaf explant of the *Vigna mungo*.

## MATERIALS AND METHODS

### Plant materials

Seeds of black gram [*Vigna mungo silvestris* (L.)], were obtained from Indian Institute of Pulse Researches (IIPR) Kanpur, U.P. India. Matured *Vigna mungo* seeds were utilized in the efficient production of in vitro *Vigna mungo* plant production experiments. Seeds were washed under continuous flashing of running tap water for 30 min and then treated with a solution of the Tween 20 (5% v/v) for 10 min and finally surface sterilized with $HgCl_2$ (0.1% w/v) for 10 min. Lastly, the seeds were washed three times with autoclaved distilled water and were germinated on solid MS medium (5**)** containing 3.0% sucrose (w/v) and 0.8% agar (w/v) (Hi-media Co., Mumbai, India) at 25° C to 28° C in the dark for the first 2 days and then transferred to a 16 h photoperiod of cool- white fluorescent light (120 µ mol $m^{-2}$ $s^{-1}$).

### Callus induction and maintenance

Primary leaves were excised from 7 days old seedlings, cut into 0.3–0.5 $cm^2$ segments and cultured on 10 ml MS medium with 3% sucrose, 0.8% agar, and different concentrations of 2,4-dichlorophenoxyacetic acid in thrice set-up for callus induction. The cultures were kept in dark condition for 24 h and then incubated at 25° C to 28° C under a 16 h light/ 8 h dark photoperiod with a light intensity of 120 µ mol $m^{-2}$ $s^{-1}$. The callusing was started after 12 days of inoculation and the pattern of the growth of callus was observed by measuring the diameter and growth percentage of the callus after every 7 days intervals by selective callus. This experiment was conducted three times in multiples of five of each 2, 4-D concentration containing tubes. Callus growth and nature of calli produced in each concentration is mentioned in Table 1.

### Culture media and conditions

All the culture media that of MS with 3% (w/v) sucrose used in the investigation were adjusted to pH 5.8 before autoclaving at 15 psi pressure 121° C for 15 min. To prepare semisolid media, agar (Sigma, St. Louis, MO) at 0.8 % was added before autoclaving. All the cultures were maintained under continuous white light (fluence density of 120 µ mol $m^{-2}$ $s^{-1}$) at 25° C.

### Statistical analysis

All the experiments were repeated three times and data on growth percentage of embryogenic calli and diameter of calli produced (mm) were statistically analyzed by set up in a completely randomized design (Table 2 and Figure 1). The effect of different concentration of 2,4-D was quantified and the level of significance was determined by analysis of variance F- value at the 5%.

## RESULT AND DISCUSSION

### Callus induction

Primary leaf explants from seven days old seedlings produced greenish white friable calli on 2, 4-D containing medium within 10–12 days of culture. The maximum proliferation and nature of calli was obtained on 6.0 µM 2, 4-D, while minimal response was noted at 0.5 µM (Table 1).

**Table 1. Response of Leaf Explant with respect to callus Induction and Nature of the callus on 2,4-D containing MS medium**

| Concentration of 2,4D (µM) | Callus induction (%) | Callus nature |
|---|---|---|
| 0.00 | – | – |
| 0.5 | + | WGF |
| 1.5 | + + | GF |
| 3.0 | + + + + | GYF |
| 6.0 | + + + + + | WGF |
| 8.0 | + + + + | YGF |
| 12.0 | + + + + | YGF |
| 36.6 | + + + | GF |
| 54.2 | + + | GF |
| 72.3 | + + | WGF |

WGF- whitish green friable; GF-green friable; GYF-greenish yellow friable; WGF- whitish green friable; YGF- yellowish green friable; ;YGF- yellowish green friable; GF-green friable; GF- green friable; WGF-whitish green friable

### Cell suspension culture

Two weeks old leaf derived greenish white friable calli were sub cultured in liquid MS medium containing different concentrations of 2, 4-D. After 10-15 days of culture on MS medium supplemented with 6.0 µM 2, 4-D, cell division and proliferation was observed. The cultures became

**Table 2. Growth pattern of calli of black gram on the basis of diameter (mm) after 25 days of inoculation**

| Concen-tration of 2,4D (µM) | Size of explant (mm) | I* (mm) | I* (mm) | I* (mm) | Mean |
|---|---|---|---|---|---|
| 0.00 | 0.5 | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.5 | 0.5 | 1.6 | 1.2 | 1.0 | 1.10 |
| 1.5 | 0.5 | 1.2 | 0.6 | 0.5 | 0.77 |
| 3.0 | 0.5 | 2.4 | 2.5 | 2.2 | 2.37 |
| 6.0 | 0.5 | 2.1 | 2.7 | 2.5 | 2.43 |
| 8.0 | 0.5 | 2.5 | 2.3 | 2.5 | 2.43 |
| 12.0 | 0.5 | 0.7 | 2.1 | 2.4 | 1.73 |
| 36.0 | 0.5 | 2.2 | 1.1 | 2.0 | 1.73 |
| 54.0 | 0.5 | 2.4 | 2.3 | 2.3 | 2.33 |
| 72.0 | 0.5 | 1.1 | 0.6 | 1.3 | 1.00 |

*- mean of five replicates on same hormonal concentration

thick, mucilaginous, and brown in color after culture for 12 days in the same medium; therefore, it was necessary to transfer the cells to fresh medium at weekly intervals. Two weeks after initiation of suspension culture, cells differentiated to form mature calli. Microscopic observation of suspension cultures showed that initial spherical cells were embryogenic, containing visible dense cytoplasm, These spherical cells were embryogenic and divided transversely resulting into two, four, and subsequently to a group of cells, that was considered to be the proliferating callus. These were transferred to fresh liquid medium containing 3% sucrose for differentiation of the callus into different shoot buds.

**Media optimization**

The effect of different concentrations of 2, 4-D (0.5–72.0 µM) in liquid MS medium was assessed on induction of shoot bud regeneration. It was observed that the frequency of calli forming increased with an increase in the concentration of 2, 4-D from 0.5 to 6.0µM (Table 2 and Figure 1). Further increase in 2, 4D concentration resulted in a decrease in calli production and recallusing. Observations based on growth percentage and size of calli was collected. Mean percent growth increased 86.67 times at concentration of 6.0 µM of 2,4D, whereas a 13.33% decrease was noted at 0.5 µM of 2, 4-D. It is observed that the highest growth percentage of calli was produced in MS media supplemented with 6.0 µM 2,4-D as shown in Table 1 and Figure 1. These results were analyzed statistically by using completely randomized block design. F-value was found to be 6.273 which indicates significance at the tabulated value (5%) of F with a Critical Difference (5%) of 25.828 ($F_{6.27}$>5%) from the ANOVA table analysis. The influence of different concentration of 2,4-D depending upon the size of calli produced was also studied (Table 2) statistically by using a completely randomized block design test and it was found to be significant with F-value of 6.457 (calculated) at critical difference (5%) of 0.779.

The use of the synthetic auxin 2, 4-dichlorophenoxyacetic acid (2, 4-D) for the induction of somatic embryos (embryoids) on cultured explants can be traced to the work of Halperin and Wetherell (6) who showed that a callus produced any vegetative part of carrot (*Daucus carota*) such as the root, petiole, or inflorescence stalk reared in a medium containing a high concentration of 2,4-D formed somatic embryos upon transfer to a medium with a reduced level of the auxin. It is observed that

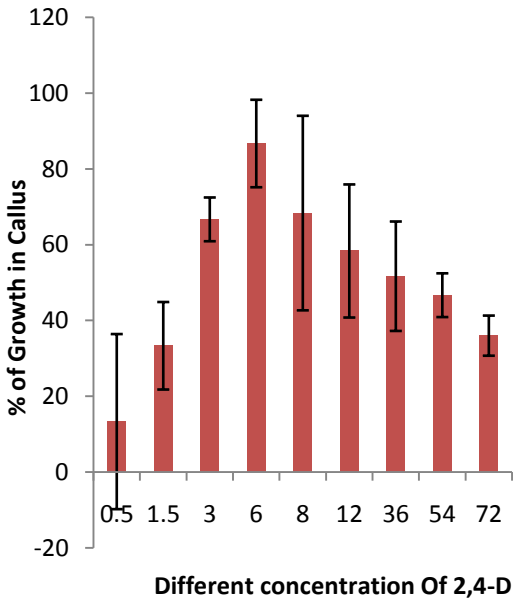


**Figure 1 Effect of different concentration of 2,4D on the % of growth in callus obtained from explants of black gram. The experiments were repeated three times.**


**Photo 1 Different form of calli of leaf explants of black gram and regeneration of plantlets (MS+2,4-D). (A,B,,E,F,G, and H- regenerative calli of explant and shoot buds and root buds from calli, H,I,J and K –regenerated plantlet and finally developed a plant (MS+ 6.0 µM 2,4-D), C and D- no induction of calli in presence of MS+NAA).**

using 2,4-D as the sole hormone, calli were initially cultured in a liquid medium containing 6.0 µM 2,4-D for 21 days to induce the formation of proliferating calli producing shoot buds followed by their transfer to an auxin containing medium for plantlet formation and regeneration of plant as shown in Figure 1. This work also showed that it was possible to obtain cell lines with continued embryogenic potential if calli derived from leaf

part were maintained on a solid medium with an increased concentration of the auxin.

A very rapid and efficient regeneration method of *Vigna mungo* L. has been established using liquid culture (7) by using a leaf explant in liquid culture medium. Christou (8) could effectively select transgenic calli after bombarding protoplasts but they failed to produce transgenic plants. The callus induction and plant regeneration of Indian soybean (*Glycine max* L.) via half seed explant culture was also carried out (9). A protocol for callus induction and plant regeneration was established by using half seed explant on $B_5$ media of *Vigna mungo* (L.) Hepper (10). A protocol of micro propagation from shoot mertistems of Turkish cow pea (*Vigna unguiculata* L.) was established by using MS media supplemented with BAP, and NAA by Muhammad. Aasim (11). In vitro techniques such as micro propagation have proved a useful tool for propagation of number of food legume crops (12, 13). Reports regarding in vitro regeneration of cowpea by tissue culture describe using primary leaves (14, 15, and 16), cotyledonary node (17, 18), mature cotyledon (19), embryonic axis (20), and mature embryo (21). However, very few reports (22, 23) describe the use of shoot mertistems/apices/tip as explant of choice.

In, present work, leaf segments were found to produce shoot and plantlet. The acquisition of embryogenic potential under auxin stimulus in such explants is manifested through a callus phase among different auxin tested. 2,4-D at 6.0 µM was most effective for inducing shoot producing cells in a liquid medium. NAA failed to induce calli and shoot buds (Photo 1), indicating that leaf segments have different sensitivity to various auxins and their concentration. Full-strength MS medium was found to be more effective than the other media used for induction and growth of calli. This may be due to the presence of a high level of nitrogen, particularly the reduced form ($NH_4PO_4$), in MS medium. In present investigation, a protocol for shoot buds and plantlet regeneration was established successfully which found to be reproducible and developed by using different concentration of 2,4-D in increasing order from 0.5 – 72.0 µM concentration in which the best proliferation and shoot buds formation was observed at 6.0 µM significantly (Figure 1) In conclusion, using plant growth regulators, the efficient callus mediated regeneration from leaf explant of black gram has been standardized. The leaves originated callus could serve as an ideal starting material for developing an efficient black gram transformation system. It was possible to produce shooting buds within three months and to regenerate plant from mature calluses in 3- 4 month. Such protocols have a great potential for improvement of this crop by biotechnological approaches such as in vitro selection, clonal propagation, genetic transformation, and production of transgenic plants.

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