DEVELOPMENT OF *IN VITRO* PLANT REGENERATION SYSTEM AND *AGROBACTERIUM* –MEDIATED GENETIC TRANSFORMATION IN MUNGBEAN (*VIGNA RADIATA* (L.)WILCZECK)

M. Phil Thesis

by

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DEVELOPMENT OF *IN VITRO* PLANT REGENERATION SYSTEM AND *AGROBACTERIUM* –MEDIATED GENETIC TRANSFORMATION IN MUNGBEAN(*VIGNA RADIATA* (L.)WILCZECK)

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHILOSOPHY IN BOTANY

BY

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Dedicated
To My
Parents and Teachers

Certificate

This is to certify that the research work embodying the results reported in this thesis entitled "**Development of *in vitro* plant regeneration system and *Agrobacterium*-mediated genetic transformation in mungbean (*Vigna radiata* (L.) Wilczeck)"** submitted by **Setara Begum**, has been carried out under our supervision in the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka. It is further certified that the research work presented here is original and suitable for submission for the degree of Master of Philosophy in Botany, University of Dhaka.

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ABSTRACT

An efficient protocol has been developed for *Agrobacterium*-mediated genetic transformation of two important mungbean (*Vigna radiata (*L.) Wilczeck) varieties cultivated in Bangladesh, namely, Binamoog-5 and BARI mung-6. Prior to transformation an efficient *in vitro* regeneration protocol was developed for these two mungbean varieties. Cotyledonary node, shoot tip and cotyledone attached decapitated embryo were used as explants for direct regeneration of shoot. MS medium with various combinations and concentrations of BAP, Kn and NAA were used for direct regeneration of shoots. MS medium supplemented with 10.0 μM BAP and 0.5 μM NAA was found to be the best towards the development of highest number of multiple shoots. Full strength of MS medium supplemented with 2.0 μM IBA was found to be the most effective for healthy root induction from the regenerated excised shoots. Following root induction the *in vitro* regenerated plantlets were successfully transplanted to soil. Transformation experiments with cotyledone attached decapitated embryo, cotyledonary node and decapitated embryo explants were conducted using *Agrobacterium tumefaciens* strain, namely, LBA4404 harboring the binary plasmid pBI121 conferring GUS (β-glucuronidase) and *nptII* (neomycin phosphotransferase II) genes. Transformation experiments were monitored through GUS histochemical assay since the strain of *Agrobacterium* contained GUS gene. Different factors influencing successful transformation such as, density of bacterial suspension, incubation and co-cultivation periods were optimized. Maximum responses towards transformation were obtained with bacterial suspension having an optical density of 0.56 at 600 nm. Moreover, 30 minutes of incubation period followed by 72 hours of co-cultivation period were found to be the most effective towards transformation as determined by transient GUS assay. Selection of transformed shoots was carried out using different concentrations of kanamycin as the *Agrobacterium* strain contained *nptII* gene. Transformed shoots were selected using 150 mg/l kanamycin. The survival of green shoots on the optimum selection medium indicated the production of transformed shoots. Stable expression of the GUS gene was detected in various parts of transformed plantlets. Putatively transformed shoots were rooted on full strength of MS medium containing 2.0 μM IBA and 100 mg/l ticrecilin. Rooted transformed plantlets were successfully transferred to soil. Genomic DNA was isolated from the putatively transformed mungbean shoots and stable integration of GUS and *nptII* genes was confirmed by polymerase chain reaction (PCR) analysis.

1. Introduction

Grain legumes are commonly known as pulses or grain legumes. The seeds of grain legumes are considered beneficial to human and animal health and these have been serving to men and animals from the pre-historic time. Grain legumes are ranked third after cereals and oilseeds in the world production and constitute a vital dietary constituent for humans and animals. Grain legumes are considered as one of the most essential crop groups in the agricultural world. They are cultivated throughout the tropical, sub-tropical and temperate zones of the world. It may be mentioned here that grain legumes are a source of dietary protein in many developing countries where malnutrition is associated with the acute shortage of animal protein production (Bressani 1973). The world demand for grain legumes is gradually rising because of the increasing population in developing countries. In Bangladesh, grain legumes have gained much importance in view of the wide prevalence of protein malnutrition and thus, the grain legumes are considered as the "meat of the poor".

The dry seeds of legumes are treated as one of the most important source of dietary protein for human, because these grains are capable to fulfill the demand of protein for the common people, who do not afford animal protein for its high price. Proteins of grain legumes are generally higher in lysine, but lower in methionine and cysteine. In addition, pulses are good source of vitamin B-complex like thiamine, niacin and folate (Gowda and Kaul 1982), minerals like Iron 14%, calcium 2%, and also contain carbohydrate in the form of starch with caloric yield comparable to cereals (Aykroyd and Doughty 1966). Moreover, pulses combined with cereals contribute hugely in making balanced diet of energy and protein. Furthermore, grain legumes have the ability to fix nitrogen and thus improve soil fertility (Bajaj and Gosal 1981). Soil bacteria of the genus *Rhizobium*, accomplish the conversion of atmospheric nitrogen to ammonia. Unlike cereal, pulses deposit significant amounts of organic matter to the soil, enriching it with much needed carbon. Thus, pulses serve in biological soil conservation. These contributions make these crops indispensable for the natural conservation of Bangladesh where soil is losing fertility at a rapid rate because of increased cropping with the inadequate inputs and the removal of most of the biomass from the field.

In Asia, at least ten different species of grain legumes are presently grown. In Bangladesh a number of pulses are grown, which includes lentil (*Lens culinaris* Medik), chickpea (*Cicer arietinum* L.), blackgram (*Vigna mungo* (L.) Hepper), mungbean (*Vigna radiata* L. Wilczek), grasspea (*Lathyrus sativus* L.), field pea (*Pisum* spp.) and cowpea (*Vigna unguiculata* L. Walp), etc. However, pulses occupy less than 5% of the total cultivated land and have been reported to contribute about 2% of the total food grain production in Bangladesh (Gowda and Kaul 1982). These crops play a significant role in the Bangladesh agricultural system with an increasing annual demand for human consumption.

Among the different pulses grown in Bangadesh, Mungbean (*Vigna radiata* (L.) Wilczek) is widely cultivated because it has protein rich edible seeds for the human consumption and this grain is regarded as a quality pulse for its high contents of carbohydrate (56.7%), protein (24.5%), fat (1.2%), minerals (3.5%) vitamin, iron (3 µg/20g) and lysine (504 mg/g). Mungbean is a rich source of amino acids in which sulpher containing amino acids like methionine, cysteine. Further, mungbean can be used as a dehulled grain in hulled, curries, salads, noodles, bread, sweets and many other culinary products. Sprouted mungbean is highly vitamin-C rich vegetable and 12 (twelve) times available iron is found in sprouted mungbean than normal mungbean. On the other hand, four times iron is found when mugbean is cooked with tomato. Besides, sprouted mungbean seeds provide a succulent and nutritious vegetable, rich in protein, minerals and vitamins and available in all seasons of the year (Poehlman 1991).

In Bangladesh, mungbean is traditionally cultivated in the winter (late October-early March) season. But due to its heat tolerance, it may be cultivated either as a summer or early kharif crop. However, yield is unstable both over locations and seasons due to the susceptibility of mungbean cultivars to the environmental stresses, diseases and insect pests. As a result, mungbeans are often grown in marginal lands with minimal inputs (Shanmugasundaram and Kim, 1996). Because of its short life span (2–3 months), mungbean is considered to be a suitable crop for rotation with cereal crops in Bangladesh as well as in Tropical Asia. Although much of the mungbean production in the field is as a sole crop, its cultivation is often mixed or intercropped with other cereals and legume crops.

The average yield of mungbean in South Asia is only around 0.4 t/ha. In local context, mungbean is cultivated in almost every district of Bangladesh. The total area under mungbean cultivation in Bangladesh in the year 2007-2008 was 59717 acres with an average production of the same period was 20628 metric tons and yield was 345 kg/acre. Barisal and Patuakhali districts are considered as the dominant mungbean growing areas of Bangladesh (BBS 2008). Where as in the year 2011-12 the total area of mungbean cultivation was 90825 acres and the production was 26240 metric tons. In this year Rajshahi district is the foremost mungbean growing area in Bangladesh (BBS- 2012).
Allthough large areas of Bangladesh are used for cultivating mungbean, the production of mungbean has not been increased substantially. On the other hand, very limited efforts have been made for the increasing the yield of mungbean in our country, although a number of varieties are available. Bangladesh Agricultural Research Institute (BARI) has released six varieties of mungbean, namely, BARI mung 1, 2, 3, 4, 5 & 6; Bangladesh Institute of Nuclear Agriculture (BINA) has also released eight varieties of mungbean, named- BINA mung 1, 2, 3, 4, 5, 6, 7 & 8 and BSMRAU has also released four varieties of mungbean named- BU mung 1,2,3 & 6 (Source: Seed Wing, Ministry of Agriculture (MOA). All the varieties mentioned above have been cultivated in different regions of Bangladesh.

The demand for mungbean as stated above has been steadily increasing in the Indian subcontinent and like other countries in South Asia, this crop has been playing an important role in fulfilling the protein requirements of population of Bangladesh. In Bangladesh, among the pulses, mungbean is ranked third in acreage production and contributes to 10-12% of total pulse production, but first in market price. Mungbean is considered as a profitable farming venture and a good source of livelihood since it does not normally require additional expenditure for its cultivation. In addition, due to high consumer's demand reportedly, Bangladesh imports a large amount of mungbean every year from neighboring countries.

The production of mungbean in Bangladesh was calculated for the last few years and it was observed that mungbean production has not been increased substantially. The average yield of mungbean is quite low in our country as compared to the average yield of other pulse growing countries in the world. The main reason for low yield of this crop is the susceptibility to various diseases including yellow mosaic, *Cercospora* leaf spot

disease, powdery mildew, root rot etc. as well as the susceptibility to insect pests, such as beanfly, aphids, pod borers, stink bugs, bruchids, etc. A total of sixteen diseases of mungbean have been recorded in Uttar Pradesh, India of which viral diseases are the most damaging to the crops (Nene 1972).

In some years, losses attained more than 50% due to incidence of above diseases. Viral disease causes major yield loss. Among the viral diseases of mungbean, yellow mosaic disease caused by Mungbean yellow mosaic virus *(*MYMV) is the most damaging one (Nariani 1960). Several factors are supposed to be responsible for the lower production of this important crop, which includes: (i) low genetic variability in native varieties, (ii) yield fluctuates due to drought and floods, (iii) losses from diseases and insect pests, (iv) photoperiod sensitivity, (v) lack of synchronous podding, (vi) poor cultural practices. Due to low yield potentials, most of the farmers of our country are giving less priority to mungbean cultivation and they allocate marginal lands for this purpose.

For the improvement of mungbean, it is essential task to improve this crop to obtain desired agronomic performance such as high productivity, enhanced nutritional value and other essential agronomic quality of this crop. Existence of narrow genetic base in natural races, however, limits the improvement of this crop species for desired characters. The use of desirable genes from its related wild and cultivated species has, therefore, been considered important in the genetic improvement of mungbean and other tropical pulses (Chavan *et al*. 1966, Verma and Singh 1986, Bhadra *et al*. 1989).

In the past, several attempts have been undertaken to develop high yielding variety of mungbean through inter-specific hybridization and mutation breeding. But classical breeding has met with limited success due to non availability of desirable disease resistant genes in the germplasm (Mirza and Tanzeen 2004). Moreover, low genetic variability of mungbean caused by high degree of self-pollination has imposed limitation for its improvement using conventional methods of breeding. Besides, conventional breeding programs are laborious and time consuming, extending over seven to eight years involving crossing and selection to desired traits.

Therefore, it is urgently needed to find out some other options to induce genetic variability in mungbean. Beside the sexual breeding techniques, there are many ways of creating variability including induction of somaclonal variation through tissue culture,

somatic hybridization and genetic engineering. According to Scowcroft *et al.* (1987), tissue culture technique can play a significant role for enrichment of genetic variability by creating variation (somaclonal variation). But these were found to have a limited application in many crop species.

Under these circumstances, it has been imperative to undertake a research program to use modern biotechnological methods for increased production of mungbean. This is the vital reason to utilize effectively genetic engineering technique to develop desirable breeding lines of mungbean. Genetic transformation, a comparatively new and exciting tissue culture based technology, has opened a new way for using recombinant DNA technology and this technology is also useful in complementing the conventional breeding programs. Moreover, this approach may accelerate the development of new plant varieties which is not possible through conventional breeding and tissue culture alone (Gardner 1993).

Gene transfer is a process of introducing defined genetic information from any living organism (related or unrelated plant or animal species) into a new host nuclear genome. This technology is regarded as a pre-breeding method that can provide a solution to certain constraints that limit crop production or quality. Thus, by incorporating specific gene/s with desired character/s can widen the genetic base of a crop. Such a crop is known as genetically modified (GM) or transgenic. Since 1987, numerous potentially useful transgenic plants of cotton, maize, potato, tobacco, rapeseed, raspberry, soybean, pea, tomato, rice etc. have been generated (Fisk and Dandekar 1993).

As genetic transformation is a tissue culture based technology, a reproducible transformation system could enable us to develop transgenic mungbean lines by inserting gene/s of interests which are unavailable in present genotypes. Thus, it has a high potency that genetic transformation method combined with tissue culture and traditional breeding techniques could possibly aid in improving both the quality and yield of mungbean. Further, for the successful crop improvement programs through genetic engineering, efficient plant regeneration system is inevitably necessary.

Several different methods are involved in transformation of higher plants (Gasser and Fraley 1989). For example, direct methods, such as particle bombardment, microinjection, electroporation, chemical methods and indirect method such as *Agrobacterium*-mediated method have been used for transformation. In dicotyledonous

species, the most efficient method used for the transfer of desired foreign gene/s is through the soil bacterium *Agrobacterium tumefaciens* (Grant *et al.* 1991 and Zambryski 1992). Currently, *Agrobacterium*-mediated plant transformation techniques are extensively utilized to generate transgenic plant.

Agrobacterium tumefaciens is the causal agent of crown gall disease (the formation of tumours) in over 140 species of dicot. It is a rod shaped, gram-negative soil bacterium (Smith *et al.* 1907). Symptoms are caused by the insertion of a small segment of DNA (known as the T-DNA or 'transfer DNA') into the plant cell, which is incorporated at a semi-random location into the plant genome. They provide the natural gene transfer, gene expression and selection systems that have been treated as nature's most effective plant genetic engineer. It has the capability to form tumorous growth - the gall at the wound site of dicotyledonous plants. The infection and tumor induction capability is due to the presence of a large Ti (Tumor inducing) plasmid in the virulent strain of *A. tumefactions.* A part of this Ti plasmid is T-DNA (Transfer DNA) that is transferred to the plant nuclear genome with the help of *vir* genes (virulence genes) situated outside the T-DNA region of Ti plasmid. In transformation experiments, specific reporter and selectable marker genes along with the genes of interest are integrated into the T-DNA. These marker genes can be recognized in plant tissues with the help of selectable agents, confirming the transformation of plant cells. However, relatively small number of individual cells incorporates the T-DNA into their chromosomes and thus becomes transgenic.

Therefore, to develop successful transformants through transformation experiments, it is essential to regenerate plants from these transgenic cells by suitable *in vitro* culture method (Gardner 1993). Thus, a well-established, reliable genotype independent regeneration protocol is essential for transformation experiments.

Although there are some reports on the development of *in vitro* plant regeneration in *Vigna radiata,* these research works did not provide sufficient information towards development of reproducible protocols for *in vitro* plant regeneration via callus (Bhadra *et al*. 1989; Bose 1991 and Begum 1994). Therefore, further improvement of this crop with superior yielding ability has not yet been achieved through *in vitro* techniques. Thus, modern techniques of biotechnology, commonly known as "genetic transformation", may be suitable and effective to apply for the improvement of this crop.

In grain legumes, the production of transgenic plants using *Agrobacterium tumefaciens* as a gene vector is limited. Nevertheless, there are some reports on transgenic plant production from leguminous crops, namely, *Glycine max* (L.) Merrill (Hinchee *et al*. 1988), *Pisum sativum* L. (Zhang *et al.* 1999, De Kathen and Jacobsen 1990, Pounti-Kaerlas *et al.* 1990, Schroeder *et al*.1993, Davies *et al.* 1993), *Cicer arietinum* L. (Fontana *et al.* 1993, Kar *et al.* 1996, Sarker and Biswas 2002); *Lens culinaris* Medik. (Sarker *et al*. 2003, Hassan *et al.* 2004); *Arachis hypogaea* L. (Sarker *et al*. 2000, Sarker and Nahar 2003) but very little has been reported regarding genetic transformation of mungbean (Jaiwal *et al.* 2001, Mahalakshmi *et al.* 2006). Available literature indicated that in Bangladesh no work on genetic transformation in mungbean has been carried out. Therefore, a strategy could be adopted through development of a transgenic plant through Agrobacterium-mediated genetic transformation. It is also necessary to apply molecular techniques in characterizing different promising varieties of mungbean to identify the variability among them.

Under these circumstances, the present set of experiments were conducted to carried out for the development of a reproducibale protocol for in vitro plant regeneration from different explants of mungbena as well as development of *Agrobacterium*-mediated genetic transformation in two cultivated variety of mungbean using marker genes such as GUS and *nptII* genes. The overall objectives of the present investigation were as follows:

(i) Establishment of an efficient, reliable *in vitro* regeneration protocol for two varieties of mungbean, namely, Binamoog-5 and BARI mung-6.

(ii) Development of *Agrobacterium*- mediated genetic transformation protocol for Binamoog-5 and BARI mung-6 varieties of mungbean using marker genes (GUS and nptII).

(iii) Confirmation of integration of foreign genes through proper molecular analysis like PCR.

(iv) Establishment of the transformed plants in soil.

2. MATERIALS

2.1 Plant materials

Following two local varieties of mungbean (*Vigna radiata* (L.) Wilczek) were used in the present investigation :

(i) Binamoog-5 (Fig. 1)

(ii) BARI mung-6 (Fig. 2)

Figs. 1&2. Seeds of Binamoog-5 and BARI mung-6 varieties respectively.

2.1.1 Source of the plant materials

Seeds of the two varieties of mungbean, namely, Binamoog-5 and BARI mung-6 were collected from Bangladesh Institute of Nuclear Agriculture (BINA) and Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur respectively. Seeds of the above mentioned materials were maintained in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka.

2.1.2 Explants

During the present study following three different types of explants, namely, cotyledon attached decapitated embryo (CADE), cotyledon attached node (CN) and shoot tip (ST) were used for multiple shoot regeneration .
CADE explants were collected from the surface sterilized overnight water soaked seeds. Cotyledon attached node and shoot tips were collected from 8 -10 days-old *in vitro* grown seedlings of the above mentioned varieties.

2.2 *Agrobacterium* strain and vector plasmids

Genetically engineered *Agrobacterium tumefaciens* strain LBA4404 was used for infection in transformation experiments. The functional map of the *Agrobacterium* strains has been presented in Fig 3. The relevant characteristics of the used strains are mentioned below:

Agrobacterium tumefaciens strain LBA4404 contains plasmid pBI121 of 14 Kbp (binary vector). This binary vector contains following genes within the right border (RB) and left border (LB) region of the construct:

- The *uid*A gene (Jefferson *et al.* 1987) encoding GUS (β-glucuronidase), driven by CaMV35S promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation.
- The *npt II* gene (Herrera-Estrella *et al*. 1983) encoding neomycin phosphotransferase II conferring kanamycin resistance.
- The bacterium also contains plasmid pAL4404 which is a disarmed Ti plasmid (132 Kbp) containing the virulence genes.

The reporter gene GUS can be used to assess the efficiency of transformation. The gene for GUS has an intron in the coding sequence which can express GUS activity in the plant cell but not in the cells of *Agrobacterium.* In this strain, the transformed tissue as well as transgenic plant can be selected by using kanamycin.

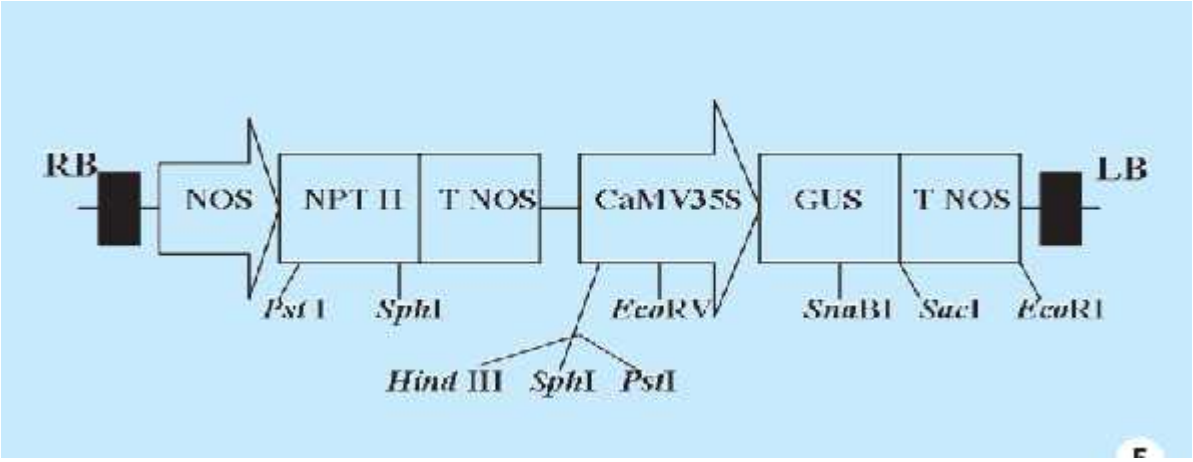

Fig 3. Diagrammatic representation of T-DNA region between left (BL) and right (BR) border of pBI121 from *Agrobacterium tumefaciens* strain LBA4404.

3. METHODS

The research work was carried out in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. The different procedures followed in the present study have been described under the following heads.

3.1 Media used

Different culture media used in the present investigation for various purposes were as follows:

3.1.1 Seed germination medium

Agar solidified 0.8% (w/v) hormone free and hormone supplemented MS (Murashige and Skoog 1962) medium with 3% (w/v) sucrose, water- agar (0.8% w/v) medium without sucrose were used for *in vitro* germination of seeds and further growth of germinated seedlings aseptically.

3.1.2 Media for shoot regeneration and elongation

For shoot initiation and development, MS medium (Murashige and Skoog 1962) supplemented with various combinations and concentrations of growth regulators viz. BAP (6- Benzyl amino purine), Kn (6-furfuryl amino purine/ Kinetin), IAA (Indole-3-acetic acid), NAA (α- Napthalene acetic acid) and GA_3 (Gibberellic acid) were used.

3.1.3 Media for root induction

For induction of roots from the base of the *in vitro* grown shoots, half strength and full strength of MS medium supplemented with various concentrations of auxins, namely, IBA (Indole-3-butyric acid) and IAA (Indole-3 acetic acid) and without auxin were used. Gelrite was used as solidifying agent for the rooting media.

3.1.4 Media for *Agrobacterium* culture

YMB (Yeast extract Mannitol Broth), YEP (Yeast Extract Peptone) and LB (Luria Broth medium) with appropriate antibiotics were used to grow genetically engineered *Agrobacterium tumefaciens*. Here two sorts of media used for each strain of

Agrobacterium, namely, maintenance and working culture medium for transformation experiments.

3.1.5 Co-culture media

For the purpose of co-cultivation, shoot regeneration medium and hormone free MS medium were used as co-culture media.

3.1.6 Selection media

The *Agrobacterium* strains LBA4404 contains *npt* II (Kanamycin resistance) gene. Therefore Kanamycin was used for selection of transformed tissue and plantlets. For this purpose, different concentrations of antibiotic (Kanamycin) gradually added to regeneration medium.

3.2 Preparation of stock solutions

Various stock solutions were required for the preparation of different culture medium. Preparation of various stock solutions has been described under the following heads:

3.2.1 Preparation of stock solutions for MS medium

Different stock solutions were prepared as the first step for the preparation of medium. The stock solutions of various constituents of the medium were prepared for ready use during the preparation of medium. As different constituents were required in different concentrations, separate stock solutions for macro and micro-nutrients, vitamins, plant growth regulators etc. were prepared.

3.2.1.1 Stock solution A (macro nutrients) for MS medium

This stock solution was made in such a way that its strength was 40 times more than the final strength of the medium in 1.0 liter distilled water. For this purpose, 40 times the weight of different salts required for 1.0 liter of medium were weighed accurately and were sequentially dissolved one after another in a 1.0 liter volumetric flask with 600 ml of distilled water. The last two salts are dissolved in 100 ml of distilled water separately and added serially at last. The final volume of the solution was made up to 1.0 liter by further addition of distilled water. The solution was filtered through Whatman No.1 filter paper to remove all the solid contaminants like dust, cotton etc. and was poured into a clean

plastic container. After labeling, the solution was stored in a refrigerator at 4˚C for several weeks.

3.2.1.2 Stock solution B (micro nutrients) for MS medium

For this constituent of the medium two separate stock solutions were prepared:

i) **Stock solution B_1 (all micro-nutrients except iron)**

This part of the stock solution was made with all the micro-nutrients except $FeSO_4.7H_20$ and Na_2-EDTA. This was made 100 times the final strength of necessary components in 500 ml of distilled water as described for the stock solution A. The solution was filtered and stored at 4˚C.

ii) **Stock solution B_2 (iron chelate solution)**

The second solution was made 100 times the final strength of $FeSO_4.7H_20$ and Na_2-EDTA in 500 ml distilled water in a conical flask and heated slowly at high temperature until the salts were dissolved completely. Finally the solution was filtered and stored in a refrigerator at 4˚C for several weeks

3.2.1.3 Stock solution C (organic constituents) for MS medium

It was also made 100 times the final strength of the medium in 500 ml of distilled water as described for stock solution B. This solution was also filtered and stored at 4˚C for future use.

3.2.2 Stock solutions for growth regulators

The growth regulators are naturally synthesized in higher plants. For the growth, differentiation and organogenesis of tissue, the growth regulators (auxins, cytokinins and additives) are important in tissue culture. The following different supplements and growth regulators were used in the present investigation:

I. Auxins

Auxins induce cell division and formation of callus. It causes cell division, cell elongation, swelling of tissues and the formation of adventitious roots. The auxins are:

(i) Indole-3-acetic acid (IAA)

(ii) Indole-3-butyric acid (IBA)

(iii) α- naphthalene acetic acid (NAA)

II. Cytokinins

Cytokinins concerned with cell division and modification of shoot differentiation in tissue culture. It induces the adventitious shoot formation. They have also been shown to activate RNA synthesis and to stimulate protein and enzyme activity in certain tissues. The most frequently used cytokinins are:

(i) 6-benzyl amino purine (BAP)

(ii) 6-furfuryl amino purine (Kinetin/Kn)

The growth regulators, their solvents, and molecular weight are listed below (Sigma Plant Cell Culture Catalogue):

Growth regulators	Solvent	Molecular weight
IAA	1N NaOH	175.2
IBA	1N NaOH	203.2
NAA	1N NaOH	186.2
BAP	1N NaOH	225.3
Kinetin	1N NaOH	215.2

To prepare any one of the previously mentioned hormonal stock solution, 50 mg of the hormone was placed on a clean-plastic weighing boat and dissolved in 1 to 2 ml of appropriate solvent (mentioned above). The mixture was then washed off with distilled water and collected in a 250 ml measuring cylinder. It was then made up to 250 ml with the addition of distilled water. The solution was then filtered and poured into clean plastic container and stored in a refrigerator at 4˚C for several weeks.

3.2.3 Preparation of stock solutions of antibiotics

Three types of antibiotics were used in different bacterial and plant regeneration media in transformation experiments.

a) Kanamycin (Duchefa, Netherlands)
b) Ticarcillin (Duchefa, Netherlands)
c) Combactam (Duchefa, Netherlands)

For the preparation of Kanamycin, ticarcillin, and Combactam stock solutions, 1.0 gm of each antibiotic was separately dissolved in 10 ml of deionized water. After filter sterilization, these solutions are stored in 1.5 ml Eppendorf tubes at -20ºC in the dark as stock.

3.3 Preparation of medium for plants using MS stocks solutions

To prepare one liter of medium the following steps were carried out successively

(i) For the preparation of MS medium, 30 gm (at 3%) of sucrose was dissolved in 750 ml of distilled water in a one liter of volumetric beaker.

(ii) 25 ml of stock solution A, 5 ml of stock solution B and 5 ml of stock solution C were added to this 750 ml distilled water and mixed well.

(iii) When increased amount of macro-salts were used as a constituent of the medium, appropriate amount of solutions from micro-salt stock were added in to the medium.

(iv) Different required amount of hormonal supplements were taken from stock solution of hormone and added to the medium either individually or in combinations and were mixed thoroughly. Since each 5 ml of the hormonal stock solution contained 1.0 mg of the chemical in 5 ml of solution, the addition of 5 ml of any hormonal stock solution will make 1.0 liter of medium resulted in 1.0 mg/l concentrations of that hormonal supplement. Different concentrations of hormonal supplements were prepared by adding required amount of the stock solution to the medium following the similar procedure described earlier.

(v) The whole mixture was then made up to 1.0 liter with further addition of distilled water.

(vi) P^H of the medium was adjusted to 5.8 with a digital pH meter (JENWAY) with the help of 1N NaOH and 1N HCl, whichever was required. Before that, the pH meter was calibrated with two buffer solution having pH 4.0 and 7.0 respectively.

(vii) To solidify medium either 8.0 gm (at 0.8%) of phytoagar (Duchefa Biocheme) or 2.5 gm (at 0.25 %) of Gelrite (Sigma,USA) was added. To dissolve solidifying agent, the whole mixture was heated in a microwave oven (National, Japan).

(viii) To make liquid medium the last step (vii) of media preparation was omitted.

3.4 Preparation of MS medium using powdered MS salts:

Occasionally commercially available dry powdered MS medium (Duchefa Biocheme) containing all the constituents of MS medium (inorganic salts, vitamins and amino acids) except sucrose and agar were used to prepare medium for plant regeneration. Required amount of powdered was dissolved in appropriate volume of distilled water (10% less than the final volume of the medium), and after adding sucrose (3%), the pH was adjusted to 5.8. To prepare solid media, agar (0.8%) was added.

3.5 Preparation of *Agrobacterium* culture medium

Liquid YMB, and YEP were used to grow the different strains of genetically engineered *Agrobacterium tumefaciens* which were used as bacterial suspension for transformation. Solid YEP were used as maintenance media for the strain.

3.5.1 Preparation of YMB (Yeast extract Mannitol Broth) medium

For the growth of *Agrobacterium tumefaciens* strains, YMB medium was prepared in the following manner:

Components	Amount needed for 100 ml medium
Mannitol	1.0 g
Yeast extract	0.04 g
$MgSO_4.H_2O$	0.02 g
NaCl	0.01 g
KH_2PO_4	0.05 g

All of these ingredients of the medium were added in 50 ml distilled water and mixed properly. Then the final volume was made up to 100 ml by adding distilled water. The pH was adjusted at 7.0 - 7.2.

For the preparation of working culture medium (to make bacterial suspension), liquid medium was used. For preparing maintenance medium 1.5 gm agar was added to the medium before autoclaving.

Liquid medium was stored at 4˚C for future use. Filter sterilized antibiotic Kanamycin was added at a rate 50 mg/l to the autoclaved liquid medium prior to bacterial inoculation

and at the same rate to autoclaved and then cooled (down to 50-55˚C) solid medium. The solid medium was then poured in Petri dishes. After solidifying, the dishes were ready for bacterial culture. It was stored at 4˚C for further use.

3.5.2 YEP medium was prepared in the following manner

Components	Amount needed for 100 ml medium
Bactopeptone	1.0 g
Bacto Yeast extract	1.0 g
NaCl	0.5 g

Preparation procedure was same as above media.

3.6 Preparation of co-culture medium:

For co-cultivation (infected mungbean tissue together with *Agrobacterium*) shoot regeneration medium i.e. solidified MS media containing 4.0 µm/l BAP medium or hormone free MS medium both at pH 5.8 were prepared.

3.7 Preparation of selection medium for putative transformed plantlets:

Regeneration medium with appropriate antibiotics was prepared for the selection of putative transformed plantlets. After preparing regeneration medium, it was autoclaved. The medium was cooled down to 50°C and required antibiotics were added at a concentration rate to such medium inside the laminar flow cabinet. Medium was then poured into suitable vials and was allowed to solidify.

3.8 Sterilization of medium:

Fixed volume of the medium was dispensed into culture vessels, (i.e. test tubes, bottles or conical flasks) or 500 ml screw capped bottles. The culture vessels were covered with aluminum foil and marked with the help of a marker to indicate the specific media with hormonal supplements. The culture vessels were then autoclaved (Hirayama, Japan) at 15 lbs/sq. inch pressure at 121°C temperature for 20 mins. In some cases, test tubes with medium were allowed to cool as slants after sterilization.

3.9 Precaution to ensure aseptic condition

All inoculation and aseptic manipulations were carried out in a laminar airflow cabinet (Labcon Co. and Forma Scientific, USA). The cabinet was switched 'on' for at least half an hour before use and cleaned with 70% alcohol to make it free from surface contaminants. The instruments like scalpels, forceps, inoculation loop, Petri dishes and materials like cotton wool, filter papers, Eppendorf tubes etc. were sterilized by steam sterilization methods. During the entire period of work in the cabinet, the scalpels, forceps, and inoculation loop were kept immersed into absolute alcohol containing in a glass jar inside the cabinet. At the time of inoculation and sub-culture these were sterilized by flaming method from time to time in the cabinet. Both the hands were rinsed with 70% alcohol. All measures were taken to obtain maximum contamination free condition during the preparation of explants. After autoclaving bacterial media were poured inside the laminar airflow cabinet to avoid contamination. Antibiotics were filter sterilized inside the laminar flow hood and stored in sterile Eppendrof tubes. Antibiotics were also added to the medium inside the laminar flow cabinet.

After each transformation experiment, used *Agrobacterium* suspension and contaminated Petri dishes, cotton wool, filter papers, instrument, glass cuvettes were autoclaved to destroy genetically engineered *Agrobacterium* as a part of 'biosafety' procedure. Any contaminants and old bacterial cultures were also autoclaved before discarding them.

3.10 Culture techniques:

The following culture techniques were employed in the present investigation:

(i) Axenic culture
(ii) Explant culture (Inoculation)
(iii) Subculture
(iv) Rooting
(v) Transplantation
(vi) *Agrobacterium* culture
(vii) Infection and incubation
(viii) Co-culture
(ix) Culture of *Agrobacterium* infected explants
(x) Control experiments

3.10.1 Axenic culture:

To reduce the level of surface organisms the mungbean seeds were washed first with detergent (Jet) under running tap water until the detergent washed out completely. The floating seeds were discarded. The seeds were then deepened in 70% alcohol for 1 minutes followed by washing with distilled water. After transferring the seeds in autoclaved flask, final surface disinfection was done with 0.1% $HgCl_2$ solution for fifteen to twenty minutes inside the laminar flow cabinet. During this period, the flask was agitated with the help of a rotatory shaker. Then the seeds were washed five times with sterilized distilled water.

The surface sterelized seeds were inoculated to sterile seed germinating medium in conical flasks. Ten to fifteen seeds were inoculated into each flask. The cultures were incubated in the dark till the germination took place. These were then maintained under a 16/8-hour (light/dark) regime. The temperature of growth room was kept at 26 ± 2^0C. *In vitro* grown 1-2 days overnight soaked seeds were used as a source of CADE explants and 10 days old *in vitro* seedlings were used as a source of CN and ST explant and thus provided contamination free inocula.

3.10.2 Explant culture (inoculation):

The germinated seeds raised in axenic culture were the source of different kinds of explants. The explants were cultured in Petri dishes or in conical flasks with different media for regeneration.

3.10.2.1 Cotyledon attached node culture

Cotyledon attached node were obtained from 8-10 days old aseptically grown seedlings and were cultured in various medium.

3.10.2.2 Cotyledon attached decapitated embryo culture

Overnight soaked seeds were taken on sterilized Petri dish. The seed coats are removed and the seeds were split open and two cotyledons were separated used as a source of cotyledon attached decapitated embryo (CADE) explants. Before inoculation the shoot and root meristems from these embryos were excised. Decapitated embryos were found to remain with one part of cotyledon.

3.10.2.3 Shoot tip culture

Shoot tips were obtained from 8-10 days-old aseptically grown seedlings. This was cultured in different media.

3.10.3 Precaution:

During culturing all the dissecting instruments (which were autoclaved earlier) namely, blades, scalpels and forceps were dipped in 70% alcohol and flamed over a spirit lamp before each time of use. The floor of the cabinet and hands were rinsed with 70% alcohol before starting the procedure.

3.10.4 Incubation of inoculated culture vessels:

The culture tube and the conical flasks containing inocula were incubated under fluorescent light of 20,000 lux intensity. The light period of the culture room was maintained at 16/8 (dark/light) hours and temperature was 25 ± 2°C.

3.10.5 Subculture

Cultures were sub-cultured to fresh media regularly, at an interval of three to four weeks for maintenance and were routinely examined.

3.10.6 Rooting

3-4.0 cm long shoots were separated and cultured on freshly prepared rooting medium containing full/half strength of MS supplements with/without different combinations and concentrations of hormones (IAA, IBA) for root induction.

3.10.7 Transplantation

The plantlets with sufficient root system were taken out from the culture vessels and the roots were washed under running tap water. The plantlets were then transplanted to small pots containing sterilized soil. Pots were then covered with transparent perforated polythene bags. Inner side of these bags was moistened with water to prevent desiccation. To reduce sudden shock, the pots were kept in growth room for two weeks, of which polythene covers were maintained for the first week and without cover for the second week. These plantlets were exposed to external environment for 2-8 hours daily and again placed in growth room for another week. Three weeks after transplantation, when the regenerated plants were fully established in the small pots, then they were transferred to larger pots for further growth and to develop flowers and fruits. After maturity the fruits from these regenerated plants were harvested to get seeds.

3.10.8 *Agrobacterium* culture

As it has been mentioned earlier, two kinds of culture media were needed for each bacterial strain: one for maintaining *Agrobacterium* stock and the other for infection of explants.

For maintenance, one single colony from previously maintained stocks was streaked into freshly prepared Petri dish containing YEP medium having appropriate antibiotics. The Petri dish was sealed with Para film and kept in incubator at 37˚C temperature for at least 48 hours. This was then kept at 4˚C to check the growth. The culture was sub-cultured regularly every week in freshly prepared media to maintain the stock.

For infection of explants, bacterial suspension was prepared. For this purpose, single streak from the bacterial stock was taken in an inoculation loop and was inoculated in a conical flask containing liquid medium (YEP or YMB) with required antibiotic. This culture was allowed to grow at 28ºC overnight on a rotary shaker to get optimum population for infection and co-cultivation of explants.

3.10.9 Infection and incubation

The overnight grown *Agrobacterium* culture was centrifuged for 10 minutes at 5000 rpm and the pellet was resuspended with liquid MS medium (pH 5.8) to make the *Agrobacterium* suspension. This *Agrobacterium* suspension was used for infection and

incubation. Prior to this "Optical Density" or OD of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). Following the determination of density, to get suitable and sufficient infection of the explants, cut explants were dipped in bacterial suspension for different incubation periods before transferring them to co-cultivation medium.

3.10.10 Co-culture

Following infection and incubation, the explants were co-cultured in regeneration medium. Prior to transfer of all explants to co-cultivation media they were soaked in filter papers for a short period of time to remove excess bacterial suspension. All the explants were maintained in co-culture medium for 2-5 days.

3.11 Culture of *Agrobacterium* infected explants and selection process for putative transformed shoots

Co-cultured explants were washed with distilled water three to four times until no opaque suspension was seen, then washed for 10 minutes with distilled water containing 300 mg/l ticarcillin and then finally washed with distilled water once again. Then explants were gently soaked with a sterile Whatman filter paper and transferred to regeneration medium with 100mg/l ticarcillin. After 2 weeks, the regenerated shoots were then sub-cultured on selection medium containing 50 and mg/l kanamycin and 100 mg/l ticarcillin. Cultures were sub-cultured regularly at an interval of 12-15 days and the concentration of selective agents was gradually increased up to 300 mg/l in case of kanamycin on selection medium. Shoots survived on selection medium were sub-cultured on rooting medium.

3.12 Experiment with negative control

As controls, non-infected explants were cultured in normal regeneration medium and after 2 weeks of shoot initiation it was sub-cultured in selection medium to detect the effect of selective agents on this control shoots. These controls were maintained with each set of transformation experiments to perform various comparative studies.

3.13 Growth room conditions:

All the cultures were maintained under fluorescent illumination with 16/8 hours dark/light cycle (except co-culture experiment) at 25 ± 2°C. The intensity of light was maintained at 20,000 lux.

3.14 GUS (β-glucuronidase) histochemical assay:

The *Agrobacterium* strain used in the present study contain GUS (*uid A*) reporter gene. The product of GUS gene is β- glucuronidase enzyme that reacts with a substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide or X-Gluc (Jefferson 1987, Gould and Smith 1989) and gives an insoluble indigo blue color at the site of GUS enzyme activity. Thus, it detects transgene expression and allows transformed tissues to be screened histochemically.

3.14 .1 Reagents for histochemical GUS assay:

(i) 0.5 M morphinoethane sulphonic acid (MES), pH 5.6

9.76 gm of MES was dissolved in 80 ml ddH_2O. pH was adjusted to 5.6 with NaOH and volume was made. This was stored in room temperatures.

(ii)Fixation solution, pH 5.6

Component	Stock concentration	Final concentration
Formaldehyde (40%)	0.75% (v/v)	0.3%
0.5 M MES	0.002% (v/v)	10 mM
Manitol	5.46% (w/v)	0.3 M

This was stored at room temperature.

(iii) 50 mM solution of Na_3PO_4, pH 7.0

Stock solutions

A. 50 mM solution of $NaH_2PO_4.2H_2O$ (0.78 gm in 100 ml)

B. 50 mM solution of Na_2HPO_4 (0.71 gm in 100ml)

For the preparation of 100 ml 50 mM solution of Na_3PO_4 (pH 7.0), 39 ml of stock solution A and 61.0 ml of B was mixed and the pH was checked for 7.0.

(iv) GUS -Buffer / X-Gluc solution

For the preparation of 10-ml X-gluc solution, 10 mg of X-gluc (5- Bromo-4-Chloro-3- indolyl β-D glucuronide) was dissolved in DMSO (dimethyl sulphonic acid) in a pyrex tube. The final volume was done with 50 mM solution of Na_3PO_4. This was stored at 20˚C.

3.14 .2 GUS (β-glucuronidase) histochemical assay of transient explants

From each batch of explants following each transformation experiment, randomly selected co-cultured tissues were examined for GUS histochemical assay. Co-cultured explants and tissues and parts of putative transformed plantlets were immersed in X-gluc (5-Bromo-4-Chloro-3-indolyl-β-D glucuronide) solution by following this method.

a) The explants were placed in fixation solution and were vacuum infiltrated briefly (for about 2 minutes).
b) The explants were then incubated for 4-5 minutes at room temperatures followed by several washes (3 times) in 50 mM Na_3PO_4 solution.
c) Explants were dipped in histochemical reagent, i.e. GUS buffer and explant were incubated at 37˚C overnight.

A characteristic indigo blue color would be the expression of GUS (β-glucuronidase) gene in the plant tissue. In each experiment explant having no *Agrobacterium* infection was used as control.

After X-Gluc treatment, explants were transferred to 70% alcohol for degreening. Degreened explants were observed under stereomicroscope (Olympus, Japan). For confirmation slides were prepared with transformed tissue and were observed under microscope.

3.15 Plant DNA Isolation for PCR

Following steps were carried out for DNA isolation

3.15.1 Collection of leaf sample

To extract genomic DNA, leaves of *in vitro* regenerated plants of BARI tomato variety 8, 9 and 14 were collected. Curling leaves of *in vitro* regenerated plantlets of three varieties were collected. Leaves were also collected from the field plants showing tomato leaf curl disease and controls were collected. The materials were washed in distilled water and dried on fresh tissue paper to remove any components of medium nutrients or dust. Then they were wrapped in aluminum foil paper and kept in -80^0 freezer until DNA isolation.

3.15.2 Preparation of Stock Solutions and Working Solutions used for DNA Isolation

For conducting the isolation procedures, the following stock solutions and working solutions were prepared.

3.15.2.1 1 M Stock Solution of Tris HCl pH 8.0 (100 ml)

12.14 gm of Trizma base (MW=121.14) was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0 by adding about 5 ml of concentrated HCl. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4º C.

3.15.2.2 0.5 M Stock Solution of EDTA pH 8.0 (100 ml)

18.61 gm of EDTA (EDTA. $2H_2O$, MW=372.24) was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 gm of NaOH pellets was added to adjust the final pH to 8.0. Final volume was adjusted to 100 ml by adding sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.15.2.3 5 M Stock Solution of NaCl (100 ml)

29.22 gm of sodium chloride (NaCl, MW=58.44) was dissolved in 75 ml of distilled water. Then it was volume up to 100 ml with distilled water. Vigorous stirring along with heating was required to dissolve NaCl. After sterilization by autoclaving it was then stored at 4º C. NaCl was added in small amount at once to be dissolved in solution.

3.15.2.4 β - Mercaptoethanol

β - Mercaptoethanol was available at strength of 14.4 M solution and was stored in a dark bottle at room temperature.

3.15.2.5 Ribonuclease A stock solution

10 mg RNAase A was dissolved in 1 ml of deionized distilled water and was store in -20^0 C.

3.15.2.6 Tris-HCl Saturated Phenol

It was prepared in following procedure,

i. The crystal phenol was melted in a water bath at 65º C for 30 minutes.
ii. Then 100 ml of melted phenol was taken and same volume of Tris-HCl (pH 8.0) was added.
iii. It was mixed with a magnetic stirrer for 10 minutes and then was left in rest for 5 minutes.
iv. At this stage, two distinct phases were visible, colorless upper phase and
v. coloured lower phase.
vi. The upper phase was removed with the help of a dropper.

This step was performed for six times which took about 3.5 hours to obtain pH 7.75. After saturation, the phenol became the half of the initial volume. As phenol is very much corrosive and highly toxic, protective measures (Apron, Gloves, and Musk) were adopted.

3.15.2.7 Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) (100 ml)

50 ml of Phenol, 48 ml of Chloroform and 2 ml of Isoamyl alcohol were added and mixed properly using vortex mixture. Mixing was done under fume hood for safety. It was prepared instantly during DNA isolation procedure. It was shaken every time before use. The Phenol: Chloroform: Isoamyl alcohol mixture is caustic and produces fumes. So, was used only in a fume hood wearing gloves and eye protection.

3.15.2.8 70% Ethanol (100 ml)

30 ml double distilled water was added in 70 ml absolute ethanol.

3.15.2.9 Stock Solution of TE (Tris-HCl EDTA) Buffer pH 8.0 (100 ml)

1 ml of 1 M Tris-HCl was added with 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.15.2.10 3 M Sodium acetate pH 5.2 (100 ml)

40.824 g of sodium acetate was dissolved in 70 ml of ddH_2O and was adjusted to 100 ml in volume adding ddH_2O and was sterilized by autoclaving.

3.15.2.11 Extraction Buffer (Homogenization buffer)

To prepare extraction buffer the following components and concentrations were used.

Chemical Names	Molecular Weight	Stock Con.	Working con.	Working Volume	
				100 ml	1000 ml
CTAB			2%	2 g	20 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (p^H 8)	372.24	0.5 M	20 mM	4 ml	40 ml
Trisbase (p^H 8)	121.1	1 M	100 mM	10 ml	100 ml
β-Mercaptoethanol		14.4 M	100 mM	700 µl	7 ml

Extraction Buffer was Prepared as the following steps (100 ml):

I. 10 ml of 1 M Tris-HCl (pH 8.0) was taken in a 250 ml conical flask.
II. 28 ml of 5 M NaCl was added to it.
III. 4 ml of 0.5 M EDTA (pH 8.0) was added next.
IV. The solution was then autoclaved.
V. After autoclaving 1 gm PVP and 2 gm CTAB was added and was stirred in the magnetic stirrer.
VI. 800µl β-mercaptoethanol was added prior to use and was mixed by glass rod.
VII. The pH of the solution was adjusted at pH 5 with HCl and was made up to 100 ml by adding sterile de-ionized distilled water

Freshly prepared extraction buffer solution was used, β-mercaptoethanol was added to the solution prior to use. The whole task was performed in the fume hood.

3.15.3 Protocol used for genomic DNA isolation

DNA was isolated using the modified CTAB method. The method is described below:

- Leaves were taken and grind in liquid nitrogen using pestle and mortar and paste was made.
- 1 ml of extraction buffer was added and grinded the leaf until it became homogenous paste.
- The paste were transferred to eppendorf tubes and incubated at 65ºC water bath for 30 min.
- After that the samples were centrifuged at 13,000 rpm for 10 min at room temperature to remove non-soluble debris.
- The supernatants were transferred to fresh tubes and an equal volume of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) was added and mixed well by slow inversion, then centrifuged the tubes at 13,000 rpm for 5 minutes. This step was repeated for two times.
- The supernatants were transferred to fresh tubes and an equal volume of Chloroform: Isoamyl alcohol (24: 1) was added and mixed well by slow inversion then centrifuged the tubes at 13,000 rpm for 5 minutes.
- The supernatants were transferred to fresh tubes and an equal volume of Chloroform was added and mixed well by slow inversion, then centrifuged the tubes at 13,000 rpm for 5 minutes
- The supernatants were transferred to fresh tubes and 0.1 vol. of 3 M sodium acetate (pH 5.2) followed by 0.6 vol. of 100% chilled Isopropanol were added and mixed slowly. In this step DNA become visible as whitish thread like network in the solution.
- The samples were centrifuged for 10 min at 13,000 rpm at room temperature. The supernatants were discarded carefully by using adjustable micropipette.
- The pellet was washed with 70% ice-cold ethanol. The washing step was repeated at least twice to thrice. The pellets were air dried on a paper towel for about 2 hour.
- The dried DNA was dissolved in 50 µl of TE buffer and treated with RNase A for 30 min at 37°C and store at -20°C.

3.16 Polymerase Chain Reaction

Following steps were carried out to perform polymerase chain reaction

3.16.1 Preparation of purified Taq DNA polymerase

- 2µl of purified Taq DNA Polymerase was transferred to an eppendorf (fresh and autoclaved).
- 4µl of storage buffer was added.
- Dilution of the enzyme was 50 fold.

Storage Buffer:

Components	MW	Concentration
Tris	121.14	50.0m M
KCL	74.50	50.0mM
EDTA	372.20	0.1 mM
DTT	154.20	1.0mM
PMSF	174.20	0.5m M
Glycerol	---------	50.0%

- 0.0174 g of PMSF was first dissolved in minimal volume of isopropanol (~lml). Then 1.21 g of Tris, 0.745 g of KCL, 0.0074 g of EDTA and 0.03084 g of DTT were added and mixed thoroughly with deionized water after adjusting the pH to 7.9. The final volume was made 75ml with ddH2O and filter sterilized through 0.2/µm Millipore.
- Meanwhile, 80% glycerol was prepared from commercially available glycerol (98%) and autoclaved. 125 ml of the 80% glycerol was added to 75ml of the mixture of storage buffer and stored at 4°C.

3.16.2 Preparation of dNTPs mixture

100 µl each of dATP, dGTP, dCTP, dTTP [their concentrations being l0 mM each] were mixed in a fresh autoclaved eppendorf tube and the final volume was made l000 µl by adding 600µl of TE solutions (l0 mM Tris-HCI, 0.l mM EDTA, pH 8.0) and dispensed as aliquots in tubes and stored at -20°C. The final concentration of each of the nucleotide in the above mixture was 1.0 mM. 2.0 mM of dNTPs mixture was also prepared.

3.16.3 Preparation of PCR reaction mixture

The following components were used to prepare PCR reaction mixture (Table 4). The total volume of PCR reaction mixture was 125 µl for 5 samples.

Component of PCR reaction mixture (for 15 reactions):

Sl. No.	Reagents	Amount per sample	Total
1	Sterile de-ionized distilled water		97.25µl
2	*Taq* Buffer A 10X (Tris with 15 mM $MgCl_2$)	2.5 µl	12.5 µl
3	Primer F	1.0 µl	5.0 µl
4	Primer R	1.0 µl	5.0 µl
5	dNTPs 2.5 mM	0.25 µl	1.25 µl
6	*Taq* DNA Polymerase	1.0 µl	5.0µl
7	Sample DNA	2.0 µl	10.0 µl
	Total	**25.0 µl**	**125.0 µl**

During the experiment, PCR buffer, dNTPs, Primers and DNA sample solution were thawed from frozen stocks, mixed well by vortexing and kept on ice.

3.16.4 Thermal cycling profile used in PCR

The thermal cycling profile that was employed in the PCR for the amplification of TYLCV gene is tabulated below:

The thermo cycler was run with the temperature profile specified below:

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	5 min	1 (first)
Denaturation	94°C	1 min	30
Annealing	55°C	30 sec	
Elongation	72°C	30 sec	
Final elongation	72°C	5 min	1 (last)

3.16.5 Preparation of the master mixture

Master mixture was prepared by mixing all of (the PCR component e.g. buffer, dNTPs, Mg^{2f} Primer-F & R, etc. except the component against which the optimization strategy was intended. In each reaction, the volume of PCR buffer was used $\frac{1}{10}$ th of the total reaction volume which was 25 µl.

After through mixing and momentary spin of the master mixture, it was transferred to different eppendorf tubes. The PCR component in question was then added. The final volume was made 25 µl by adding varying amounts of sterilized ultra-pure water. Taq DNA Polymerase was added just before the start of the reaction. Finally, the tubes were subjected to momentary spin and transferred to thermo cycler for the amplification reaction (eppendorf mastercycler gradient).

3.17 Gel electrophoresis of the amplified products

3 µl of DNA dye was added to the PCR amplified DNA. The amplified products were separated electrophoretically on 1% agarose gel. After a momentary spin the PCR products were loaded in wells of agarose gel containing ethidium bromide. The gel was prepared using 1.0 g agarose powder containing ethidium bromide and 100 ml 1×TAE buffer. Agarose gel electrophoresis was conducted in 1× TAE buffer at 50 Volts and 100 mA for 1.5 hours. DNA bands were observed on UV-transilluminator and photographed by a gel mocumentation system.

3.18 Documentation of the DNA Sample

After electrophoresis, the gel was visualized under UV transilluminator and photographs were taken in gel documentation system (Biosens SC Series-645) for documentation.

4. RESULTS

The present investigation was carried out to develop an efficient *in vitro* regeneration protocol as well as *Agrobacterium*-mediated genetic transformation for mungbean [*Vigna radiata* (L.) Wilczek]. Two varieties of locally grown mungbean, namely, Binamoog-5 and BARI mung-6 were used for this purpose. For transformation *Agrobacterium* strain LBA4404 containing binary plasmid pBI121 were used (For detail information see section 2.2).

The experiments of this study were carried out in three phases. In the first stage, experiments were conducted to obtain a transformation compatible regeneration protocol for both the varieties of mungbean. In the second phase, various strategies influencing transformation efficiency were optimized through several experiments to establish a successful transformation protocol. Thirdly, *Agrobacterium*-mediated genetic transformation was carried out for the development of transgenic mungbean plants.

4.1 *In vitro* regeneration of shoots.

A suitable regeneration system is a prerequisite for plant genetic transformation. Therefore, emphasis was undertaken to develop an *in vitro* plant regeneration system for the two varieties of mungbean using selected explants. Three different explants such as cotyledon attached decapitated embryo (CADE), cotyledon attached node (CN) and shoot tip (ST) were used for *in vitro* regeneration. Several parameters such as percentage of responsive explants towards shoot induction, days required to shoot initiation, number of multiple shoots per explant and shoot length for each set of experiment were recorded. Shoots were cultured for *in vitro* root formation and regenerated plantlets were acclimatized in soil and transplanted to grow under field condition.

4.1.1 Seed germination for obtaining explants

Surface sterilization of the seeds were carried out for obtaining aseptic explants under *in vitro* condition.

4.1.1.1 Surface sterilization of seeds with $HgCl_2$ in controlling contamination

Mungbean seeds were surface sterilized using 0.1% $HgCl_2$ prior to inoculation. The sterilization period of seeds with $HgCl_2$ has been found to be important in obtaining seedlings germination. Therefore, a series of experiments were conducted to examine the effects of surface sterilization in controlling contamination. In both varieties, optimum responses towards regeneration were achieved when the seeds were surface sterilized for 15 minutes. The responses of the surface sterilized seeds towards germination has been presented in Table 1.

Table 1. Effects of surface sterilization periods with $HgCl_2$ in controlling the contamination of seeds of two mungbean varieties.

Sterilization period (min)	Number of seeds inoculated	% of contaminated seeds		Rate (%) of responsive seeds for germination	
		Binamoog-5	BARImung-6	Binamoog-5	BARImung-6
7	50	32	41	100	98
9	50	18	22	99	97
11	50	10	12	99	97
13	50	5	7	98	96
15	**50**	**2**	**4**	**98**	**96**
17	50	0	0	80	72

Variety (Binamoog-5):

Zero percentage of contamination (0%) was observed with 17 minutes of surface sterilization period and maximum contamination (32%) was observed with 7 minutes of surface sterilization. Percentage of responsive seeds for regeneration was highest (100%) with 7 minutes of surface sterilization and was lowest (80%) with 17 minutes of surface sterilization (Table1). However, considering the reduced number of contaminants (2%) as well as high rate of responsive *in vitro* regeneration (98%) optimum time period for surface sterilization was 15 minutes.

Variety (BARI mung-6):

Minimum percentage of contamination (0%) was observed with 17 minutes of surface sterilization period and maximum contamination (41%) was observed with 7 minutes of surface sterilization. Percentage of responsive seeds for regeneration was highest (98%)

with 7 minutes of surface sterilization and was lowest (72%) with 17 minutes of surface sterilization (Table1). However, optimum time period for surface sterilization was 15 minutes in considering reduced number of contaminants as well as high rate of responsive in regeneration.

4.1.1.2. Seed germination and seedling development

The experiment was conducted to find out the optimum condition for *in vitro* seed germination. 0.8% agar solidified ½MS media containing 3% sucrose, MS medium containing 3% sucrose with 0.8% agar and MS medium supplemented with BAP containing 3% sucrose with 0.8% agar were used for germination and seedling development. Seed germination rate and time required for germination were more or less identical for both varieties used in this experiments. Figures 4 and 5 are presented to show the germination of seed on agar solidified medium.

The results of these experiments were shown in Table 2. Seed germination rate was higher on MS medium supplemented with BAP containing 3% sucrose with 0.8% agar after 4 - 5 days. In agar solidified MS medium seedling development was found to be less than BAP supplemented medium whereas on agar solidified ½ MS medium germination rate was the lowest among three medium and it required 2-3 days. It was observed that the percentage of germination of seeds was 100% for Binamoog-5 and 98% for BARI-6 variety on MS medium supplemented with BAP. On agar solidified MS medium 92% Binamoog-5 and 80% BARI-6 variety seeds germinated whereas comparatively only 84% Binamoog-5 and 78 % BARI-6 variety seeds germinated on agar solidified ½ MS medium. Varietal difference was observed between the two varieties that germination rate of Binamoog-5 was better than BARI-6 on three medium (Fig 6).

Table 2. Comparison of seed germination of two varieties of mungbean.

Media		Mungbean varieties	
		Binamoog-5	BARImung-6
Agar solidified ½ MS medium	No. of seeds inoculated	50	50
	No. of seeds germinated	42	39
	Days to seed germination	2-3	2-3
Agar solidified MS medium	No. of seeds inoculated	50	50
	No. of seeds germinated	46	40
	Days to seed germination	4-5	4-5
MS medium supplemented with BAP	No. of seeds inoculated	50	50
	No. of seeds germinated	50	49
	Days to seed germination	4-5	4-5

4.1.2 Determination of suitable explants for direct regeneration

During the present investigation, experiments were carried out to regenerate shoots directly without the intervention of callus using various explants from both the varieties. Cotyledonary nodes (CN), shoot tips (ST) and hypocotyls (HC) from *in vitro* seedlings and decapitated embryo (DE), cotyledon attached decapitated embryo (CADE) and single cotyledon (C) from overnight soaked surface sterilized seeds were used as explants for the present set of experiments. All kinds of explants were inoculated to a medium (MS + 5.00 μM BAP) where regeneration of shoots was obtained through direct organogenesis. Effect of different explants for this experiment towards regeneration of shoots is shown in Tables 3

Table 3. Effect of different explants from both mungbean varieties on MS medium supplemented with 4 μM BAP for multiple shoot regeneration.

Type of explants Explant	No. of explant inoculated	No. of responsive explant	% of responsive explant	Time to start regeneration (days)	No. of shoots per explants after 60-70 days
		Binamoog-5			
Cotyledonary node (CN)	50	49	98%	8-10	25-30
Shoot tips (ST)	50	46	92%	10 - 12	4-5
Hypocotyls (H)	50	0	0%	-	-
Decapitated embryo(DE)	50	32	64%	7 - 10	5-7
Cotyledon attached Decapitated,embryo (CADE)	50	48	96%	12-15	18-20
Single cotyledon (C)	50	0	0%	-	-
		BARI mung-6			
Cotyledonary node (CN)	50	46	92%	10-12	20-22
Shoot tips (ST)	50	42	84%	12 - 15	3-4
Hypocotyls (H)	50	0	0%	-	-
Decapitated embryo (DE)	50	29	58%	7 - 10	5-6
Cotyledon attached Decapitated embryo (CADE)	50	45	90%	15-16	15-16
Single cotyledon (C)	50	0	0%	-	-

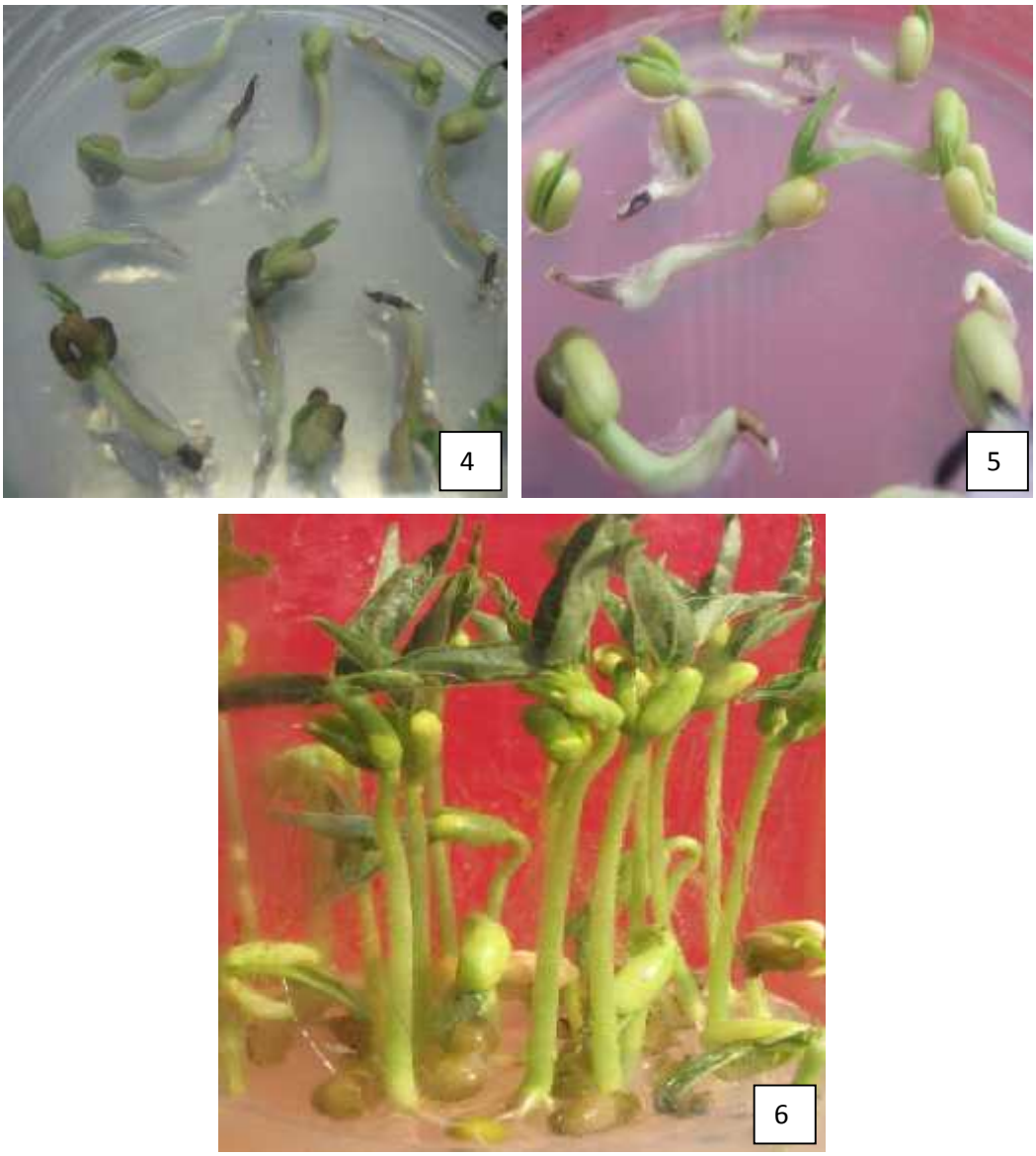

Fig. 4. Germination of seeds of BARI mung-6 variety;

Fig. 5. Same as Fig. 4 but in case of Binamoog-5 variety;

Fig. 6. *In vitro* grown seedlings of Binamoog-5

4.1.3 Effect of different growth regulators on multiple shoots regeneration

In the present investigation, MS medium supplemented with various concentrations and combinations of different cytokinins, such as, BAP (2 μM – 10 μM), Kn (0.5 μM – 1.5 μM) and auxins, such as, NAA (0.1 μM – 1.0 μM) were used for direct multiple shoot regeneration and elongation of shoots from three different explants.

4.1.3.1. Effect of different concentrations of BAP in MS medium on regeneration of multiple shoots from different explants of Binamoog-5 and BARI-6 mung varieties.

MS medium supplemented with different concentrations of BAP (2 μM – 10 μM) were used to examine the effects of BAP on multiple shoot induction and their subsequent development from different explants. Results of these experiments were presented in Table 4. Among different concentrations MS medium supplemented with 4.0 μM/L of BAP was found to be best in response multiple shoot regeneration for both varieties of mungbean .It is observed from the table that the maximum number of shoots in an average of **20.5** shoots per cotyledonary node, 4.5 shoots per shoot tip and 8.3 shoots per CADE explants were observed on MS medium supplemented with 4.0 μM BAP for Binamoog-5 variety (Figs 7-9.), whereas in the same concentrated medium maximum number of shoots was 16.5 from CN, 4.5 from ST and 7.3 from CADE explants for BARI-6 variety. In case of Binamoog-5, 96% of CN and CADE explants were responsive towards shoot initiation, whereas 94% of BARI mung-6 explants. Shoot initiation started from 10-15 days old explants but maximum number of shoots were observed after 60-70 days of inoculation. Multiple shoots developed on this medium were found green and healthy.However the length of shoots developed using these hormonal supplements was 4.5cm from CN and 4.3 from CADE in case of Binamoog-5 and for BARI-6 it was 4.2 from CN and 4.1 from CADE explants.

Second highest response was observed on MS medium supplemented with 2.00 μM BAP for both the varieties. Among the two varieties, Binamoog-5 regenerated maximum number of shoots.

Table 4. Effect of different concentrations of BAP in MS medium on regeneration of shoots from different explants of two varieties of mungbean.

Explant	Conc. of BAP (μM/L)	No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoots/explant after 60-70 days	Average length of shoots (cm)
			Binamoog-5			
CN		25	94	10-12	10.5	3.6
ST	2.00	25	84	8-9	4.3	2.8
CADE		25	92	12-15	6.3	3.2
CN		**25**	**96**	**11-12**	**20.5**	**4.5**
ST	**4.00**	**25**	**88**	**10-11**	**4.5**	**4.0**
CADE		**25**	**94**	**14-15**	**8.3**	**4.3**
CN		25	92	15-16	15.2	3.6
ST	6.00	25	80	12-13	3.2	2.5
CADE		25	82	12-13	6.5	3.5
CN		25	76	13-14	10.4	3.0
ST	8.00	25	72	12-15	2.8	2.2
CADE		25	80	12-14	6.2	3.0
CN		25	74	16-18	8.6	2.2
ST	10.00	25	60	18-19	2.2	1.5
CADE		25	68	19-20	6.0	2.0
			BARI mung-6			
CN		25	92	10-12	8.5	3.2
ST	2.00	25	80	8-9	4.3	2.3
CADE		25	88	12-15	5.3	3.1
CN		**25**	**94**	**11-12**	**16.5**	**4.2**
ST	**4.00**	**25**	**84**	**10-11**	**4.5**	**4.0**
CADE		**25**	**90**	**14-15**	**7.3**	**4.1**
CN		25	88	15-16	15.2	3.2
ST	6.00	25	76	12-13	3.2	2.5
CADE		25	78	12-13	5.5	3.0
CN		25	72	13-14	8.2	3.0
ST	8.00	25	64	12-15	2.8	2.0
CADE		25	84	13-14	4.6	2.9
CN		25	68	15-18	6.0	2.1
ST	10.00	25	64	16-18	2.0	1.3
CADE		25	68	19-20	4.2	2.0

CN= Cotyledonary node, ST=Shoot tip, CADE= Cotyledone attached decapitated embryo.

4.1.3.2 Effect of different concentrations and combinations of BAP and Kn in MS medium on multiple shoot regeneration from different explants of mungbean variety Binamoog-5

MS medium supplemented with different concentrations and combinations of BAP (2.0 and 4.0 µM) and Kn (0.5– 1.5 µM) were used for multiple shoot induction directly from different explants of Binamoog-5 variety (Table 5). Shoots were found to initiate within 10 – 15 days of inoculation. Among all the concentrations and combinations of BAP and Kn used, best response towards multiple shoots initiation and development was observed on MS medium supplemented with 4.0 µM/L BAP and 0.5 µM/L Kn, In this media combinations, Binamoog-5 showed 90% response from CN and 88% from CADE explants towards shoot initiation where as showed 85% response from ST explants and mean number of shoots obtained per explant **7.5**, 5.6 and 4.3 in case CN, CADE and ST explants, respectively (Table 3).

Besides, MS medium supplemented with same concentration of BAP (4.0 µM/l) and 1.0 µM/l Kn, CN, CADE and ST explants were cultured and mean number of shoots obtained per explant was 4.1,4.2 and 3.6, respectively. Therefore, MS medium supplemented with 4.0 µM/l BAP and 0.5 µM/L Kn was found to be the most effective for multiple shoots regeneration in case of Binamoog-5 (Figs 10&11). It was observed that, the overall regenerated shoots were elongated and healthy.

Table 5. Effect of different concentrations and combinations of BAP and Kn in MS medium on regeneration of multiple shoots from different explants of mungbean variety Binamoog-5.

Explant	Conc. of BAP (μM/l)	Conc. of Kn (μM/l)	No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoots/ explant after 30-40 days	Average length of shoots after eight weeks (cm)
CN			50	64	12-14	2.5	3.4
ST	2.00	0.5	50	58	12-14	2.0	2.2
CADE			50	68	12-14	2.8	3.2
CN			50	66	13-15	2.5	3.5
ST	2.00	1.0	50	60	13-15	2.6	2.6
CADE			50	64	13-15	3.0	3.8
CN			50	70	12-13	3.1	4.6
ST	2.00	1.5	50	68	12-13	2.4	3.0
CADE			50	78	12-13	3.0	4.4
CN			**50**	**90**	**10-15**	**7.5**	**5.3**
ST	**4.00**	**0.5**	**50**	**85**	**10-15**	**4.3**	**4.1**
CADE			**50**	**88**	**10-15**	**5.6**	**5.1**
CN			50	86	10-12	4.1	5.0
ST	4.00	1.0	50	80	10-12	3.6	3.2
CADE			50	84	10-12	4.2	4.2
CN			50	70	13-14	2.6	3.6
ST	4.00	1.5	50	64	13-14	2.4	2.2
CADE			50	66	13-14	2.6	3.4

CN= Cotyledonary node, ST=Shoot tip, CADE= Cotyledone attached decapitated embryo.

4.1.3.3 Effect of different concentrations and combinations of BAP and Kn in MS medium on multiple shoot regeneration from different explants of mungbean variety BARI-6.

A number of combination and concentration of BAP and Kn were also used in MS medium to examine their effects on of multiple shoot induction and their development in case of variety BARI mung-6. The results of these experiments are presented in Table 6. Maximium percentage of shoot induction from the CN, CADE and ST was on MS

medium supplemented with 4.0 μM/l BAP and 0.5 μM/l Kn and these were 88%,86% and 84%, respectively . It was observed from the table 6, the maximum number of shoot proliferation were 6.5, 4.6 and 3.3 from CN, CADE and ST explants, respectively. On the other hand, the lowest response (an average of 2.2 shoots from CN, 2.0 shoots from ST and 2.4 shoots from CADE explants) was observed on MS medium supplemented with 4.0 μM/L BAP, 1.5 μM/L Kn. With increasing and decreasing the concentration of Kn with same concentration of BAP, percentage of shoot response and mean number of multiple shoots decreased . Among all combinations and concentrations of BAP and Kn used, best response towards multiple shoot induction was observed on MS medium supplemented with 4.0 μM/L BAP and 0.5 μM/L Kn after 60-70 days of culture. It is noticed from the table that multiple shoot induction was not very satisfactory on this media combination. Regenerating shoots using this hormonal supplement is presented in Fig 12.

Table 6 Effect of different concentrations and combinations of BAP and Kn in MS medium on regeneration of multiple shoots from different explants of BARI-mung-6 variety.

Explant	Conc. of BAP (µM/l)	Conc. of Kn (µM/l)	No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoots/ explant after 30-40 days	Average length of shoots after eight weeks (cm)
CN			50	63	12-14	2.4	3.1
ST	2.00	0.5	50	55	12-14	2.2	2.0
CADE			50	64	12-14	2.6	3.0
CN			50	68	13-15	2.8	3.1
ST	2.00	1.00	50	66	13-15	2.4	2.2
CADE			50	62	13-15	3.0	3.4
CN			50	68	12-13	3.2	3.6
ST	2.00	1.5	50	65	12-13	2.4	2.6
CADE			50	78	12-13	3.4	4.0
CN			**50**	**88**	**10-15**	**6.5**	**5.1**
ST	**4.00**	**0.5**	**50**	**84**	**10-15**	**3.3**	**3.5**
CADE			**50**	**86**	**10-15**	**4.6**	**5.0**
CN			50	82	10-12	4.0	4.6
ST	4.00	1.00	50	80	10-12	2.6	3.0
CADE			50	84	10-12	3.2	4.0
CN			50	72	13-14	2.2	3.2
ST	4.00	1.5	50	66	13-14	2.0	2.0
CADE			50	64	13-14	2.4	3.3

CN= Cotyledonary node, ST=Shoot tip, CADE= Cotyledone attached decapitated embryo.

Fig. 7. Initiation of shoot from CN explants of Binamoog-5 variety on MS supplemented with 4.0 µM BAP.

Fig. 8. Same as Fig.7 but showing elongation of shoots.

Fig. 9. Same as Fig. 8 but showing the development of multiple shoots.

Fig. 10. Multiple shoot development from CN explants of Binamoog-5 on MS + 4.0 µM BAP + 0.5 µM Kn.

Fig. 11. Same as Fig 10 but showing multiple shoot development.

Fig 12. Same as Fig. 11 but in case of BARI mung-6.

4.1.3.4 Effect of different concentrations and combinations of BAP and NAA in MS medium on multiple shoot regeneration from different explants of Binamoog-5 variety.

MS medium supplemented with different combination and concentration of BAP and NAA were used to observe their effect on multiple shoot induction and their subsequent development. The results are presented in Table 7. It is observed from the table that the maximum number of shoots were developed after 60-70 days (an average of 29 shoots per CN, 20.2 shoots per CADE and 10.6 shoots per ST) on MS medium supplemented with 10.0 μM BAP and 1.0 μM NAA. Shoots were healthy, deep green and leaves were large (Fig. 13). Maximum shoot length (an average is 5.2 cm from CN, 5.1 cm from CADE and 2.5 cm from ST explants developed in 60-70 days of culture) was observed on the same medium. Proliferated and elongated multiple shoot obtained from this culture is presented in (Figs. 14 and 15).

It is noteworthy that, before collecting explants (CN, CADE and ST) seeds were germinated on 4.0 μM BAP supplemented MS medium .Then explants were inoculated on MS medium supplemented with 10.0 μM BAP and 1.0 μM NAA. After 30 days, explants were subcultured on MS medium supplemented with 4.00 μM BAP and after 60-70 days of culture maximum number of multiple shoots (shown in Table 7)were observed. It was the best regeneration medium for all explants (CN, CADE and ST) among different combination used in the present experiments

Table 7. Effect of different concentrations and combinations of BAP and NAA in MS medium on regeneration of shoots from different explants of mungbean variety Binamoog-5.

Explant	Conc. of BAP (µM/l)	Conc. of NAA (µM/l)	No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoots/ explant after 60-70 days	Average length of shoots after eight weeks (cm)
CN			50	64	12-13	2.3	3.0
ST	2.00	0.1	50	55	12-14	2.0	2.0
CADE			50	66	12-14	2.4	3.0
CN			50	68	13-15	4.4	3.1
ST	4.00	0.2	50	56	13-15	2.2	2.3
CADE			50	52	13-15	3.0	3.3
CN			50	64	12-13	4.2	3.6
ST	6.00	0.3	50	66	12-13	1.4	2.5
CADE			50	76	12-15	5.4	3.0
CN			50	86	10-12	16.5	4.1
ST	8.00	0.4	50	80	10-12	6.3	3.5
CADE			50	84	10-12	14.6	5.0
CN			**50**	**98**	**8-10**	**29.0**	**5.2**
ST	**10.00**	**0.5**	**50**	**88**	**8-10**	**10.6**	**2.5**
CADE			**50**	**94**	**8-10**	**20.2**	**5.1**
CN			50	74	13-14	14.0	3.3
ST	12.00	1.0	50	64	13-14	9.2	2.0
CADE			50	60	13-14	10.4	3.2

CN= Cotyledonary node, ST=Shoot tip, CADE= Cotyledone attached decapitated embryo.

4.1.3.5 Effect of different concentrations and combinations of BAP and NAA in MS medium o multiple shoot regeneration from different explants of BARI mung-6 variety.

In case of variety BARI mung-6, MS medium supplemented with six different combinations of BAP and NAA were used to distinguish their effect on stimulation of multiple shoots and their successive development from the CN, CADE and ST explants. Results of these experiments are presented in Table 8. Seed germination and explants inoculation steps explained in 4.1.4.4 chapter were followed as similar as BARI mung-6 variety. It is apparent from the table that the maximum number of shoots was observed after 60-70 days (an average of 26 shoots per CN, 18.2 per CADE and 10.2 shoots per ST explants) on MS medium supplemented with 10.0 μM BAP and 1.0 μM NAA. The shoots were healthy, green and leaves were large (Figs 16 and 17). Maximum shoot length (an average of 5.0 cm from CN, 4.6 cm CADE and 2.2 cm from ST developed in 60-70 days of culture) was observed on same medium. Among all the combination used in the present study, this medium was found to be the best for BARI mung-6 variety.

Table 8. Effect of different concentrations and combinations of BAP and NAA on MS medium for regeneration of shoots from different explants of BARI mung-6 variety.

Explant	Conc. of BAP (µM)	Conc. of NAA (µM)	No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoots/ explant after 60-70 days	Average length of shoots after eight weeks (cm)
CN			50	62	12-13	2.8	2.9
ST	2.00	0.1	50	54	12-14	2.4	2.0
CADE			50	64	12-14	2.5	3.1
CN			50	60	13-15	4.6	3.0
ST	4.00	0.2	50	54	13-15	2.8	2.2
CADE			50	54	13-15	3.4	3.1
CN			50	62	12-13	4.4	3.2
ST	6.00	0.3	50	64	12-13	1.2	2.6
CADE			50	72	12-15	5.4	2.4
CN			50	84	10-12	16.6	4.0
ST	8.00	0.4	50	80	10-12	6.4	3.2
CADE			50	82	10-12	14.4	4.4
CN			**50**	**96**	**8-10**	**26.0**	**5.0**
ST	**10.00**	**0.5**	**50**	**84**	**8-10**	**10.2**	**2.2**
CADE			**50**	**92**	**8-10**	**18.2**	**4.6**
CN			50	76	13-14	15.0	3.2
ST	12.00	1.0	50	62	13-14	8.2	2.0
CADE			50	64	13-14	10.4	3.1

CN= Cotyledonary node, ST=Shoot tip, CADE= Cotyledone attached decapitated embryo.

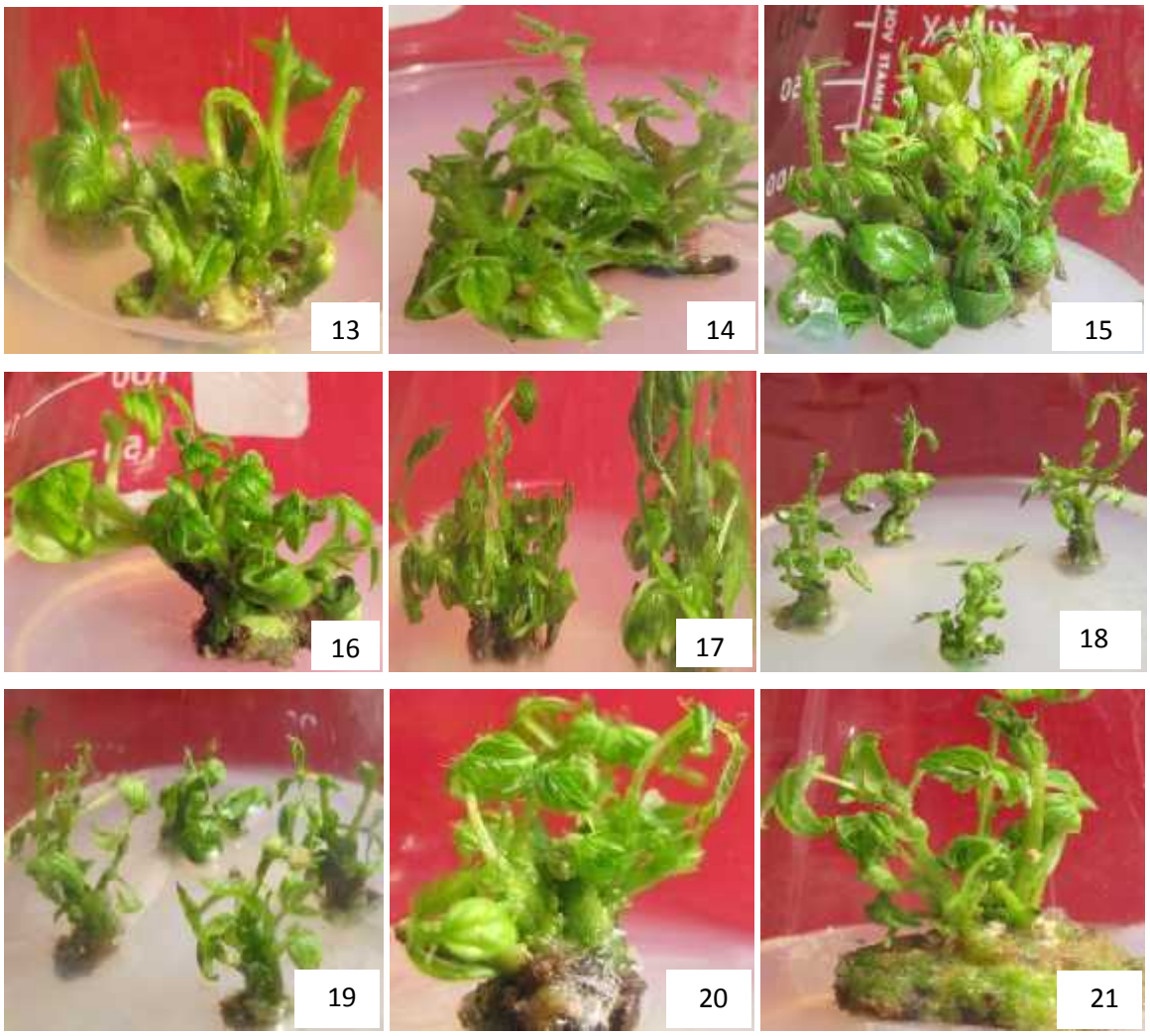

Fig. 13. Initiation of shoots from CN explants of Binamoog-5 variety on MS medium supplemented with 10.0 µM BAP and 0.5 µM NAA.

Fig. 14. Same as Fig. 13 but showing elongation of shoots.

Fig. 15. Same as Fig. 13 but showing development of multiple shoots.

Figs. 16-17. Development of multiple shoot from CADE explants of BARI mung-6 on same medium as mentioned in Fig. 13.

Fig. 18. Initiation of shoot from shoot tip explants of BARI mung-6 on same medium as mentioned in Fig. 13.

Fig. 19. Initiation of shoot from shoot tip explants of Binamoog-5 on same medium mentioned in Fig. 13.

Fig. 20-21 Multiple shoot development from CN explants of Binamoog-5 on MS medium supplemented with 4.0 µM BAP, 0.5 µM Kn and 0.5 µM NAA.

4.1.3.6 Effect of different concentrations and combination of BAP, Kn and NAA in MS medium on regeneration of multiple shoots from different explants of both mungbean varieties.

In this experiments MS medium supplemented with different combinations of BAP, Kn and NAA were used to examine the effects on multiple shoot induction and their consequent development from different explants of two mungbean varieties. Results of these experiments are presented in Table 9.

The regeneration ability of different explants of two varieties of mungbean were tested on MS medium supplemented with different concentrations and combinations of BAP (4.0 μM), Kn (0.5 μM) and NAA (0.5μM) that were found best individual combination. All the varieties showed almost same response regarding shoot responsive percentage . In this experiment, callus was found from the three types of explants after 15-20 days and shoots were regenerated from the callus after 30-40 days. When explants were cultured on MS medium supplemented with 4.0 μM BAP, 0.5 μM Kn and 0.5μM NAA, mean number of shoots per explant was 6.2 from CN, 5.2 from CADE and 4.2 from ST in case of Binamoog-5 and 3.4 from CN, 3.0 from CADE and 2.5 from ST in case of BARI mung-6 variety. Shoot elongation was obtained on the same media combinations (Figs 20-21).

Table 9. Effect of different concentrations of BAP, Kn and NAA on MS medium on regeneration of multiple shoots from different explants of two varieties of mungbean.

Explant	Conc. (μM) BAP + Kn + NAA	No. of explants inoculated	%explants	Days to callus induction	Mean no. of shoots/explant after 30-40 days	Average length of shoots after 60-70 days (cm)
			Binamoog-5			
CN	4.00 + 0.50 + 0.5	25	60	15-20	6.2	3.2
ST	4.00 + 0.50 +0 .5	25	80	15-20	4.2	2.0
CADE	4.00 + 0.50 + 0.5	25	84	15-20	5.2	4.5.
			BARI mung-6			
CN	4.00 + 0.50 + 0.5	25	66	15-20	3.4	2.0
ST	4.00 + 0.50 + 0.5	25	77	15-20	2.5	1.5
CADE	4.00 + 0.50 + 0.5	25	83	15-20	3.0	2.5

CN= Cotyledonary node, ST=Shoot tip, CADE= Cotyledone attached decapitated embryo.

4.1.4. Induction of roots from regenerated shoots

For induction of roots from regenerated shoots various experiment were tried. For this purpose

3-5 cm long shoots were excised and cultured on MS and half strength of MS medium for root induction. Various auxins namely IAA, IBA and NAA were used for the induction of roots from the *in vitro* grown shoots. Results of the study are presented in Tables 10,11 and 12. From the tables it is clearly visible that root induction was good on MS medium in compared to ½ MS medium and among the two varieties Binamoog-5 variety showed better root induction.

4.1.4.1 Effects of different auxins on root induction

Three auxins namely IAA, IBA, and NAA were used to examine their effect on root induction from the *in vitro* grown shoots of two mungbean varieties. For this purpose 2.0 μM IAA, 2.0 μM IBA and 2.0 μM NAA were added separately with MS medium to induce roots. Table 12 shows the effect of IAA, IBA and NAA on mungbean varieties towards root production. Among these three auxins, IBA found to be more suitable for rooting. Maximum number of healthy roots were obtained with IBA (Fig. 22).

4.1.5 Establishment of plantlets

Plantlets with adequate roots developed of two mungbean varieties were successfully transplanted into small plastic pots containing autoclaved soil (Fig. 23). The transplantation procedure has been described in the section 3. Using this method, the survival rate of the transplanted plantlets was found to be about 90%. The survived plantlets were transferred to larger clay pots for their further growth and establishment.

Table 10. Effects of MS and half strength of MS on root formation in two varieties of mungbean.

Media	Variety	Days to initiate root	Mean number of roots per shoot	Days requireed to get well developed roots	Remarks
MS	Binamoog-5	13-15	17	30	Healthy and medium size
	BARI-mung-6	13-15	12	30	
½ MS	Binamoog-5	13-15	11	35-40	Thin and short
	BARI-mung-6	13-15	08	35-40	

Table 11. Effects of different auxins on root formation in three varieties of mungbean.

Variety	Concentrations of growth regulators (µM/l)	% of shoots forming roots	Days to initiate roots	Mean number of roots/shoot	Days required to get well developed roots
Binamoog-5	2.0 IAA	10	20-25	5	30-32
	2.0 IB A	25	13-15	19	30-32
	2.0 NAA	12	30-32	7	30-32
BARI-mung-6	2.0 IAA	10	22-25	4	35-40
	2.0 IBA	24	15-17	09	35-40
	2.0 NAA	11	32-35	6	35-40

Table 12. Effects of different concentrations of IBA on root formation in mungbean varieties.

Variety	Concentrations of IBA (μM/l)	% of shoots forming roots	Days to initiate roots	Mean number of roots/shoot	Days required to get well developed roots
Binamoog-5	1.0	…	….	….	….
	1.5	…..	….	….	….
	2.0	25	13-15	19	30-32
	2.5				
BARI mung-6	1.0	…	…	…..	…..
	1.5				
	2.0	25	13-15	09	35-40
	2.5				

Fig. 22. Development of roots from excised regenerated shoots of BARI mung-6 on full strength of MS medium supplemented with 2.0 µM IBA.

Fig. 23. *In vitro* regenerated plantlets of BARI mung-6 transferred to plastic pots containing soil.

4.2 Genetic transformation

In this phase of investigation a series of experiments were carried out to establish a suitable protocol for *Agrobacterium*-mediated genetic transformation of two mungbean varieties (Binamoog-5, BARI mung-6) cultivated in Bangladesh. For this purpose a genetically engineered *Agrobacterium tumefaciens* strain, namely, LBA4404/pBI121 was used (section 2.2). Transformation experiments for the two mungbean varieties were conducted by using cotyledon attached decapitated embryo (CADE), decapitated embryo (DE), shoot tip (ST) and cotyledonary node (CN) as explants. Transformation ability of various explants was detected using GUS histochemical assay. For transformation experiments screenable markers were used to find out the transformation ability of various explants. Generally transient assay for such marker genes were routinely performed as a preliminary step to identify the conditions that will allow efficient DNA transfer. In the present study GUS gene (β-glucuronidase) was used as screenable marker and several experiments were conducted to identify the transient expression of GUS genes.

4.2.1 Determination of the compatibility of *Agrobacterium* strain towards transformation of mungbean

Responses of various explants following *Agrobacterium*-mediated genetic transformation was detected using histochemical assay as there was gus gene in strain. Infection of desired explants with the specific *Agrobacterium* strain is an important step for successful transformation, so that the genes of interest can be inserted into the host cells. After incubation for 40 minutes and co-cultivation for 72 hrs with *Agrobacterium* strain, transformation ability was monitored through histochemical assay of the activity of GUS reporter gene in explant tissues. Following the incubation of the co-cultured explants in X-gluc buffer, GUS (β-glucuronidase) expression was characterized by the formation of indigo blue colour on the explant tissue (Figs. 24-27) Cotyledonary node, decapitated embryo, cotyledon attached decapitated embryo and shoot tip explants of Binamoog-5 and BARI mung -6 varieties hence subjected to *Agrobacterium*-mediated transformation experiments. Microscopic observations were also carried out to study the expression of GUS genes in various explants. Control explants were always maintained in each set of

experiment and were subjected to GUS histochemical assay in order to understand the difference between transformed and non transformed tissue. Results of these experiments are presented in Table 13. Co-cultured explants with *Agrobacterium* strain LBA4404 showed positive to GUS staining. GUS positive regions were visualized at the cut surfaces as well as within the internal tissues of various explants.

From Table 13 it was evident that, cotyledon attached decapitated embryo explants showed the best response towards transformation with LBA4404 strain and the percentage of GUS positive cotyledon attached decapitated embryo explants for Binamoog-5 and BARI mung-6 was 90.40 and 81.00 respectively (Figs. 26 and 27). Next to decapitated embryo explants showed better responses towards transformation and percentage of GUS positive explants for Binamoog-5 and BARI mung-6 was 73.33 and 68.75 (Figs. 24 and 25). Prominent blue colored (GUS +ve) zones within co-cultured explants were visualized following stereomicroscopic observation. Besides cotyledonary node explants showed good result towards transformation and percentage of GUS positive explants for Binamoog-5 and BARI mung-6 was 56.50 and 52.60 respectively. In case of shoot tip explants percentage of GUS positive explant for Binamoog-5 and BARI mung-6 was 39.16 and 35.64 which was lowest among the four explants studied.

As regeneration capacity for decapitated embryo and transformation efficiency of shoot tip explants low, therefore, these explant were excluded from further transformation experiments. Alternatively, in spite of maximum regeneration ability of cotyledonary node explants further transformation experiments to optimize the factors were not carried out because transformation rate of this explant was not satisfactory in compare with cotyledon attached decapitated embryo explants. So, further transformation experiments such as optimization of different parameters were done with cotyledon attached decapitated embryo explants.

Table 13. Responses of various explants from Binamoog-5 and BARI mung-6 towards genetic transformation with *Agrobacterium* strain LBA4404 containing the binary plasmid pBI121 analyzed by transient GUS histochemical assay

Variety	Explant	No. of explants assayed for GUS	No.of GUS+ve explants	% of GUS+ve explants
Binamoog-5	CN	200	113	56.50
	CADE	250	226	90.40
	DE	150	110	73.33
	ST	120	47	39.16
BARI mung-6	CN	150	79	52.60
	CADE	200	162	81.00
	DE	112	77	68.75
	ST	101	36	35.64

CN = Cotyledonary node, DE = Decapitated embryo, CADE = Cotyledon attached decapitated embryo, ST=Shoot tip

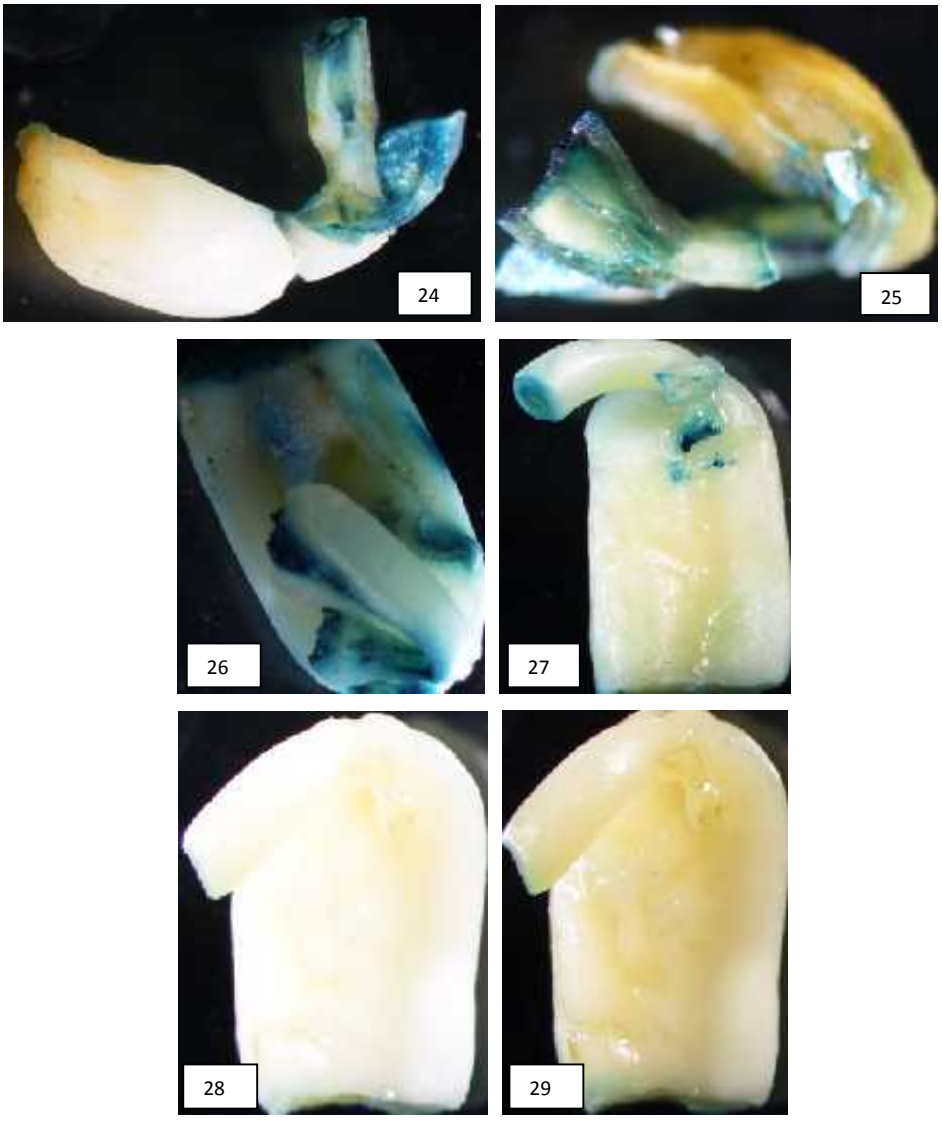

Fig. 24. Stereomicroscopeic view of GUS expression (blue coloured zone) following histochemical assay of decapitated embryo of Binamoog-5 mungbean variety.

Fig. 25. Same as Fig. 24 but in case of BARI mung-6 variety.

Fig. 26. Same as Fig. 24 but in case of cotyledon attached decapitated embryo explants of Binamoog -5 variety.

Fig. 27. Same as Fig. 26 but in case of BARI mung-6 variety.

Fig. 28. Same as Fig. 26 but controlled explant of Binamoog-5 showed no expression of GUS gene.

Fig. 29. Same as Fig. 28 but in case of BARI mung-6 .

4.2.2 Optimization of different parameters influencing transformation efficiency

Different parameters influencing transformation efficiency are optical density (OD) of *Agrobacterium* suspension culture, duration of incubation period of explants in bacterial suspension and co-cultivation period, on which the transformation efficiency depends significantly. These parameters were optimized during this study. *Agrobacterium* strains LBA4404 containing binary plasmid pBI121 was used in this experiment to transform decapitated embryo attached cotyledon explants of Binamoog-5 and BARI mung -6 variety of mungbean . Optimizations of these conditions were done by monitoring transient expression of the GUS reporter gene after co-cultivation .

4.2.2.1 Influence of optical density of *Agrobacterium* suspension on transformation of decapitated embryo attached cotyledon explants of two mungbean variety

Optical density of *Agrobacterium* suspension is one of the most important influencing factor of transformation efficiency. *Agrobacterium* suspension prepared from the overnight grown *Agrobacterium* culture was used to infect the explants. The relationship between optical density and transformation efficiency of decapitated embryo attached cotyledon explants of two mungbean varieties were studied. For this purpose optical density (O.D.) was measured at 600 nm and bacterial suspension with optical density of 0.39 ,0.56 0.82, 1.0, 1.2 and 1.3 were used in these experiments.

Above mentioned six types of O.D. of bacterial suspension were used, maximum transformation efficiency was observed 100% and 88% at 0.56 O.D of bacterial suspension for Binamoog-5 and BARImung-6 respectively. Therefore, to optimize other factors and for all the transformation experiments *Agrobacterium* suspension with OD 0.56 was used (Table 14).

4.2.2.2 Influence of incubation period on transformation of two mungbean variety

Second important parameter is the incubation period for the explants. During this period the explants were incubated into bacterial suspension for a definite duration. For this purpose different incubation periods, such as 20, 30, 40 and 50 minutes were applied using bacterial suspension with a constant optical density of 0.56. The results of this

study are presented in Table 15 From the result it is proved that, maximum percentage (100%) of the transformed explants was observed with an incubation period of 30 minutes. Therefore, in *Agrobacterium* suspension of the strain incubation period of 30 minutes was found to be best for the explants of two mungbean varieties.

4.2.2.3 Influence of co-cultivation period on transformation of two mungbean variety

Duration of co-cultivation is another factor which influences the transformation efficiency significantly. Different periods from 2 - 4 days were tried to find out the optimum co-culture period .It was observed that, at a constant optical density (0.56) of bacterial suspension and a constant incubation period (30 minutes), percentage of transformation could be increased with the increase of co-cultivation period. But, during long co-cultivation period (more than 3 days), bacteria were found to grow heavily on the co-culture medium which was not suitable for the growth and survival of co-cultured explants and finally failed to regenerate. Therefore, co-cultivation period of 3 days was found to be the most suitable when transformation experiment was performed under optimum condition (Table 16).

Table 14. Influence of optical density (measured at 600 nm) of *Agrobacterium* (strain LBA4404/ pBI121) suspension on transformation of cotyledon attached decapitated embryo explants analyzed by transient GUS histochemical assay for two mungbean varieties

Varieties	OD_{600}	Number of explants infected	Number of explants assayed for GUS	Number of explants +ve for GUS	% of GUS +ve explants
Binamoog-5	0.39	50	25	8	32
	0.56	**50**	**25**	**25**	**100**
	0.82	50	25	21	84
	1.00	50	25	18	72
	1.20	50	25	14	56
	1.30	50	25	12	48
BARI mung- 6	0.39	50	25	7	28
	0.56	**50**	**25**	**22**	**88**
	0.82	50	25	19	76
	1.00	50	25	15	60
	1.20	50	25	12	48
	1.30	50	25	10	40

Table 15. Influence of different incubation period on transformation of cotyledon attached decapitated embryo explants analyzed by transient GUS histochemical assay for two mungbean varieties

Varieties	Incubation Period(min)	Number of explants infected	Number of explants assayed for GUS	Number of explants +ve for GUS	% of GUS +ve explants
Binamoog-5	20	50	25	20	80
	30	**50**	**25**	**25**	**100**
	40	50	25	19	76
	50	50	25	15	60
BARI mung- 6	20	50	25	18	72
	30	**50**	**25**	**22**	**88**
	40	50	25	16	64
	50	50	25	13	52

Table 16. Influence of different co-cultivation period on transformation of cotyledon attached decapitated embryo explants analyzed by transient GUS histochemical assay for two mungbean varieties

Varieties	Co-cultivation period	Number of explants infected	Number of explants assayed for GUS	Number of explants +ve for GUS	% of GUS +ve explants
Binamoog-5	2	50	25	17	68
	3	**50**	**25**	**25**	**100**
	4	50	25	15	60
BARI	2	50	25	16	46
mung- 6	**3**	**50**	**25**	**22**	**88**
	4	50	25	14	65

4.2.3 Optimum conditions for *Agrobacterium* transformation

For *Agrobacterium* strain (LBA4404/pBI121), bacterial suspension having an OD of 0.56 with 30 minutes of incubation and 3 days of co-cultivation period was found to be optimum for transformation of cotyledon attached decapitated embryo of two varieties of mungbean.

4.2.4 Shoot regeneration from co-cultured explants

After co-cultivation, the explants were transferred to regeneration medium in obtaining regeneration of shoots. Experiments were conducted with cotyledon attached decapitated embryos and cotyledonary nodes of Binamoog-5 and BARI mung -6 variety of mungbean using *Agrobacterium* (LBA4404/pBI121). After performing transient GUS expression the remaining explants following co-cultivation were washed with 300 mg/l ticarcillin for 10 minutes and then transferred to suitable regeneration medium (MS medium with 4.0 BAP μM).

In the present study, the selection pressure was not applied immediately after co-cultivation. Shoot regeneration media supplemented with 100 mg/l ticarcillin were used for the elimination of all unwanted bacteria but no selectable agents were used at this stage. When the regenerating shoots attained a height of 2.0-3.0 cm, the shoots were sub-cultured in the same media but with selectable agents. Kanamycin was used as selective

agent. In each set of experiments, regenerated control explants were also maintained to perform various comparative studies between transformed and non transformed tissues. It was observed that when kanamycin was applied immediately after co-cultivation, most of the explants even failed to survive in presence of kanamycin. Regeneration of shoots was found to initiate after 14-15 days from the co-cultured explants in kanamycin free regeneration medium. Then the regenerating explants were sub-cultured on the same medium supplemented with lower concentration of kanamycin (100 mg/1) .

Table 17. **Effects of kanamycin towards initiation of regeneration on MS medium containing 4.0** μM/l **BAP after co-cultivation in case of both the varieties**

Kanamycin cone. (mg/1)	Total no. of explants infected	% of explants showing regeneration responses	Colour of explants
Binamoog-5			
Without kanamycin	25	96	green
100	25	10	yellow
150	25	0	Deep brown
BARI-mung-6			
Without kanamycin	25	88	green
100	25	10	yellow
150	25	0	Deep brown

4.2.5. Elimination of non transformed shoot

Elimination of non transformed shoots was necessary for the selection of putative transformed shoots. Elimination of non transformed shoots was done using selective agent kanamycin because in this experiment the strain (LBA4404 containing pBI121 plasmid) used for transformation possessed kanamycin resistance gene (nptII).

To eliminate all the non transformed shoots, the transformed shoots was first initiated on a medium with a low concentration of selective agent (50 mg/l kanamycin) but this concentration was gradually increased with each subculture at 12-15 days of interval. During each subculture, the albino and deep brown dead shoots were discarded and only green shoots were sub-cultured on fresh medium containing the next higher concentration of kanamycin. During this period the number of non transformed shoots was also recorded.

4.2.6. Determination of optimum kanamycin concentration for selection

As kanamycin resistant gene was transformed, the shoot developed from the transformed tissues was supposed to survive in the selection medium supplemented with kanamycin. On the other hand, shoots regenerated from the non transformed tissues could not survive in selection medium since they did not possess the kanamycin resistant gene.

The shoots developed from both transformed and non transformed (control) decapitated embryo attached cotyledon explants were transferred to selection medium. To determine the optimum selection level non infected explants (those served as negative control) were subjected to selection pressure. For this experiment after the initiation of regeneration the explants were transferred to suitable regeneration medium containing kanamycin, similar to those explants used for transformation experiments. Then the concentration of kanamycin was increased gradually in the following manner: 50, 100, 150, 200, 250 and 300 mg/l.

From this experiment it was revealed that with the increase of kanamycin concentration the percentage of survived shoots were found to decrease. Due to effect of kanamycin the shoots first became albino and finally died. In this study all the non infected explants (negative control) died in presence of 150 mg/l kanamycin for within 15-20 days .

Table 18 The Effect of selective agents (kanamycin) on the regenerating shoots from infected explants of two mungbean varieties

Varieties	No. of infected explants	No. of shoots survived in selection medium					
		(50 mg/l kanamycin)	(100 mg/l kanamycin	(150 mg/l kanamycin)	(200 mg/l kanamycin	(250 mg/l kanamyci)	(300 mg/l kanamycin
Binamoog-5	500	488	450	410	321	212	164
BARI mung-6	500	474	433	401	303	195	123

4.2.8 Selection of transformed shoots

For successful transformation experiments towards the development of transformed shoots, it is important to select the transformants from the regenerating plantlets. Selection of transformed plants was done using kanamycin, since kanamycin resistance gene was used as component of the plasmid of the engineered *Agrobacterium*. In this case kanamycin was used as a selectable marker.

During these experiments the shoots were transferred to selection medium containing 50 mg/l kanamycin. After 14 days, only green and healthy shoots were subcultured in fresh medium with 100 mg/l kanamycin. To select transformed shoots, kanamycin concentration was raised up to 150 mg/l in the third subculture and 200 mg/l in the fourth subculture and then 300mg/l in the fifth subculture transformed shoots survive in this high concentration of kanamycin containing medium. The results of this experiment are presented in Figs. 30-35

Finally those shoots that survived in this selection medium containing 300 mg/l kanamycin for 15 days and remained green and healthy were selected as possible transformed shoots .A good number of decapitated embryo attached cotyledon derived shoots were recovered after such selection. Results are shown in the Table 18. A total of 164 for Binamoog-5 and 123 for BARI mung-6 kanamycin resistance shoots out of approximately 500 for Binamoog-5 and 500 for BARI mung-6 infected shoots were recovered in final selection medium with LBA4404 strain of *Agrobacterium* containing binary plasmid pBI121.

Fig. 30. Elongation of putatively transformed shoots BARI mung-6 variety produced from *Agrobacterium* infected CN explants on 150 mg/l kanamycin containing MS medium where the non-transformed shoots were failed to survive.

Fig. 31. .Same as Fig. 30 but in case CN explant of Binamoog-5.

Fig. 32. .Same as Fig. 30 but in case CADE explant of BARI mung-6.

Figs. 33-34. Same as Fig 30 but in case CADE explants of Binamoog-5.

Fig. 35. Same as Fig 30 but in case CN explant of BARI mung-6.

4.2.9 Histochemical GUS assay of the transformed plantlets developed under selection pressure

Experiments were carried out to monitor the stable expression of GUS gene in the developing shoots which were under selection pressure. For this purpose, at the time of each sub-culture, developing stem and leaves were subjected to GUS-assay to detect the incorporation of foreign genes.

Further stable expression of GUS genes within the various organs of the developing putative transformed plants in presence of higher concentration of kanamycin (300 mg/1) were tested through GUS - histochemical assay. These tests were carried out regularly for more than three months and the results of these experiments are presented in Table 19.

Following the selection pressure of 300 mg/1 kanamycin 25 shoots were tested randomly for histochemical GUS localization. It was found that, out of 25 shoots and 25 leaves 18 shoots and 22 leaves showed characteristic expressions for GUS gene. Conspicuous blue color was found in the leaf (Fig. 36(a-i)).

Table 19. Stable GUS expressions in the putative transformed plantlets following three months of selection in presence of higher concentration of kanamycin (250-300 mg/1)

Plant organs tested	Total No. of organs tested	No. of GUS positive organs	Percentage of GUS positive organs
Leaf	25	22	88
shoot	25	18	72

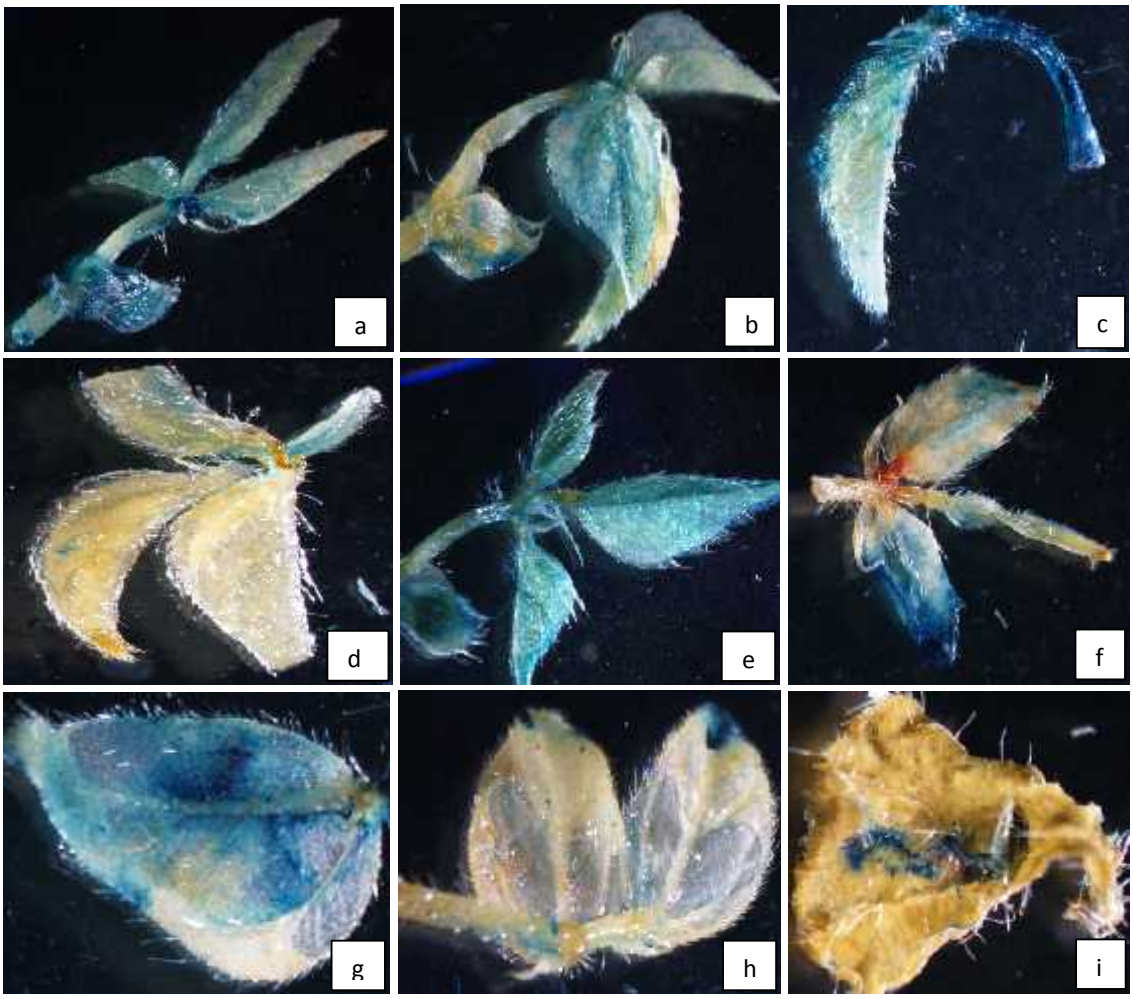

Fig 36(a-i). Expression of GUS (blue coloured zone) gene in the regenerated shoots obtained from CADE explants in different varieties of mungbean. Variable responses of GUS expression was noticed at different optical density (O.D.) as well as variable incubation periods of *Agrobacterium* suspension.

a ,b. OD-0.56, 30 mins of incubation

c. OD-1.2 with 20 min of incubation period in case of CADE explants

d. OD-1.3 with 25 min of incubation period in case of CADE explants

e. OD- 1.2 with 30 mins of incubation period in case of cotyledonary node explants

f. OD-1.0 with 30 mins of incubation period in case of from CADE explants

g. OD- 1.2 with 20 mins of incubation period in case of CADE explants

h. OD-1.4 with 25 mins of incubation period in case of CADE explants

i. OD-1.4 with 20 mins of incubation period in case of CADE explants.

4.2.10 Root induction from the transformed shoots and establishment in the soil

The transformed shoots (2-3 cm in length) survived in the selection medium were separated and transferred to MS medium containing 2.0 μM/l IBA + 100 mg/l ticrecilin . Ticrecilin was used in the rooting medium to avoid bacterial growth. It was found that, 22-25 days was required to initiate rooting. Fig. 37 shows the transformed shoot producing roots in MS with 2.0 μM/l IBA and 50 mg/1 ticrecilin. These transformed plantlets were then transplanted to soil (Fig 38). These plants were found to survive following proper acclimatization.

Fig 37. Root formation of transformed shoot on MS + 2.0 μM IBA in case of Binamoog-5 variety.

Fig 38. Establishment of transformed shoot of Binamoog-5 in soil.

4.4 Analysis of transgene integration

The genomic DNA from the leaf of transformed shoots as well as from control shoots were isolated and visualized through agarose gel electrophoresis (Fig. 39). At the same time DNA of a maintained engineered *Agrobacterium* strain containing GUS and *nptII* gene was isolated and visualized to use as positive control during polymerise chain reaction (PCR).

Genomic DNA of all the kanamycin resistant plants was analyzed by PCR to detect the presence of the GUS and *nptII* gene.

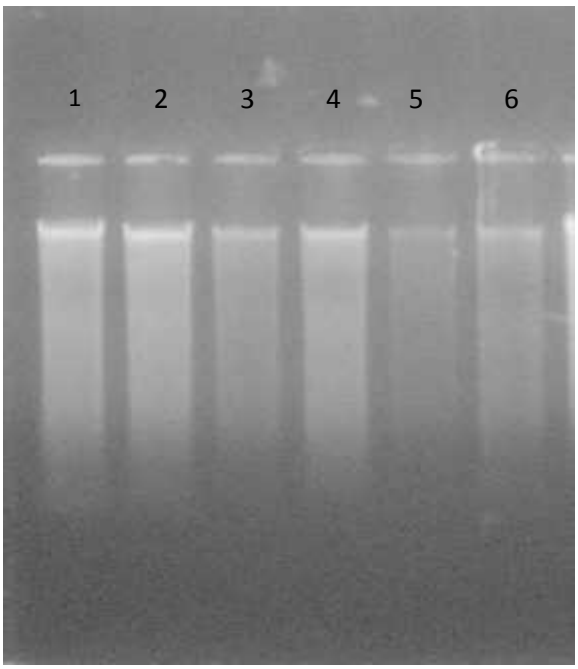

Fig 39. Lane 1-6 : Genomic DNA of transformed shoots of mungbean.

4.4.1 PCR for the amplification of *nptII* gene

The DNA isolated from leaves of both transformed and non-transformed mungbean shoots were subjected to PCR for the amplification of *nptII* gene.The Primers that were used for amplification were forward 5'-ATAAACCCAGCGAACCATTTGAGGT-3'and reverse 5'-TACGCAGCGGTATTTTTCGATCAGT-3. For the amplification of DNA through PCR 30 cycles were maintained. After 30cycles the amplified DNA was again analyzed through agarose gel electrophoresis. Fig. 40 is presented to show the results obtained following PCR analysis. From the gel it was observed that bands formed in six transformed plants were identical to DNA obtained from *Agrobacterium* strain LBA4404-pBI121 . Therefore the six mungbean plants that were subjected to PCR were identified as transformed plant. This result indicated that the *nptII* gene was inserted in the genomic DNA of 6 transformed mungbean plants.

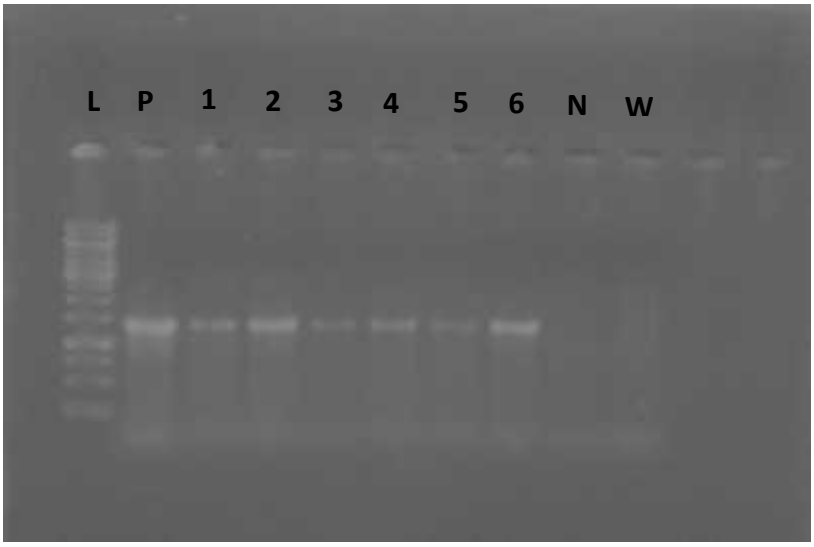

Fig. 40. PCR amplification of *nptll* gene of putative transformants. Lane L-ladder, lane P - positive control, lanes 1-6-genomic DNA from transformed shoot of mungbean, Lane N- negative control, lane W- water control.

Lane	Genomic DNA	Amplification
P	Positive control	+ve amplification
1-6	Transgenic mungbean	+ve amplification
N	Negative control	No amplification
W	Water control	No amplification

4.4.2 PCR for the amplification of GUS gene

In the case of GUS, the isolated DNA both from transformed and nontransformed plants was subjected to PCR amplification. The primers that used for amplification were GUSA-10: 5'-CCT GTA GAA ACC CCA ACC CG -3' and reverse GUS-769: 5'-TGG CTG TGA CGC ACA GTT CA-3'. For the amplification of DNA through PCR 30 cycles were maintained. The PCR amplified DNA were analyzed through agarose gel electrophoresis. From the gel it was found that one transformed plant produced the corresponding band after amplification, identical to the band of DNA obtained from *Agrobacterium* strain LBA4404-pBI121 (Fig. 41.). On the other hand as control plant did not have any gus gene it showed no band after visualizing the PCR amplified DNA .

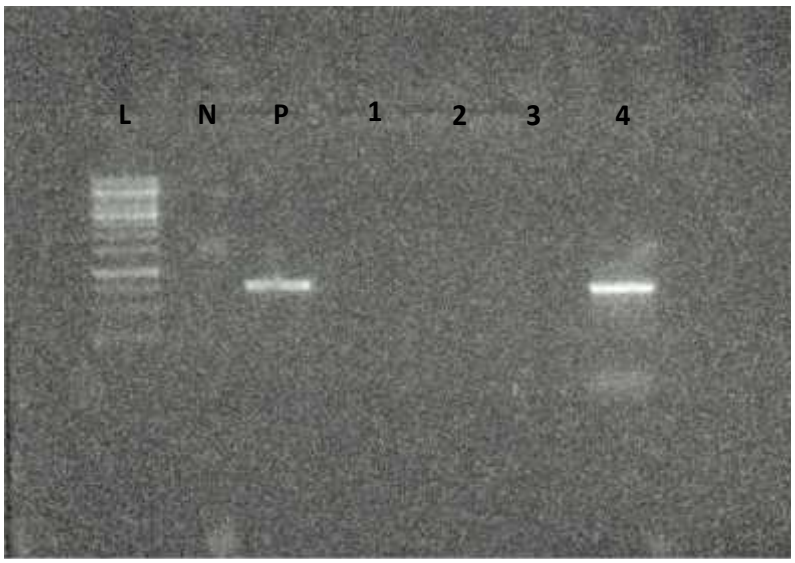

Fig. 41. PCR amplification of GUS gene putative transformants. Lane L- ladder, lane N- negative control, lane P -positive control, lane 4 -genomic DNA from transformed shoot of mungbean.

Lane	Genomic DNA	Amplification
N	Negative control	No amplification
P	Positive control	+ve amplification
4	Transgenic mungbean	+ve amplification

5. DISCUSSION

The present investigation was undertaken to establish an efficient and reproducible *in vitro* regeneration and *Agrobacterium*-mediated genetic transformation protocol for mungbean (*Vigna radiata* (L.) Wilczek) varieties growing in Bangladesh. Two different varieties of mungbean, namely, Binamoog-5 and BARI mung-6 were used in this study. *Agrobacterium* strain, namely, LBA4404 harboring binary vector plasmid pBI121 was used for transformation experiments. The strain contain screenable marker β-glucuronidase (GUS) and kanamycin resistance *nptII* gene as selectable marker. In the first phase of the investigation, an efficient *in vitro* regeneration protocol was established for the two mungbean varieties. In the second phase, steps were taken to standardize the various factors involved in the transformation experiments. The third phase was aimed to develop *Agrobacterium*-mediated genetic transformation protocol for the two varieties of mungbean. Various explants were also examined to find their transformation efficiency. The transformants developed through this study were characterized through PCR analysis to study the introgression of foreign genes. Steps were also taken to establish transgenic mungbean plants in soil.

Prior to the regeneration experiments, investigations were carried out to determine the effects of the duration of surface sterilization for the mungbean seeds using 0.1% mercuric chloride ($HgCl_2$) solution. In both the varieties of mungbean, percentage of contaminated seeds decreased with increased sterilization period. However, an increase of sterilization period also caused a decreased rate in the regeneration capacity of surface sterilized seeds. Islam (1990) and Mila (1991) applied a sterilization period of 20 minute using 0.1% $HgCl_2$. Khan and Rabbani (1999), Sarker and Mustafa (2002) and Sultana (2005) used a sterilization period of 15 minute for sterilizing explants in case of different potato varieties before regeneration. In the present investigation an optimum response in terms of reduced percentage of contamination and increased rate of responsive seeds was observed with surface sterilization period of 15 minutes with 0.1% $HgCl_2$ for both the two varieties of mungbean.

An efficient and reproducible *in vitro* regeneration system is a prerequisite for the development of transgenic plants through transformation (Gardner 1993). Therefore, regeneration from appropriate tissue of the whole plantlets is important in developing a genetic transformation protocol. Several attempts were made in establishing a suitable regeneration protocol for mungbean. Among the various important crops, grain legumes have been considered as recalcitrant due to their passiveness to *in vitro* techniques (Bajaj and Ghosal 1981, Mroginski and Karth 1984). As a result, success of transformation in leguminous crops is limited (Nisbet and Webb 1990). In spite of this fact, several reports have been published on *in vitro* regeneration of plantlets from different grain legumes including pea, chickpea, common bean, cowpea, soybean, mungbean and peanut (Schroeder *et al.* 1993, Jayanand *et al.* 2003, Aragão *et al.* 2002, Kartha *et al.* 1981, Ikea *et al.* 2003, Hinchee *et al.* 1988, Jaiwal *et al.* 2001, Wang *et. al.* 1998, Sarker *et al.* 2003). Although many legumes have been regenerated using tissue culture techniques, very few efficient regeneration protocols are presently available to use them in transformation experiments. Moreover, in mungbean reports of success towards *in vitro* regeneration are few compared to other crops.

From the available literature it is known that not much work has been done in the improvement of mungbean mainly through the application of tissue culture. Successful programs on genetic improvement of legumes based on cell and tissue culture technology depend largely on efficient plant regeneration system. In general plant regeneration of *Vigna* sp. is very limited compared to those of other grain legumes. Shoot regeneration via callus formation was tried by several previous workers using various concentrations and combinations of hormonal supplements. However, their attempts to regenerate shoots from the induced callus were not successful (Bhadra *et al.* 1989, Avenido and Hautea 1990, Bose *et al.* 1992, Sarker and Siddiqua 2004). Mathews and Rao (1984) and Mathews (1987) also failed to regenerate shoots from established callus of mungbean.

In the present investigation, the mode of regeneration adopted here was direct organogenesis (without intervention of callus) as the callus phase and its duration are negatively correlated with the regeneration ability of explants. Moreover, somaclonal variation can influence the phenotype of regenerated plants in case of indirect

organogenesis (Fontana *et al.* 1993). Therefore, in this study emphasis was given on developing a direct regeneration protocol for mungbean.

For this purpose explants were collected from aseptically germinated seeds and seedlings. 0.8% agar solidified ½MS media containing 3% sucrose, MS medium containing 3% sucrose with 0.8% agar and MS medium supplemented with BAP containing 3% sucrose with 0.8% agar were used for seed germination and seedling development. Seed germination rate and time required for germination were more or less identical for both varieties used in this experiments. Uniform Seed germination rate was higher and consequent development of seedlings were on MS medium supplemented with BAP containing 3% sucrose with 0.8% agar after 4 - 5 days.

During the present investigation, experiments were carried out to regenerate shoots using various explants from both the varieties. Cotyledonary nodes (CN), shoot tips (ST) and hypocotyls (HC) from *in vitro* seedlings and decapitated embryo (DE), cotyledon attached decapitated embryo (CADE) and single cotyledon (C) from overnight soaked surface sterilized seeds were used as explants. All kinds of explants were inoculated to a medium containing MS + 5.00 µM BAP where regeneration of shoots was obtained. It was observed that among all explants such as cotyledonary node (CN), cotyledon attached decapitated embryo (CADE), shoot tip (ST), hypocotyls (HC), decapitated embryo (DE) and single cotyledon (C) explants, cotyledonary node was found to be the best explant towards the number of regenerated shoots. Whereas hypocotyls (HC), decapitated embryo (DE) and single cotyledon (C) were not suitable as explants for regeneration. In mungbean Afrin (2009) found best shoot regeneration from CADE explants which is similar to present study but Zahan (2007) found best result from cotyledon explant which is dissimilar to present study.

The different concentrations and combinations of auxin and cytokinin under the *in vitro* condition have been reported to greatly influence the morphogenesis of plants (Skoog and Miller 1957). In the present study, different concentrations of BAP, Kn, and NAA were used either singly or in combinations in MS medium to evaluate their effects on shoot regeneration and ultimately to find out the proper combinations and concentrations of growth regulators for initiating and developing multiple shoots from three different

explants. MS medium has been found to be effective for plant regeneration. This is an agreement with that of the findings of Reddy and Reddy (1993) in *Arachis hypogaea*, Kartha *et al.* (1981) in soybean, cowpea, peanut, chickpea and bean, Narasimhulu and Reddy (1983) in *Arachis hypogaea*, Zahan (2007) in *Vigna radiata*.

BAP is the most widely used and most effective cytokinin in legumes, including *Vigna* species (Gulati and Jaiwal 1994, Sahoo *et al.* 2002, Saini and Jaiwal 2003). Therefore, in the present investigation MS medium supplemented with different concentrations of BAP (2. μM – 10 μM) were used to examine the effects of BAP on multiple shoot induction and their subsequent development from different explants. It was observed that among different concentrations of BAP, MS medium supplemented with 4.0 μM of BAP was found to be best in response multiple shoot regeneration for both varieties of mungbean. Afrin (2009) reported more or less identical BAP concentration (4.44 μM) for shoot regeneration from CADE explant. Increasing BAP concentration did not produce more number of shoots, rather decreased the regeneration efficiency. Zahan (2007) reported similar findings in shoot regeneration from cotyledon explants. From present study, the maximum number of shoots were observed on MS medium supplemented with 4.0 μM BAP for Binamoog-5 variety, in an average of 20.5 shoots per cotyledonary node, 4.5 shoots per shoot tip and 8.3 shoots per CADE explants. In case of BARI-6 variety the maximum number of shoots were 16.5 from CN, 4.5 from ST and 7.3 from CADE explants in the same concentrated medium. In this study, 96% of CN explants were responsive BINA mung-5 towards shoot initiation, whereas 94% of BARI mung-6 explants. Shoot initiation started from 10-15 days old explants but maximum no. of shoots were observed after 60-70 days of inoculation. Multiple shoots developed on this medium were found green and healthy. However the length of shoots developed using these hormonal supplements was 4.5cm from CN and 4.3 from CADE in case of BINA-5 and for BARI-6 it was 4.2 from CN and 4.1 from CADE explants .

Different concentrations and combinations of BAP (2.0 and 4.0 μM) and Kn (0.5– 1.5 μM) on MS medium were used for multiple shoot induction directly from different explants of BINA mung-5 and BARI mung-6 varieties. Among all the concentrations and combinations of BAP and Kn, best response towards multiple shoots initiation and development was observed on MS medium supplemented with 4.0 μM BAP and 0.5 μM

Kn for both varieties. In this media combination, BINA mung-5 showed 90% response from CN and 88% from CADE explants towards shoot initiation whereas 85% response from ST explants and mean number of shoots obtained per explant was 7.5, 5.6 and 4.3 in case of CN, CADE and ST explants, respectively. In case of variety BARI mung-6 maximum percentage of shoot induction from the CN, CADE and ST were 88%, 86% and 84%, respectively. It was observed that the maximum number of shoot proliferation were 6.5, 4.6 and 3.3 from CN, CADE and ST explants, respectively. It is noticed that multiple shoot induction was not very satisfactory on this media combination. Zahan (2007) found best response in BARI mung-5 and Binamoog-5 on MS supplemented with 0.5 mg BAP and 0.5 mg Kn and for BARI mung-3 on MS supplemented with 0.5 mg BAP and 0.1 mg Kn. It was also more or less identical result with the this study. As for BAP and Kn combination, MS medium supplemented with 4.0 µM BAP and 0.5 µM Kn was found best in the present study.

MS medium supplemented with different combination and concentration of BAP and NAA were used to observe their effect on multiple shoot induction and their subsequent development. It is observed that for BINA mung-5, the maximum number of shoots were developed in an average of 29 shoots per CN, 20.2 shoots per CADE and 10.6 shoots per ST after 60-70 days on MS medium supplemented with 10.0 µM BAP and 1.0 µM NAA . On this medium shoots were healthy, deep green and leaves were large. Maximum shoot length in an average is 5.2 cm from CN, 5.1 cm from CADE and 2.5 cm from ST explants was observed on the same medium. In case of variety BARI mung-6, after 60-70 days the maximum number of shoots were observed in an average of 26 shoots per CN, 18.2 per CADE and 10.2 shoots per ST explants on MS medium supplemented with same concentration and combination of BAP and NAA (10.0 µM BAP and 1.0 µM NAA). The shoots were healthy, green and leaves were large. Maximum shoot length in an average of 5.0 cm from CN, 4.6 cm CADE and 2.2 cm from ST was observed on same medium .It is noteworthy that , before collecting explants (CN, CADE and ST) seeds were germinated on 4.0 µM BAP supplemented MS medium. Then explants were collected and inoculated on MS medium supplemented with 10.0 µM BAP and 1.0 µM NAA. After 30 days, explants were subcultured on MS medium supplemented with 4.00 µM BAP and after 60-70 days of culture maximum number of multiple shoots were observed. Yadev et al (2010) reported the similar observation but they did not mention subculture media

concentration. In the present study it was found that the best subculture medium was MS medium supplemented with 4.00 μM BAP. It was found to be the best regeneration medium for all explants (CN, CADE and ST) of both varieties among different combination used in the present experiments.

Induction of healthy root from the regenerated shoot is an essential part for successful development of plantlets. For root induction, regenerated shoots were cultured on both full and half strength of MS medium supplemented with IAA and IBA. It was observed that root induction occurs rarely when half strength of MS medium with different concentrations of auxins was used for mungbean varieties. Full strength of MS medium containing 2.0μM IBA was found to be effective for root induction and for its subsequent development for both varieties of mungbean tested. The present findings showed similarities in terms of hormone concentration with the findings of Bose *et al.* (1991) and Zahan (2007) but dissimilar in terms of strength of MS medium. However, Siddiqua (2004) reported that full strength of MS medium containing 4.90 μM IBA was found to be the best for rooting which is similar in strength of MS medium but dissimilar in hormonal concentration. This differential response towards root induction may be due to the difference of genotypes used by Siddiqua (2004) and in the present investigation.
The overall results of the present study indicate that the mungbean varieties used in the investigation are very responsive to tissue culture techniques. Therefore, it is recommended to incorporate desired genes by transformation to improve their agronomic or qualitative properties (An *et al.* 1986; Sheerman and Bevan 1988 and Visser *et al.* 1989).

The last phase of these present investigation deals with the genetic transformation for mungbean varieties grown in Bangladesh. Among the different approaches, *Agrobacterium*-mediated genetic transformation has been considered as the most common and successful method used in various leguminous crop plants such as soyabean (Hinchee *et al.* 1988; Meurer *et al.* 1998), chickpea (Fontana *et al.* 1993; Kar *et al.* 1996), peanut (Mc Kently *et al.*1995, Sarker *et al.* 2003), wheat (Sarker *et al*. 2002).

Mungbean is considered to be an under exploited crop since limited research has been done so far to improve mungbean varieties compared to other pulse crops. There are a

few reports of the production of transgenic mungbean plants using *Agrobacterium*-mediated genetic transformation (Jaiwal *et al.* 2001, Mahalakshmi *et al.* 2006). There is also report on transient GUS assay using various infected explants of local variety BARI mung-3 and Binamoog-5 (Sarker and Siddiqua 2004, Zahan 2007, Islam *et al*, 2010)

Agrobacterium-mediated genetic transformation procedure is be1ieved to be influenced by several factors (Mansur *et al.* 1993). Factors that influence successful transformation such as *Agrobacterium* strain, genotype of the host, explant type, incubation period of the explants, density of the bacterial suspension were also optimized during the present investigation. The introgression of GUS reporter gene was monitored through GUS histochemical assay in the present study.

Evaluation of *Agrobacterium* strain and mungbean variety compatibility is an important step in the establishment of an efficient transformation protocol. Therefore, compatibility of the *Agrobacterium* strain with the two mungbean varieties, namely, Binamoog-5 and BARI mung-6 were examined. It was found that *Agrobacterium* strain, LBA4404 containing the binary plasmid pBI121 showed positive interaction with both varieties of mungbean.

In the present study, transformation experiments were performed using four explants, namely, cotyledonary node (CN), cotyledon attached decapitated embryo (CADE), decapitated embryo (DE) and shoot tip (ST) for both varieties to determine their transformation ability. Among the four explants, CADE and CN showed better response towards transformation than DE explants and ST. Yadev *et al* (2010) reported similar effective result from double cotyledonary node with *Agrobacterium* strain and Mahalakshmi *et al.* (2006) found genetic transformation from primary leaf explants (cut at the node) with *Agrobacterium* strain. As regeneration capacity for decapitated embryo and transformation efficiency of shoot tip explants low, therefore, these explants were excluded from further transformation experiments. Alternatively, in spite of maximum regeneration ability of cotyledonary node explants further transformation experiments to optimize the factors were not carried out because transformation rate of this explant was not satisfactory in compare with cotyledon attached decapitated embryo explants. So,

further transformation experiments such as optimization of different parameters were done with cotyledon attached decapitated embryo explants.

During optimization of regulatory factors maximum number of explants found to be transformed with bacterial suspension having an optical density of 0.56 at 600 nm for both the varieties. Moreover, 30 minutes incubation period followed by 72 hours of co-cultivation were found to be most effective towards transformation. In *Vigna radiata* Jaiwal *et al*. (2001), Tazeen and Mirza (2004) reported that optical density of 1.0 and 3 days of co-cultivation were considered optimal , Islam and Islam (2010) found that 45 minutes incubation and OD of 1.3 at 600nm and 72 hours cocultivation was best. In chickpea, Krishnamurthy *et al.* (2000) were able to develop transgenic chickpea when they incubated mature embryo explants for 20 minutes followed by co-cultivation for three days. Tewari-Singh *et al.* (2004) were also able to develop transgenic chickpea following the same co-cultivation period of three days. However, they incubated explants in bacterial suspension for 1 - 2 hours. Lecardonnel *et al.* (1999) used 40 minutes for infection and co-culture was maintained for 3 days in potato. Influence of regulatory factors on the transformation efficiency of various explants was also observed by Islam (1998) in potato and by Mansur *et al.* (1993) in peanut.

Agrobacterium strain LBA4404 have *nptll* gene within their T-DNA and this gene confers kanamycin resistance to the transformed cells. Therefore, selection of the transformants was carried out using various concentrations of kanamycin. However, presence of kanamycin greatly hampers growth of the explants and as a result many putative transformants may be lost. For this reason immediately after co-cultivation kanamycin was not applied for selection, rather, co-cultivated explants were first allowed to regenerate in regeneration media without any selective agents. After 15-20 days the transformed explants with small shoots (2.0- 3.0 cm) were subjected to selection pressure. This observation is similar to the results obtained in other plant species including flax, alfalfa, peanut and chickpea, where a preculture period and/ or a delayed selection with kanamycin were used in obtaining regeneration from explants with high transformation frequency (Pezzotti *et al.* 1991, McHughen *et al.* 1989, Cardi *et al.* 1992).

In the present investigation higher concentrations of kanamycin were applied to screen the transformed shoots effectively. For this purpose kanamycin concentration was gradually increased from 50 to 300 mg/1. During this investigation, it was found that all the control shoots failed to survive at 150 mg/1 kanamycin within 15-20 days. Sarker *et al.* (2003) also increased kanamycin concentration gradually from 50 - 200 mg/l in BM-2 and BM-4 varieties of lentil. In chickpea, Kar *et al.* (1996) applied two concentrations of kanamycin for the selection of transformed shoots (25 and 50 mg/l). Therefore, the shoots survived in the medium containing 150 mg/1 kanamycin were considered as transformed. Shoots that survived in the final selection pressure were subjected to rooting. It was found that these shoots failed to regenerate roots in rooting media supplemented with kanamycin of different concentrations. Therefore, rooting of transformed shoots was carried out without selection pressure during the present study. In chickpea, Fontana *et al.* (1993) and Kar *et al.* (1996) used media without selectable agents for rooting of putative transformed shoots.

Histochemical GUS assay (Jefferson 1987) was performed to detect the expression of GUS gene in the transformed explants and plantlets. Such histochemical assay of selected shoots demonstrated the presence of blue coloured zones on few selected shoots and leaves indicating the typical expression of GUS gene. Expression of the GUS gene in the plantlets obtained following selection indicated the stable integration of the inserted gene. In the present investigation, the plant parts (shoots and leaves) of the transformed plantlets showed presence of GUS positive blue colour in their respective tissues indicated the stable integration of GUS gene. However, in several occasions chimeric expression of GUS gene was also observed in a number of transformed shoots. A number of workers reported this kind of phenomenon in other crops. Ying *et al.* (1992) in *Carthamus* and Ottavani *et al.* (1993) in potato, found expression of GUS gene only in some resistant calli. This observation indicate that the expression of GUS activity in the regenerated shoots was not directly correlated with kanamycin resistance. The lack of GUS expression in kanamycin resistant shoots may be due to alternation or less expression of GUS gene resulted from rearrangement of the coding sequence or methylation of the gene (Battraw and Hall 1990, Ottavani *et al.* 1993).

After sufficient development of roots from the selected shoots, the plantlets were successfully transplanted to soil. Transformed plantlets were transferred to soil. But the survival rate of these plantlets was low compared to the controls. The low survival rate of the transformed plantlets in the soil is most likely due to the lack of adequate root development or the proper acclimatization of the plantlets. Histochemical localization of GUS was also performed with the fully developed plantlets growing in soil.

In the present investigation the transgenic nature of the transformed plantlets were confirmed through the application of specific molecular techniques like polymerase chain reaction (PCR) analysis. The DNA isolated from both of transformed and non-transformed shoots was subjected to PCR for the amplification of GUS and *nptII* gene. Amplified DNA was analyzed through agarose gel electrophoresis. From the gel it was observed that the single band formed in each of the six transformed plantlets were identical to the amplified DNA of *Agrobacterium* strain LBA4404 pBI121. This result indicated that the gene GUS and *nptII* was inserted in the genomic DNA of transformed plantlets.

Here, it may be pointed out that the selection procedure developed during this study has been found to be effective in recovering transformed plantlets. This selection technique can be used in future experiments to develop transgenic mungbean plants more efficiently. Moreover, the protocol developed here for the regeneration of plantlets from the co-cultured explants requires very simple hormonal supplements and appears to be less complicated. Therefore, this transformation compatible regeneration system can also be used in the future transformation experiments.

The protocol of A*grobacterium*-mediated genetic transformation developed through the present investigation can be used for the production of transgenic mungbean plants for specific purpose. In this case the protocol was primarily developed using screenable marker gene like GUS and selectable marker gene like *nptII.* Using this protocol, in future agronomically important gene/s can be transferred to the mungbean varieties grown in Bangladesh. Particularly for the development of yellow mosaic virus disease, insect and pest resistant mungbean variety this technique of transformation can be exploited.

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7. APPENDIX - A

ABBREVIATIONS

The following abbreviations have been used throughout the text:

A.	:	*Agrobacterium*
BAP	:	6-benzyleaminopurine
BARI	:	Bangladesh Agriculture Research Institute
BINA	:	Bangladesh Institute of Nuclear Agriculture
BBS	:	Bangladesh Bureau of Statistics
C	:	Centigrade / Celsius
$CaCl_2$	:	Calcium chloride
CaMV	:	Cauliflower Mosaic Virus
CH	:	Casein hydrolase
cm.	:	Centimeter (s)
CRB	:	Commodity Research Bureau
e.g.	:	Example gratia, for example
et al.	:	et alil and others
etc.	:	et cetra, and the rest
Fig/s	:	Figure / Figures
g/gm	:	gram (s)
GR	:	Growth regulators
GUS	:	ß-glucoronidase
he (s)	:	Hectore
hr (s)	:	Hour (s)
HCl	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
IAA	:	Indole- 3 – acetic acid
i.e.	:	id est = which to say in other words
Kbp	:	kilo base pair
Kg	:	Kilogram

Kn	:	Kinetin (6- furfuryl amino purine)
KNO_3	:	Potassium nitrate
l	:	Litre
lb / sq. inch	:	Pound per square inch
m	:	Meter (s)
M	:	Molar
mM	:	Millimolar
mg	:	Milligram
mg / l	:	Milligram per liter
min (s)	:	Minute (s)
ml (s)	:	Milliliter (s)
MS	:	Murashige and Skoog Medium 1962
MT	:	Murashige and Tucker Medium
NAA	:	α- naphthalene acetic acid
NaOH	:	Sodium hydroxide
Na_2 – EDTA	:	Sodium salt or ferric ethylene diamine tetra acetate
NH_4NO_3	:	Ammonium nitrate
No.	:	Number
NOS	:	Nopaline synthase
nm	:	Nanometer
nptII	:	Neomycine phosphotransferasae II
OD	:	Optical density
pH	:	Negative logarithm of Hydrogen
sec.	:	Second
Sp. / Spp.	:	Species
STAT	:	Statistical report
T- DNA	:	Transfer DNA
UV	:	ultraviolet wavelength
var. (s)	:	Variety (s)
vir	:	virulence region
viz	:	Namely
v / v	:	Volume by volume
Wt.	:	Weight

w / v	:	Weight by volume
X – gluc	:	5-bromo-4-chloro-3-indolyl glucoronide
YMB	:	yeast extract Mannitol Broth
µ	:	Micron
µM	:	Micromole
µl	:	Micro liter
µg	:	Microgram
I n	:	I Normal
%	:	percentage
+ve	:	Positive

APPENDIX-B

Murashige and Skoog (MS) Medium 1962

Constituents	**Concentrations**
Macronutrients	**(mg/l)**
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micronutrients	
$FeSO_4.7H_2O$	27.80
Na_2-FeEDTA	37.30
$MnSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Glycine	2.00
Nicotinic acid	0.50
Pyrodoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30,000.00

pH adjusted to 5.8 before autoclaving