DEVELOPMENT OF YELLOW MOSAIC VIRUS RESISTANT MUNGBEAN [*VIGNA RADIATA* (L.) WILCZEK] LINE THROUGH GENETIC TRANSFORMATION

Ph.D. THESIS

Submitted

BY

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BANGLADESH

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SUJAY KUMAR BHAJAN

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DEPARTMENT OF BOTANY, UNIVERSITY OF DHAKA
BANGLADESH

JULY, 2020

Dedicated

To

My Respected Parents

And

Beloved Family

CERTIFICATE

This is to certify that the thesis entitled "**Development of yellow mosaic virus resistant mungbean [*Vigna radiata* (L.) Wilczek] line through genetic transformation"** submitted by Sujay Kumar Bhajan has been carried out under our supervision in the Plant Breeding and Biotechnology Laboratory of the 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 Doctor of Philosophy in Botany, under the University of Dhaka.

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– The Author

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Mungbean [*Vigna radiata* (L.) Wilczek] yellow mosaic virus (MYMV) is responsible for the yellow mosaic disease causing maximum yield loss of mungbean in Bangladesh. RNAi-based antiviral strategy has been used with the purpose of generating yellow mosaic virus resistant transgenic mungbean lines through targeting coat protein (*CP*) and silencing suppressor gene (*AC2*). MYMV coat protein (*CP*) and silencing suppressor (*AC2)* gene specific primers were designed from the conserved regions after alignment of the available *CP* and *AC2* gene sequences in NCBI database. MYMV *CP* and *AC2* genes were amplified through PCR using gene specific primers. Sequence analysis of PCR amplified DNA confirmed the presence of MYMV coat protein and silencing suppressor gene. Amplified *CP* gene (750 bp) and *AC2* gene (450 bp) were cloned in an antisense orientation under CaMV35S promoter of pBI121 vector replacing the *GUS* gene by using BamHI and SacI restriction recognition sites in antisense orientation resulting pBI121CP-AC2 construct. The cloned construct was transferred to *Agrobacterium tumefaciens* strain LBA4404. The transformation efficiency of this newly developed antiviral gene construct was checked using tobacco as a model plant. Putatively transformed tobacco plants were recovered following *Agrobacterium*-mediated transformation and the *transgene* integration in tobacco plants was confirmed by polymerase chain reaction (PCR) analysis. Following these results a protocol for *Agrobacterium*-mediated genetic transformation was developed for locally grown mungbean varieties (BARI mung-3 and Binamoog-5) using *Agrobacterium* strain LBA4404 harboring binary plasmid pBI121 containing *GUS* (β–glucuronidase) and *nptII* (neomycin phosphotransferase II) genes (Construct I). Among the explants studied cotyledon attached decapitated embryo (CADE) explant of mungbean was found to be suitable for transformation. Best response (80%) towards multiple shoots regeneration from CADE explant was achieved on MS medium containing B5 vitamins supplemented with 5.0 µM BAP following 28 days of culture. Bacterial suspension having an O.D of 0.6 (at 600 nm) in an incubation period of 30 minutes with 3 days of co-cultivation period was found to be optimum for transformation of CADE explants. Transformed shoots were selected using 200 mg/l kanamycin. Non-transformed shoots became albino and died within 5 weeks due to this selection pressure. Using this protocol *Agrobacterium*-mediated transformation of mungbean was further carried out using the newly developed antiviral gene construct, pBI121CP-AC2 and

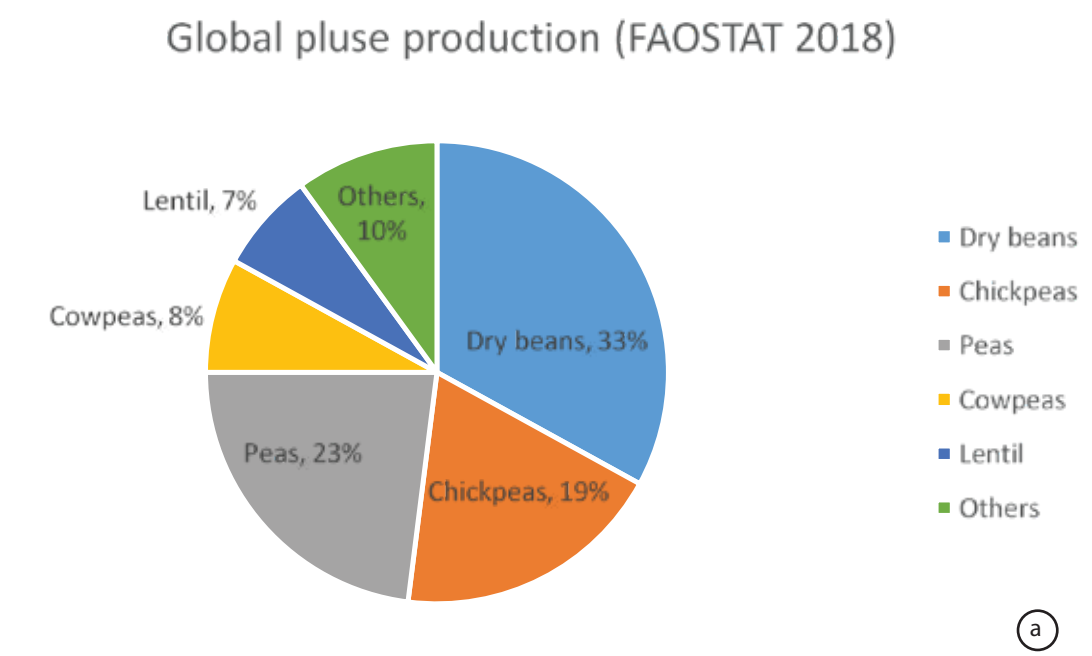
consequently transformed shoots of mungbean were recovered using CADE explants. However, *in vitro* regenerated shoots produced low number (26.66 %) of roots on both full and half strength of MS medium supplemented with different concentrations and combinations of auxins. To overcome the problems of *in vitro* rooting, an alternative approaches of root development i.e., *in vitro* micrografting technique was applied using regenerated shoots to obtain complete plantlets. The best response (55%) towards successful grafting was obtained when 3.0 cm long scions and 14-days old *in vitro* mungbean rootstocks were utilized. Following proper hardening successful micro-grafted plants produced flowers and set viable seeds. On the other hand, in a separate set of experiments the putatively transformed shoots developed roots when cultured on half strength of MS medium supplemented with 2μM IBA. Following the development of roots putative plantlets were hardened and successfully acclimatized in soil. Integration of antisense *CP-AC2* gene in the transgenic mungbean plants was confirmed by polymerase chain reaction (PCR) using CP forward and AC2 reverse gene specific primers. Following proper hardening the T_0 and T_1 plants produced flowers and viable seeds.

1. INTRODUCTION

1.1 General information about legumes and pulses

Legumes, commonly known as pulses, are the major source of dietary proteins in Asia and constitute an important supplement to its predominantly cereal based diet (Sahoo and Jaiwal 2008). In diet, more balanced amino acid can be provided by legume which is not sufficient in cereals. As a major food crop, legumes are popular in agriculture and as a source of feed for the domestic animals. Presently agriculture continues to depend on leguminous crops because they all have high energy and high protein content for human and animal nutrition. Moreover, for sustainable agriculture, the symbiotic ability of legumes to fix atmospheric nitrogen for plant growth makes it most acceptable crop in the subcontinent.

Grain legumes or pulses are the plants belonging to the family Leguminosae which is also known as Fabaceae. The leguminosae is a diverse and important family of angiosperms (Young et al. 2003). Pulses are cultivated in tropics, sub-tropics and temperate regions of the world. Recent information indicates that they were cultivated globally in 95720189 hectares of land with a production of 92277859 tons and having an average yield of 9640 kg/ha (FAOSTAT 2018). Among the major food crops in the Asia-Pacific regions, particularly South, East and Southeast Asia, pulses play an important role in improving the diet of the people as a source of nutrition. The countries of this region grow a dozens of summer and winter pulses to meet the dietary requirements. The low income farmers in developing countries use pulses in various ways i.e. food alternative, livestock feed etc. Beans represented the largest percentage (33%) of all pulse production in the world (almost 92 million tons), followed by chickpeas (19%), peas (23%), and lentils (7%) (FAOSTAT 2018). Production of various pulses has been presented in Fig. 1. Economically legumes represent the second most important crop plants after Poaceae (grass family), accounting for approximately 27% of the world's crop production (Smykal et al. 2020). In many developing countries of the world, grain legumes have gained much importance in view of the acute shortage in the production of animal proteins and the wide prevalence of protein malnutrition. This makes the grain legumes to be considered as the "Meat of the poor". The major legumes in Asia are lentils, peas, chickpeas, broad beans, mungbean etc.



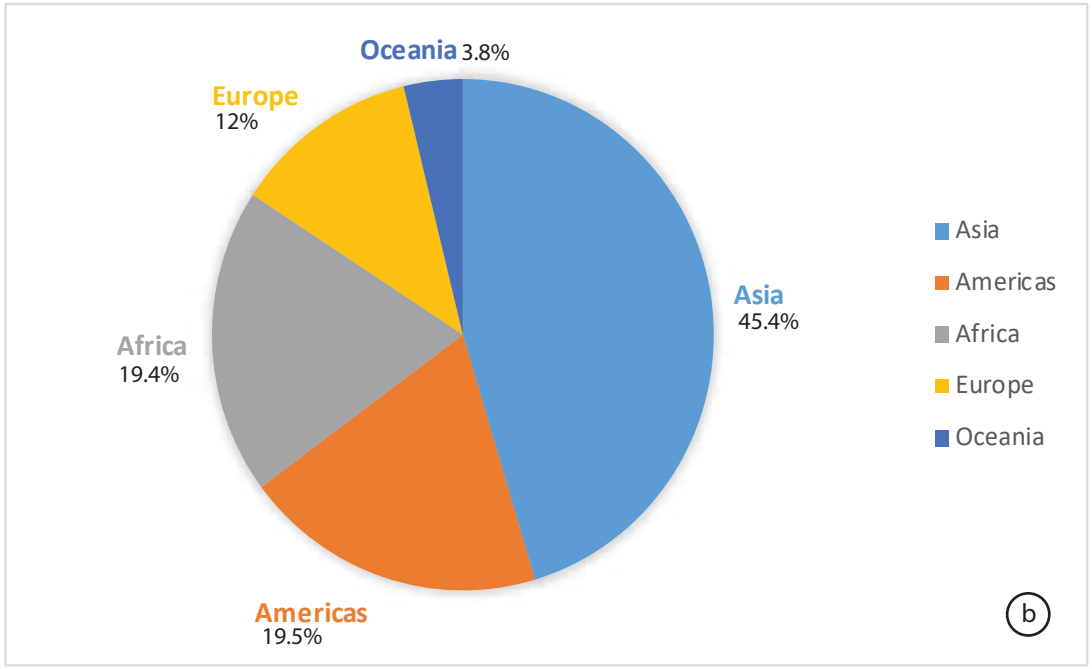


Fig.1 (a-b) : Production of different pulses in different regions of the world. (a) Different pulse production in the world (FAOSTAT 2018); (b) Production of pulses in different region of the world (FAOSTAT 2018).

1.2 Mungbean: an important pulse crop

Mungbean [*Vigna radiata* (L.) Wilczek] commonly known as green gram is one of the most important pulse crops growing in many parts of the world including Bangladesh. Mungbean has tremendous value in agriculture as a good source of plant protein for its high digestibility, good flavor and high protein content (Ahmaed et al. 1978). It is a good source of sulphur containing amino acids like methionine, cysteine etc. Sprouted mungbean is a highly Vitamin-C rich vegetable and 12 times more iron is available in sprouted mungbean than normal mungbean seeds (Mendoza et al. 1988). This crop is widely cultivated all over the world prevalent in south Asia because of its protein rich edible seeds for human consumption. It 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), lysine (504 mg/g) and dietary fibers (Keatinge et al. 2011). The seeds proteins are easy to cook, easy to digest and lack flatulence factors in contrast to other legumes. It stands next to soybean in its dietary protein content. Mungbean can be consumed as dehulled grain, hulled, curries, salads, noodles, bread, sweets and many other culinary products.

Mungbean is a self-pollinating diploid plant with 2n = 2x = 22 chromosomes and a genome size of 515 Mb/1C (Parida et al. 1990). It is a short duration plant, taking about 55 to 75 days to complete its life cycle. In Bangladesh it is grown for three seasons in a year with an average yield of 368 kg/acre. (BBS 2018). Being a short duration and low water requirement plant, it can be used in crop rotation practices. Moreover, it contributes to improve the soil fertility by fixing atmospheric nitrogen and depositing significant amounts of organic matter to the soil. Other properties like easy digestibility and low proportions of flatulence factor also add to its value among the pulse crops.

In Bangladesh, several pulses are cultivated, and these are considered as a vital component in diversification of predominantly rice-based cropping system in Bangladesh. The main pulses in terms of production in Bangladesh are grass pea, lentil, chickpea, black gram and mungbean. In Bangladesh, among the pulses, mungbean ranks third in acreage and contributes 10 -12% of total pulse production, but first in market price. It is worth to mentioning here that 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). There are a few varieties of mungbean available for cultivation in Bangladesh. Bangladesh Agricultural Research Institute (BARI) has so far

released six varieties of mungbean, named- BARI mung 1, 2, 3, 4, 5 and 6; Bangladesh Institute of Nuclear Agriculture (BINA) has also released eight varieties of mungbean, named- Binamoog 1, 2, 3, 4, 5, 6, 7 and 8 and Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) has also released four varieties of mungbean named- BU mung 1, 2, 3 and 4 (www.dhcrop.bsmrau.net). All the above-mentioned varieties have been cultivated in different regions of Bangladesh. The demand for this crop has been steadily increasing in the Indian subcontinent and it has been playing an important role in fulfilling the nutritional requirements for the people of Bangladesh.

1.2.1 Origin and distribution of mungbean

This is an ancient crop cultivated in India and the plant is not found in a wild state. The mungbean is thought to have originated from the Indian subcontinent where it was domesticated as early as 1500 BC. It is probably derived from *Phaseolus radiatus* L, which occurs wild throughout India, Myanmar and is occasionally cultivated (Ligon 1945). Currently green gram has been widely cultivated in India and adjacent regions for several thousand years, and to have spread early into other Asian countries and to northern Africa. Cultivated mungbeans later spread from India to China and Southeast Asian countries. Cultivated mungbeans were introduced to southern and eastern Asia, Africa, Austronesia, the Americas and the West Indies. It is now widespread throughout the tropics and is found from sea level up to an altitude of 1850 m in the Himalayas (Lambrides et al. 2006, Mogotsi 2006). Its present wide distribution throughout the tropics and subtropics of Africa, the west of India, north of America and Australia is comparatively recent. Currently, green gram is the most important seed legume in Thailand and the Philippines; it ranks second in Sri Lanka and third in each of India, Myanmar, Bangladesh and Indonesia. It is a minor crop in Australia, China, Iran, Kenya, Korea, Malaysia, the Middle East, Peru, Taiwan and the USA (Summerfield and Roberts 1985). Mungbean is known by various names in different parts of the world. The most common names are Mug (Bengali), Mungbean (English), Maash (Arabic), Mash (Turkey), Mungo (Spanish), Mung (Hindi) and Green bean (Japanese).

1.2.2 Botanical description of mungbean

Green gram (*Vigna radiata*) is a small herbaceous annual plant growing to a height of 30 to 120 centimeters with a slight tendency to twining in the upper branches. The central stems are more or less erect while side branches are semi erect. A description of mungbean have been presented in Fig. 2 as published by Baldev (1988). The leaves are 5 -10 cm long trifoliate with long petioles. Both the stems and leaves are covered with short hairs, generally shorter than those found in urad bean (black gram). The crop begins flowering 50 to 60 days after sowing and then continue flowering for a few weeks; the leaves dry down but may not drop off completely. From 10 to 25 flowers are born in axillary clusters or racemes. The flowers are greenish to bright yellow, with a gray tinged keel, 1.0 to 1.75 cm in diameter. The pods are cylindrical, straight to strongly curved, pointed at the tip and radiate horizontally in whorls.

Pods mature in about 20 days after flowering. When mature, the pods are glabrous or have short hairs, light brown to black, 5 to14 cm long and 4 to 6 mm wide and may burst open when dry shattering the seeds. Seeds, born 8 to 20 per pod, are globose; glossy or dull; with green, yellow, tawny brown, black or mottled testa. Dull seeds are coated with a layer of the pod inner membrane which may be translucid or pigmented and which covers a shiny testa. Seeds vary in weight from 15 to 85 milligrams, generally averaging 25 to 30 thousand seeds per kilogram. The hilum is round, flat (non-concave) and white. Flowers are self-fertile and highly self-pollinated.

1.2.3 Growth habits of mungbean

In India and Bangladesh, they are grown during two seasons i.e., Rabi season (November-February) and Kharif season (March-September). Mungbean is a tropical or sub-tropical crop and requires warm temperatures (optimally round 30 - 35°C). Due to its heat tolerance, it may be cultivated either as a summer or early kharif crop. But in Bangladesh mungbean is traditionally cultivated in the winter season. In Rabi season, it is mostly cultivated in the south-eastern districts of Bangladesh. Seed can be planted when the minimum temperature is above 15°C. Mungbeans are responsive to daylight length. Short days result in early flowering, while long days result in late flowering. However, mungbean varieties differ in their photoperiod response. The plant is heat and drought tolerant in nature. Adequate rainfall is required from flowering to late pod fill for purposes of ensuring good yield. Mungbeans grow best on fertile, sandy loam soils with good internal drainage and a pH in the range of 6.3 - 7.2. They require slightly acid soil

for best growth. If they are grown in rotation, lime is added to attain optimum pH for mungbean cultivation. Root growth can be restricted on heavy clays. Mungbeans do not tolerate saline soils and can show severe iron chlorosis symptoms and certain micronutrient deficiencies on more alkaline soils.

1.2.4 Chemistry of mungbean

Grain legumes, being a rich and economical source of proteins, complex carbohydrates (dietary fiber), minerals, and vitamins are considerably important in Asian and African vegetarian diets (Rege 1981, Kumar et al. 2002, Salunke et al. 2005). They are rich in phosphorus and provitamin A. The high protein levels and high lysine/low methionine amino acid profile of mungbean complement the high carbohydrate and low lysine/high methionine content of cereals to form a much-balanced amino acid diet (Jaiwal et al. 2001).

Moreover, the mungbean protein is rich in some essential amino acids, including aromatic amino acids, leucine, isoleucine and valine, as well as glutamic acid (Tang et al. 2009). Therefore, mungbeans can be considered as a potential protein source of human diets due to its high contents of amino acids and are rich in nutritional value. During germination, seed storage carbohydrates are also hydrolyzed to small molecules and sugar, resulting in an increase in the amount of reducing sugar (Randhir et al. 2004). Chemical composition includes proteins, fatty acids, carbohydrates, Vitamin B_1, Vitamin B_2, beta-carotene, folic acid, calcium, phosphor and irons. Proteins are rich in lysine, leucine, threonine, but not rich in methionine, tryptophane, and tyrosine. Mungbean skin contains 21 kinds of minerals, rich in phosphor and irons. 100 grams mungbean seed contains 22.1 grams protein, 0.8 grams fat, 59 grams carbohydrate, and 332 calorie heat energy.

1.2.5 Human consumption of mungbean

Mungbean is consumed in different forms and ways, for example, as a viand, boiled, or cooked with vegetables or meat, as well as a dessert or incorporated in bread or cake. It can be used to make sprouts for egg rolls and other vegetable dishes (Mendoza et al. 2001). Mungbeans are considered “one of the most cherished foods” in the ancient Indian practice this has been a traditional form of medicine since roughly 1500 BC. Dehulled mungbeans can also be used in a similar fashion as whole beans for the purpose of making sweet soups.

Mungbean is often eaten as a product “Dhal”, which is a split and de-hulled seed used as a main dish, side dish or salads. Only red cotyledon type is used as food in Bangladesh, where it is boiled into soup-like “Dhal” and eaten with flat bread (roti) or rice. Khichuri is another popular dish, which is made from a mixture of split mungbean seeds and rice. Together rice and mungbean make a quickly prepared meal that is well balanced nutritionally. Mungbean seeds can also be fried or seasoned. Mungbeans produce an edible sprout that’s crisp and described as nutty tasting. These sprouts are substantial enough to stand-up to stir-frying, but they are often used raw in salads and on sandwiches. Mungbean sprouts are low in calories, have fiber and B vitamins, and deliver a boost of vitamins C and K.

1.2.6 Mungbean production in the world and in Bangladesh

Mungbean is mainly produced in Asia (90%) of which India is the largest producer (Vikhe and Nehul 2020). India produces about 2.01 million tons of mungbean annually from about 4.26 million hectares land (2017-2018), with an average productivity of 472 kg/ha (Ministry of Agriculture & Farmers Welfare, India 2018). Myanmar is the main exporter and its production increased by 22% in the year 2018-2019. The global mungbean cultivation area is about 7.3 million ha, and the average yield is 721 kg/ha and total production is 5.3 million tons (Nair et al. 2020). India account for 30% of global output of 5.3 million tons. Other large producers are China, Myanmar, Indonesia, Pakistan, Thailand and Bangladesh (Fig. 3). Currently, mungbean is cultivated in Bangladesh in 102109 acres of land and annual total production is 33951 MT (BBS 2019).

1.3 Constraints of mungbean production: biotic and abiotic stress

Low productivity of mungbean is due to abiotic and biotic constraints, poor crop management practices and non-availability of quality seeds of improved varieties to farmers (Chauhan et al. 2010, Pratap et al. 2019). Abiotic stresses affecting mungbean production include waterlogging, salinity, heat, and drought stress (Hanumantharao et al. 2016, Singh and Singh 2011). The major biotic factors include diseases such as yellow mosaic, anthracnose, powdery mildew, Cercospora leaf spot (CLS), dry root rot, halo blight, and tan spot, and insect-pests especially bruchids, whitefly, thrips, aphids, and pod borers (Lal 1987, Singh et al. 2000, War et al. 2017 and Pandey et al. 2018). In order to improve productivity and stabilize crop production, there is a need to develop varieties

resistant to biotic and abiotic stress factors (Nair et al. 2019). Breeding information on the biotic and abiotic stresses in mungbean and on the influence of environmental stresses at different plant development stages is essential to identify the sources for tolerance traits expressed at the right stage. Among biotic stresses, mungbean yellow mosaic virus (MYMV) is the most influential virus which causes maximum yield loss.

1.3.1 Viruses as a major constraint in pulse production

Plant viruses are obligate, intercellular parasites which have emerged as a major global threat causing severe diseases and yield losses in economically important plants. In fact, 47% of all emerging infectious diseases of plants have been reported to be caused by viruses (Anderson et al. 2004, Yadav et al. 2011). Most viruses infect all types of plant tissues and use sieve elements in the phloem as the route of long-distance movement and systemic infection in plants (Vuorinen et al. 2011, Jiang et al. 2012). The virus replicates its genome inside the host cell to establish and spread disease (Singh et al. 2007). Management of viral diseases is much more difficult than that of diseases caused by other pathogens because viral diseases have complex disease cycle, efficient vector transmission and no effective viricide against them. Plant viral diseases cause serious economic losses in many major crops by reducing seed yield and quality (Kang et al. 2005). Among the various diseases, the mungbean yellow mosaic virus (MYMV) disease was given special attention because of severity and ability to cause yield loss up to 85 per cent (AVRDC 1998). Host resistance to the disease or the vector has therefore, been considered as the only solution to control this important disease (Kang et al. 2005). The host range of the virus was reported to be largely confined to the plants belonging to the family *Leguminosae*. The legumes like black gram, mungbean, moth bean and pigeon pea are the hosts for the mungbean yellow mosaic virus.

A total of sixteen diseases have been reported to be associated with mungbean in Bangladesh (Gowda and Kaul 1982). Among the diseases, mungbean yellow mosaic virus (MYMV) is the most damaging disease of mungbean in Bangladesh and Southeast Asia (Jalaluddin and Shaikh 1981, Nariani 1960). It has been mentioned that the viral diseases are the most damaging to this crop (Nene et al. 1972). It may be pointed out that information is available on the inheritance of resistance to MYMV using inter and intra specific crosses (Khan et al. 2007, Sudha et al. 2013).

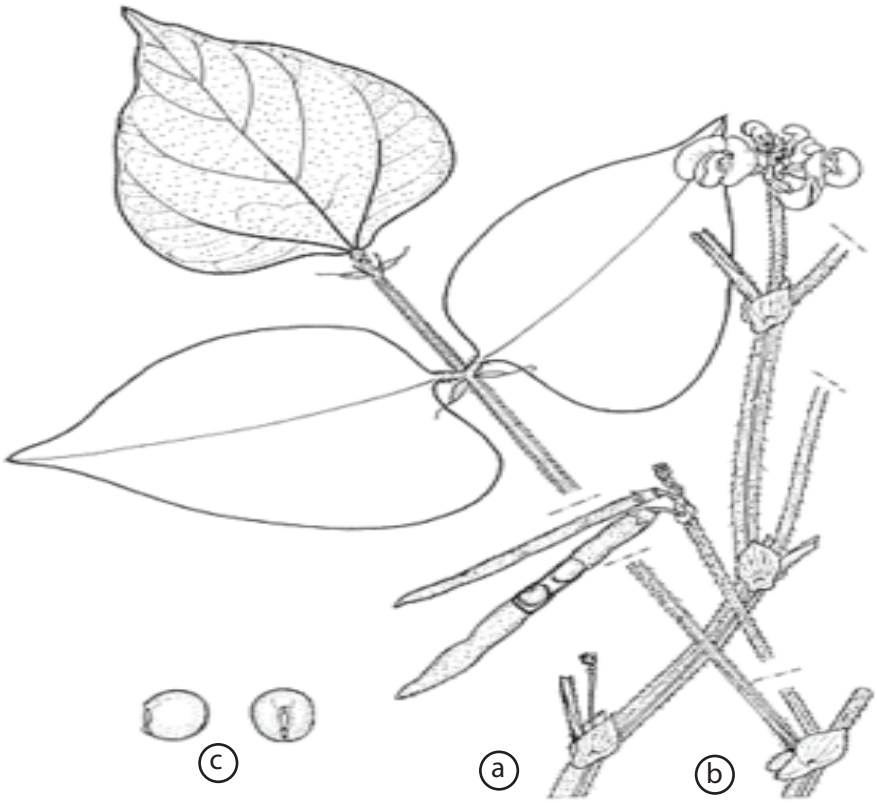


Fig. 2: A typical mungbean *(Vigna radiata)* plant. (a). part of flowering branch; (b). part of fruiting branch; (c). seeds. Source: PROSEA [https://uses.plant- net-project.org/en/*Vigna radiata* (PROTA)]

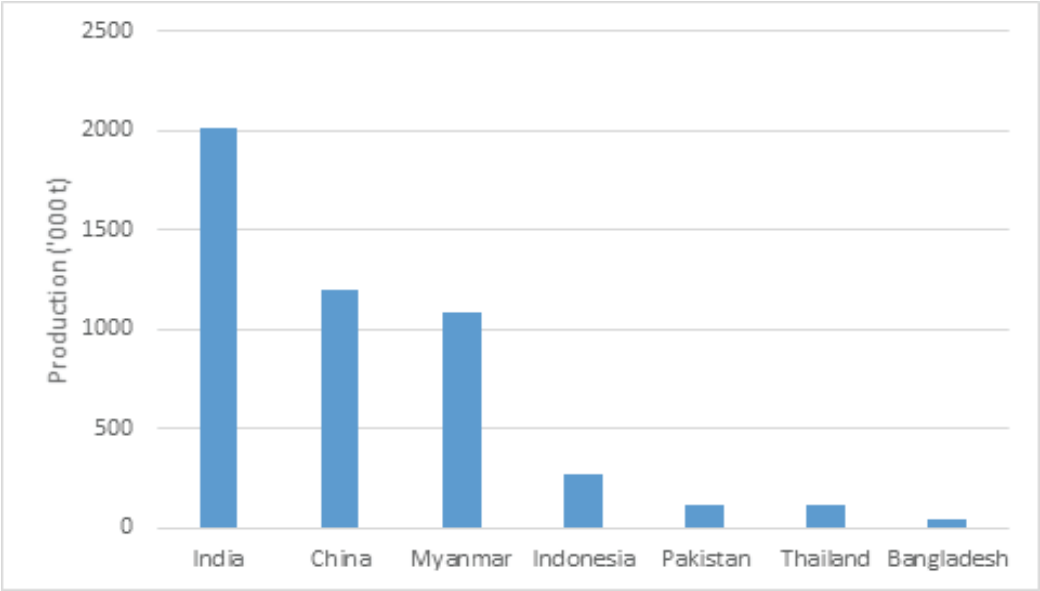


Fig 3: Production of major mungbean producing countries. (Source: Ministry of Agriculture & Farmers Welfare, India 2018, Pakistan bureau of statis tics 2018, Bangladesh bureau of statistics 2018 and avrdc.org/intl-mungbean network 2018).

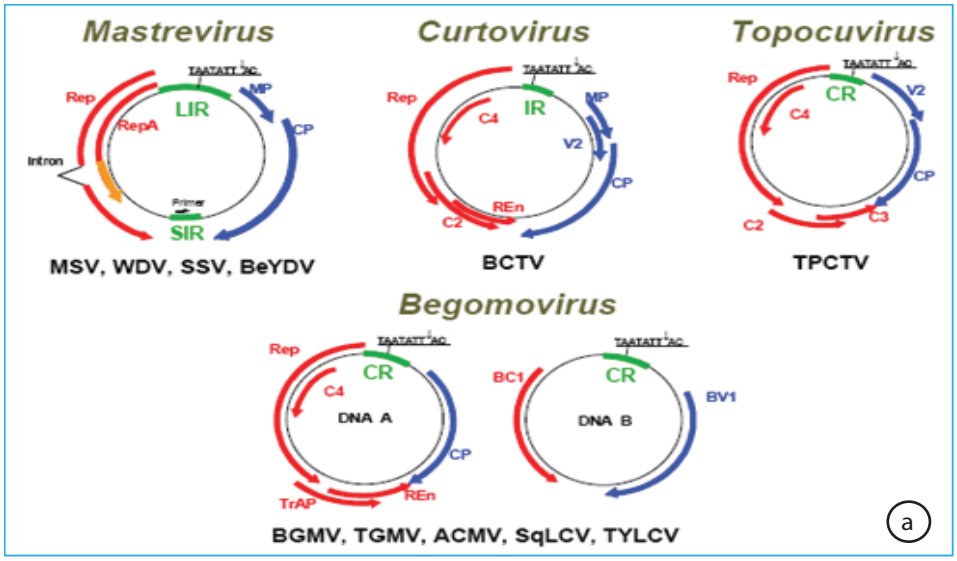
Very little information is available regarding sources of resistance to MYMV and molecular based techniques for the development of resistant line of mungbean. It may be mentioned here again that a good number of mungbean varieties have been developed by various research institutes in Bangladesh. The yield potential of these varieties is better and some of them are considered as MYMV tolerant. However, none of these varieties have MYMV resistance.

1.3.2 Mungbean yellow mosaic virus (MYMV)

MYMV is a plant pathogenic geminivirus which belongs to the genus, begomovirus of the family Geminiviridae. Geminiviruses are a group of plant viruses that infect a wide variety of crop plants causing great economic losses worldwide. Geminiviruses encode only a few proteins for their replication and recruit most from their plant hosts (Suyal et al. 2013). Based on genome organization, host range and vector specificity, the members of the family Geminiviridae are classified into four genera: Begomovirus, Masterovirus, Curtovirus and Topocuvirus (Fig. 4a). The genus Begomovirus is the largest and consists of more than 180 species and several unassigned isolates (Fauquet et al. 2008). Based on the nucleotide sequence identity analyses, four species of bipartite begomoviruses (family Geminiviridae) causing yellow mosaic disease in legumes have been recognized in southern Asia (Qazi et al. 2007 and Haq et al. 2011a). These are: Mungbean yellow mosaic India virus (MYMIV), Mungbean yellow mosaic virus (MYMV), Dolichose yellow mosaic virus (DYMV) and Horsegram yellow mosaic virus (HgYMV). MYMIV and MYMV are considered as most important among these viruses (Fauquet et al. 2003).

MYMV is a notorious geminivirus having twin icosahedral particles carrying monopartite or bipartite, circular, single stranded DNA genome (Thomas et al. 1986, Navot et al. 1991). They are characterized by their geminate (twinned) particles (Fig. 4c), ssDNA circular genomes which replicate by a rolling circle mechanism. The virus is transmitted by only one species of whitefly (Fig. 4b), *Bemisia tabaci* (Rishi 2004, Singh et al. 2007, Usharani et al. 2004, Qazi et al. 2007, Haq et al. 2011a). Whitefly adapts easily to new host plants and geographical regions. In South Asia, mungbean yellow mosaic virus (MYMV) severely constrained mungbean expansion and production.

It causes yellow mosaic disease (Fig. 5) in *Vigna radiata*, *Vigna mungo* and *Vigna unguiculata* (Maiti et al. 2011, Singh et al. 2013a).



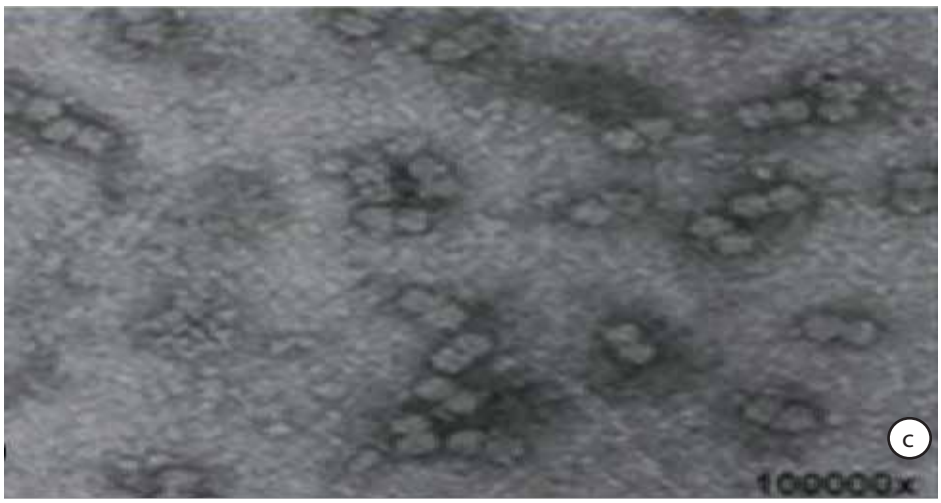


Fig. 4 (a-c): Genetic organization of the four genera of the geminiviridae family. (a)The vector white flies and geminivirus particles. (b) white flies which transmit the geminivirus. (c) Electron microscopic view of geminivirus.

It is a widespread and most destructive viral disease of mungbean and depending upon the magnitude of infestation, loss in yield may vary from 10 -100 % depending upon the stage of infection (Chenulu et al. 1979, Marimuthu et al. 1981).

The yield loss can be up to 100% if infection occurs at seedling stage (Varma et al. 1992, Ghafoor et al. 2000). Yellow mosaic disease (YMD) was first reported from western India in the late 1940s in lima bean and later in mungbean in the experimental farm at IARI, New Delhi (Capoor and Varma 1948, Nariani 1960). Later this disease was also reported from other parts of India, Pakistan, Bangladesh and Sri Lanka (Qazi et al. 2007). An epidemic outbreak of yellow mosaic disease of mungbean was also identified in Thailand in 1980s which was caused by a sap transmissible geminivirus and was found to be different from Indian isolates of the virus, which were whitefly transmitted.

1.3.3 Mungbean yellow mosaic virus genome organization

MYMV is characterized by bipartite (two components, namely DNA-A and DNA-B) single stranded genome (Fig. 6) and double icosahedral particle morphology. The DNA-A and DNA-B are similar in size (2.7 -3.0 kb) but differ in sequence, except for a 200–250 bp region of high sequence homology known as the common region (CR). The CR is a part of large intergenic region (IR) that contains origin of replication and divergent promoters for transcription (Yadava et al. 2010). Both genomes are essential for infectivity of the virus. The invariant TAATATTAC sequence, located in the intergenic region is indicated together with the initiation site for rolling circle DNA replication (Gutierrez et al. 2004). The bipartite nature of MYMV has been confirmed by agro-infection with infectious clones of DNA-A and DNA-B (Mandal et al. 1997, Jacob et al. 2003, Karthikeyan et al. 2004). DNA-A typically has six open reading frames (ORFs): AV1/V1 (coat protein, CP) and AV2/V2 (AV2/V2 protein) on the virion-sense strand, and AC1/C1 (replication initiation protein, Rep), AC2/C2 (transcriptional activator, TrAP), AC3/C3 (replication enhancer, REn) and AC4/C4 (AC4/C4 protein) on the complementary-sense strand (Padidam et al. 1996, Choudhury et al. 2006, Shunter and Bisaro 1992), DNA-B has two ORFs encoding movement proteins: BV1 (nuclear shuttle protein, NSP) on the virus-sense strand and BC1 (movement protein, MP) on the complementary-sense strand (Rojas et al. 2005, Seal et al. 2006) which act cooperatively to move the virus cell-to-cell within plants.

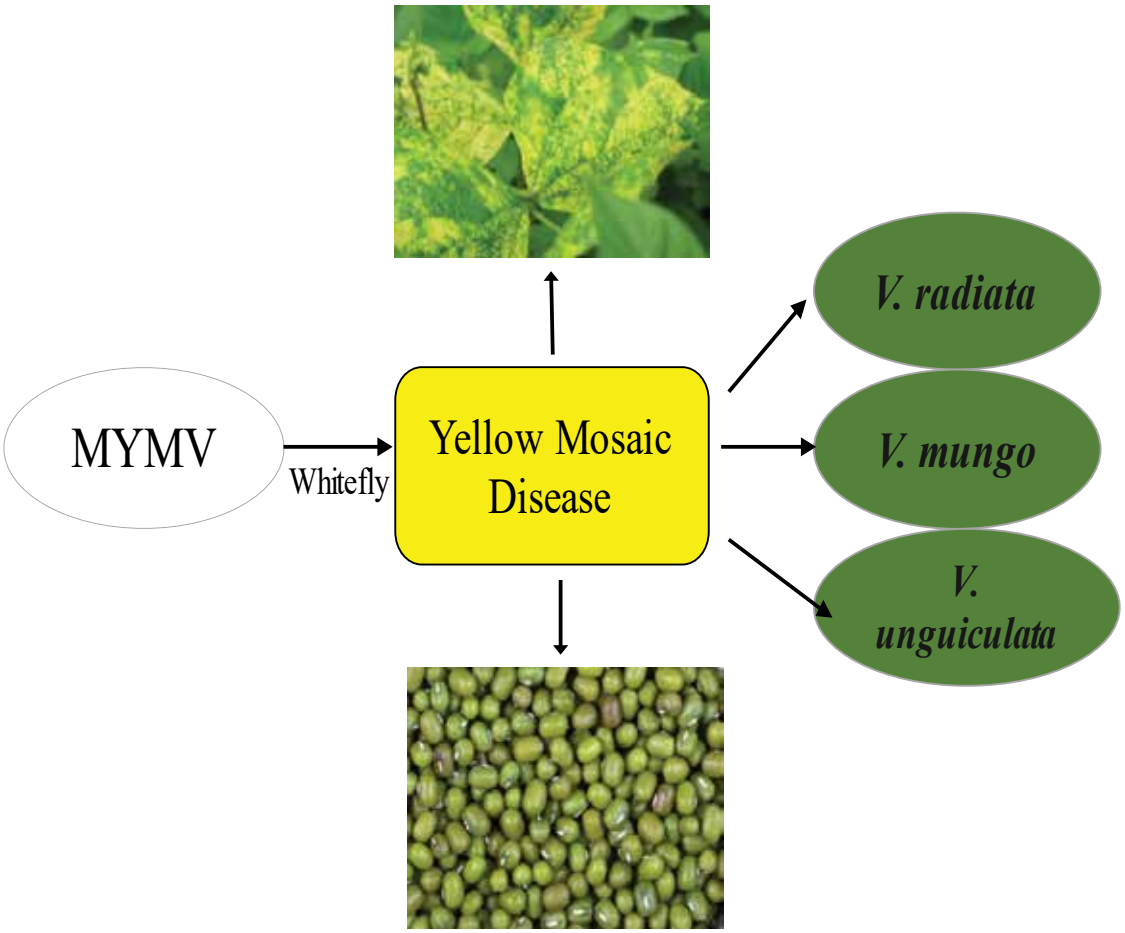


Fig. 5: *Vigna* species affected by yellow mosaic disease caused by mungbean yellow mosaic virus

1.3.4 MYMV replication

MYMV replicates predominantly via dsDNA intermediates following rolling circle mode of DNA replication inside the nucleus of the infected host cell. The replication process of the virus can be divided in two stages. First, upon entry inside the nucleus, the viral ssDNA is converted into dsDNA (replicative form, RF) with the help of only the host cellular factors. This dsDNA is transcriptionally active and serves as a template for the production of various viral factors. In the second phase, the viral factors along with the cellular factors synthesize ssDNA using the dsDNA as a template via rolling circle replication. These ssDNA can again either (i) re-enter the DNA replication pool, (ii) associate with CP for production of virions or (iii) be transported outside the nucleus by nuclear shuttle proteins (NSPs) or outside the cell with the help of virus encoded movement proteins (MPs) and associated host factors. The MYMIV-CR comprises a highly conserved stem loop structure, which also contains the conserved sequence, TAATATT↓AC, where the viral Rep protein acts to initiate the rolling circle replication. Virus replicate its small genome through a rolling circle mechanism with a dsDNA intermediate, and gene transcription is bidirectional from the common region (Yadava et al. 2010).

1.3.5 Symptomology caused by MYMV

MYMV produces typical yellow mosaic symptoms (Fig. 5). The symptoms appear in the form of small irregular yellow specs and spots along the veins which enlarge until leaves were completely yellowed. Diseased plants are stunted, with fewer flowers and pods that bear smaller, occasionally shriveled seeds in severe cases, other plant parts also become completely yellow. Symptoms caused by legume yellow mosaic viruses were largely dependent on host species and susceptibility. In severe infections the entire leaf may become chlorotic. In blackgram, the chlorotic areas sometimes turn necrotic. It can cause up to 100 per cent yield loss if infection occurs three weeks after planting. Loss may be less if infection occurs after eight weeks from the day of planting. Initially, symptoms appear on young leaves in the form of mild scattered spots. The proportion of yellow areas goes on increasing in the new growth and some of the apical leaves turn completely yellow. The infection not only drastically reduces yield but also severely impairs the grain size and quality. The leaves showed slight puckering with reduction in size.

1.4 Strategies for controlling mungbean yellow mosaic virus

Strategies for the management of viral diseases normally include control of vector population, whiteflies by contact or systemic high toxicity insecticide with the concomitant problems of development of pesticide-resistant forms, low cost-benefit ratio and environmental concerns. Chemicals used for the control of insect pests are often expensive and non-specific in their action thus killing beneficial organisms. Chemical control may have undesirable effect on human health and environment (Manczinger et al. 2002). Other methods such as avoidance of source of infection, use of virus-free propagating material and eradication of infected material are laborious and do not seem to be practical. However, each of the above methods has its own drawback.

1.4.1 Problem associated with the improvement of mungbean through conventional breeding techniques

Genetic improvement of legumes through conventional breeding has been limited due the lack of desirable and satisfactory levels of variability in their germplasm. Despite nearly 25 years of resistance breeding efforts, none of the known varieties of legumes is fully resistant to yellow mosaic virus. Resistance is often unsatisfactory and commercial cultivars are susceptible to early, moderate or severe infection (Morales and Jones 2004, Seo et al. 2004, Atif et al. 2013). Most of the released varieties in mungbean have a narrow genetic base, mainly because of limited pre-breeding efforts and repeated use of a handful of genetic resources in hybridization programs (Kumar et al. 2005). The only promising option left may be to transfer desirable genes from other sources for their quantitative and qualitative improvements through *in vitro* regeneration and genetic transformation techniques. These are the application of biotechnology including more precise and more rapid ways as compared to traditional mendelian methods. So, there is a strong need for engineered resistance to fight against the virus.

1.4.2 Application of biotechnology: an efficient alternative against viruses for improvement of mungbean

Genetic engineering provides the opportunity to create virus resistance in existing plant cultivars. There are mainly two approaches for developing transgenic resistance (Fig. 7) depending on the source of the genes used. The genes can be either from the pathogenic virus itself or from any other source.

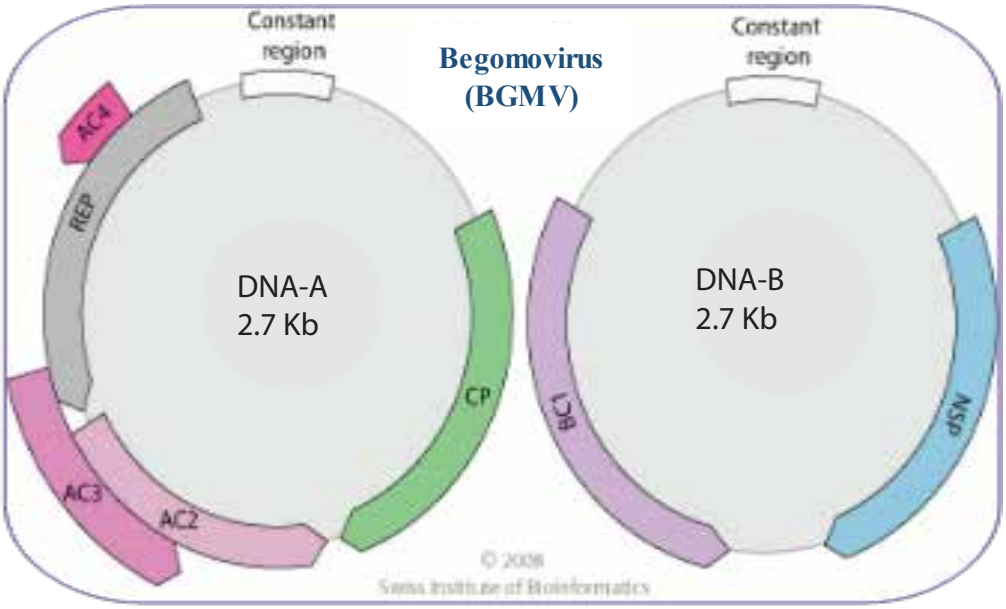


Fig. 6: Bipartite begomovirus genomic components are reffered as DNA-A and DNA-B which contains single strands genomes of approximately 2.7 kb.

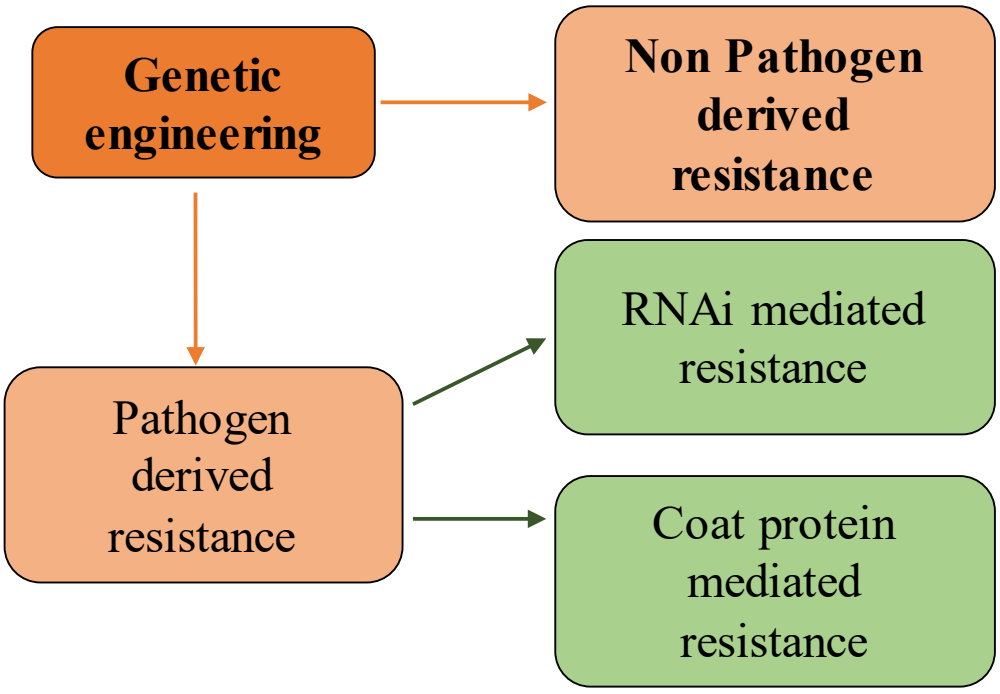


Fig. 7: Genetic engineering approaches for development of virus resistance transgenic plants.

The development of successful transgenic viral resistance is based on "pathogen-derived resistance" (PDR) concept (Sanford and Johnston 1985). For PDR, a part or a complete viral gene is introduced into the plant which subsequently interferes with one or more essential steps in the life cycle of the virus. Because the entire viral genome is needed to cause disease, integrating a viral gene fragment into a host genome does not cause disease, instead plant's natural antiviral mechanism gets activated and cause targeted degradation of genome of the virus. This was first illustrated in tobacco by the group of Powell 1986, who introduced the coat protein gene (*CP*) of tobacco mosaic virus (TMV) into tobacco and observed TMV resistance in the transgenic plants. PDR mainly includes coat-protein mediated resistance and RNAi-mediated resistance (Wani and Sanghera 2010).

1.4.3 Coat protein mediated resistance (CPMR)

In CPMR viral coat protein gene (*CP*) is transferred into plants to make them resistant against the virus from which the gene for coat protein was taken. This was first demonstrated for tobacco mosaic virus (TMV) in tobacco (Powell et al. 1986). Ferreira et al. (2002) developed transgenic papaya resistant to Papaya ring spot virus (PRSV) which has been commercially grown in the USA. The success of CPMR has prompted the production of transgenic plants expressing multiple *CP* genes from more than one virus. Several important crops have been engineered for virus resistance using CPMR approach and released for commercial cultivation. These include tomato resistant to TMV, tomato mosaic virus (ToMV) and cucumber mosaic virus (CMV), cucumber resistant to CMV, squash resistant to zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus (WMV), cantaloupe resistant to ZYMV, WMV and CMV; potato resistant to PVX, potato virus Y (PVY) and potato leaf roll virus (PLRV) (Dasgupta et al. 2003). Previously, it has been reported that inoculation of viral constructs having N terminal deletion of 75 and 150 amino acids from coat protein region of mungbean yellow mosaic India virus (MYMIV) in cowpea, mungbean, blackgram and French bean that decrease the symptoms of yellow mosaic disease (Haq et al. 2011b).

Geminiviruses encodes the small protein AC2 which is a trans activator, enhancing the transcription of late viral genes (Haley et al. 1992). Daniela et al. (2006) reported that MYMV originated AC2 played a role as a viral promoter trans activator. It is a suppressor protein which interferes with different steps of the RNA silencing pathway (Dunoyer et al. 2004). AC2 ensures efficient viral replication in host cells and thus systemic infection occurs in the plants.

1.4.4 RNAi-mediated resistance

The phenomenon of RNAi-mediated resistance came into spotlight in 1998, when Andrew Fire, Craig Mello and colleagues announced their discovery of RNAi (Fire et al. 1998). First report on RNA silencing dates back in 1928 (Wingard et al. 1928, Baulcombe 2004). The unexpected results of over expression of chalcone synthase (chs A) transgene in petunia (Napoli et al. 1990, Vanderkrol et al. 1990) and finally the silencing of gene expression by dsRNA molecules in nematode worms (Fire et al. 1998), made it clear that RNAi is an extremely potent experimental tool for learning the functions of genes. RNA-interference (RNAi) strategy has emerged as an efficient means to control begomoviruses infection in crops including legumes (Kumar et al. 2017). RNA silencing also called as Post-Transcriptional Gene Silencing (PTGS), in which the degradation of target RNA occurs in a sequence-specific manner. RNAi-derived transgenic resistance has been accomplished by targeting the *CP* and *AC2* gene of different geminiviruses.

1.4.5 Mechanism of RNAi

RNAi is an evolutionary conserved, sequence specific mechanism of inhibition of gene expression in eukaryotic organisms. Broadly, this mechanism depends on the formation of dsRNA whose antisense strand is complementary to the transcript of a targeted gene. Genetic and molecular analyses have confirmed the existence of at least three pathways for the working of RNA silencing at different levels in plants. These pathways include cytoplasmic silencing by dsRNA cleaving mRNA, silencing endogenous mRNA by miRNAs resulting in either RNA cleavage or inhibiting protein translation and silencing associated with DNA methylation and suppression of transcription (Baulcombe et al. 2004, Mansoor et al. 2006). However, the unifying mechanism involves dicing of long dsRNA into short 21-25 nucleotide duplex RNA fragments by an enzyme Dicer which is a member of the RNAse III family of dsRNA specific endonuclease (Zamore et al. 2000, Bernstein et al. 2001). An illustration of RNAi mechanism has been presented in Fig. 8. These small RNAs are broadly defined as siRNAs and miRNA (Ramchandran et al. 2008, Voinnet 2009) that are generated via processing of longer dsRNA and stemloop precursors respectively. These small RNAs are incorporated into RISC (RNA-induced silencing complex) and RISC like complexes, which have Argonauts the only protein component of RISC which cleaves the target RNA. ATP-activated RISC then unwinds the ds siRNA and distinguishes between the different strands of the siRNA.

The sense strand-the strand having exactly the same sequence as a target gene is degraded. The antisense strand of the siRNA then hybridizes to mRNA as a guide and the RISC cleaves mRNA near the center of the siRNA destroying it (Novina and Sharp 2004). The process shares many of the same components and is closely related to post transcriptional gene regulation by microRNAs (miRNA) where the translation initiation is inhibited (Price and Gatehouse 2008). However, the discoveries of these small RNAi pathways have revolutionized the way we study gene regulation and developmental control in plants and animals (Eamens et al. 2008).

1.4.6 Antisense RNA technology

Various approaches have been used to produce dsRNA triggering gene silencing in plants. Initially, it was achieved by transforming plants separately with constructs to produce sense and antisense RNA and subsequently crossing these to induce dsRNA formation (Waterhouse et al. 1998). Antisense technology provides protection either by inhibiting gene expression or viral replication, when mRNA forms a duplex (Fig. 9) with a complementary antisense RNA sequence and hence translation is blocked (Auer and Frederick 2009). The antisense RNA mediated resistance in geminiviruses has been attributed to RNAi technology (Vanitharani et al. 2004, Praveen et al. 2005).

During the last decade, our knowledge of RNA-mediated functions has been greatly increased with the discovery of small non-coding RNAs which play a central part in RNA silencing. Initially, antisense viral-derived RNA sequences have been used to suppress gene expression artificially with high specificity. Because of the specificity of RNAi, there is a great interest in application of this mechanism for crop improvement (Petrick et al. 2013). RNAi has revolutionized the possibilities for creating custom “knock-downs” of gene activity and holds immense promise in development of virus resistant transgenic plants (Wani et al. 2010). RNAi broadly involves two types of single-stranded small RNAs as effector molecules known as siRNA and miRNA. RNAi decreases or eliminates gene expression by cleaving targeted mRNA molecules or by interfering with translation (Lindbo 2012). There are two major ways in which RNA silencing thwarts viral multiplication in plants. The first is to limit accumulation of viral RNA in initially infected cells. This is achieved by processing viral dsRNAs into viral siRNAs, which are then used to target destruction of additional viral RNA molecules. The second is to prime distant tissues for a rapid antiviral response by producing a systemic silencing signal that

moves along the same routes as the virus, spreading silencing to all parts of the plant (Mlotshwa et al. 2008).

1.5 *In vitro* regeneration and genetic transformation of mungbean varieties

The major prerequisite for the application of biotechnology, particularly for the exploration of plant genetic transformation is the availability of an effective *in vitro* regeneration system for the plant of interest. *In vitro* regeneration and genetic transformation procedures of mungbean are not well developed among the grain legumes.

1.5.1 *In vitro* regeneration of mungbean

An efficient genetic transformation system in plants requires, (i) a rapid, reliable, and responsive plant regeneration system, (ii) an efficient mean of delivering DNA into target cells and (iii) a system of selecting or identifying transformed cells (Somers et al. 2003). Genetic transformation of mungbean like other large seeded grain legumes is difficult and challenging because of their recalcitrant nature towards *in vitro* regeneration (Jaiwal and Gulati 1995, Somers et al. 2003). Legumes in general are recalcitrant to tissue culture and are highly genotype specific (Somers et al. 2003). Efficient *in vitro* plant regeneration system is required for successful crop improvement programs through genetic engineering. Successful plant regeneration through *in vitro* technique for grain legumes is rather limited (Mroginski and Kartha 1984). Several attempts have been made to establish *in vitro* regeneration protocols for mungbean. There are some reports on *the in vitro* plant regeneration in mungbean using different explants (Singh et al. 1980, Goel et al. 1983, Mathews 1987, Gulati and Jaiwal 1990, 1994, Mendoza et al. 1992, Chandra and Pal 1995, Amutha et al. 2003, Khatun et al. 2008, Yadav et al. 2010, Patra et al. 2018).

Regeneration of mungbean is appeared to be difficult than most other legumes (Khatun et al. 2008). Regardless of the regeneration method employed, all methods depend on root formation for recovery of plants from culture. After selection of *in vitro* regenerated shoots, the formation of well-developed root and frequency of survival of hardened plants into soil is inevitable. The loss of regeneration potential due to failure to form strong and effective root system during the *Agrobacterium*- mediated transformation results in very low efficiency of plant transformation (Shuangxia et al. 2006).

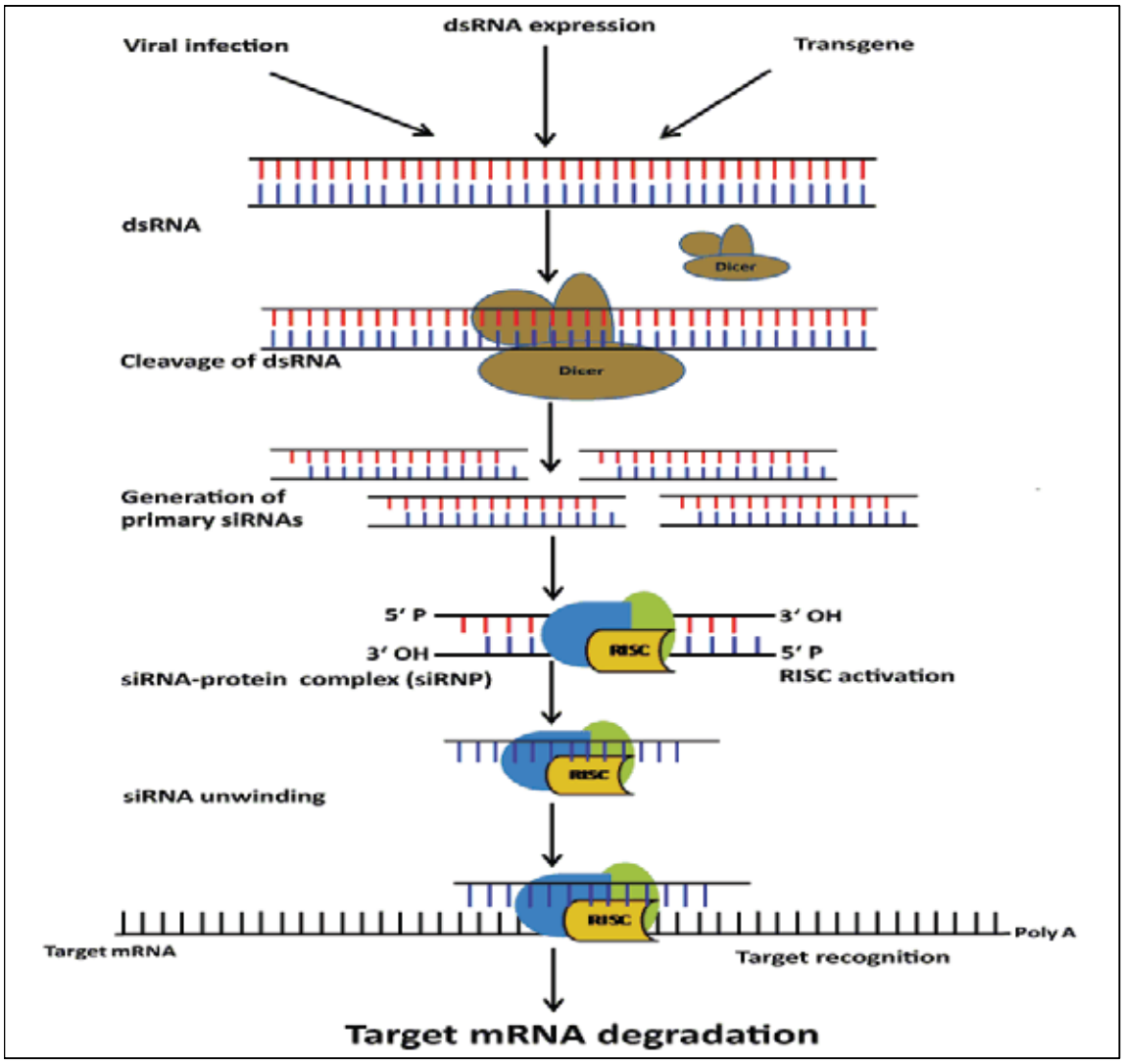


Fig. 8: Schematic representation of RNAi mechanism. Mechanism of RNAi which depends on the formation of dsRNA whose antisense strand is complementary to targeted gene. Double stranded RNA (dsRNA) molecule binds to a Dicer protein, which is a dsRNA specific endonuclease that cleavages of long dsRNA into short siRNAs. These small siRNAs are incorporated into RISC (RNA-induced silencing complex). Then ATP-activated RISC unwinds the ds siRNA. The sense strand is degraded and the antisense strand of the siRNA hybridizes to mRNA and degrades target mRNA.

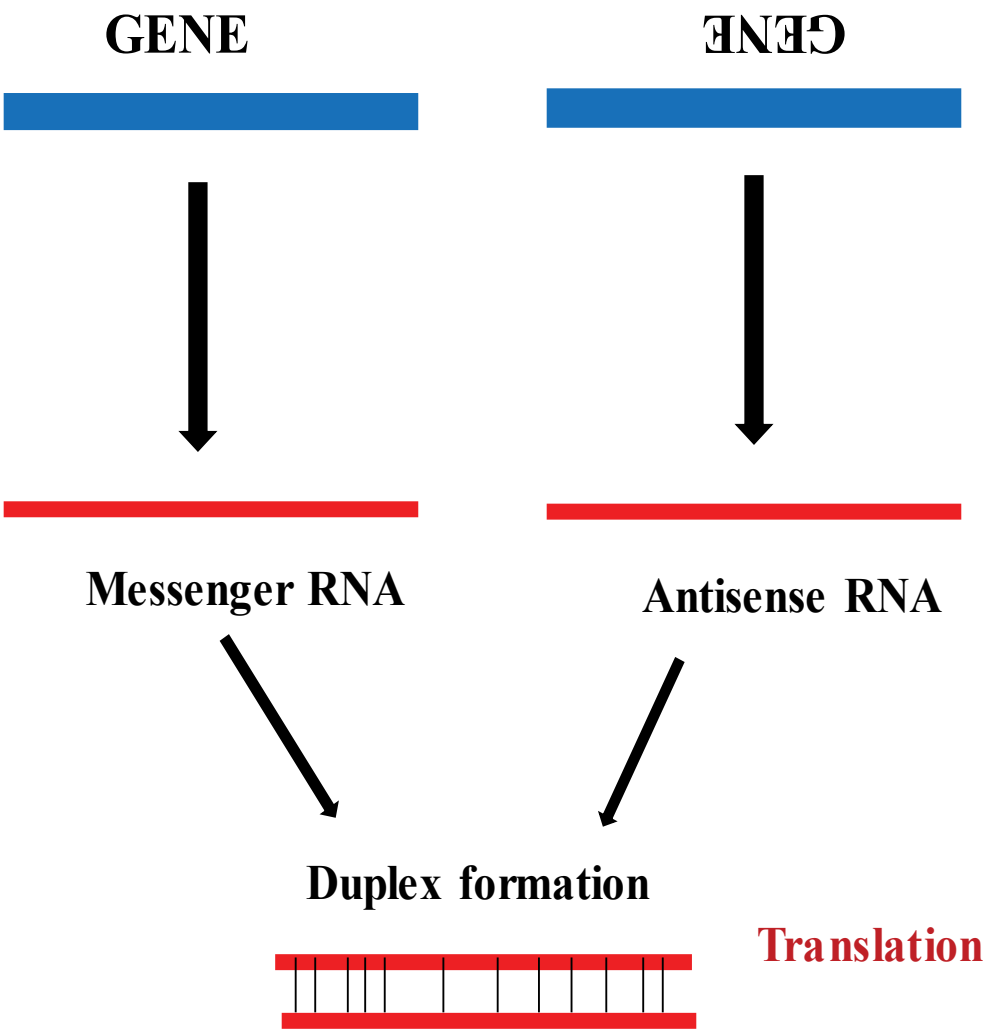


Fig 9: A schematic representation of antisense RNA technology mechanism. When mRNA forms a duplex with a complementary antisense RNA sequence and translation is blocked.

The major limiting factor in the establishment of a successful plant regeneration system from *in vitro* regenerated shoots for mungbean is the low frequency of rooting. Under these circumstances, micrografting could be an alternative options of effective *in vitro* root development in mungbean.

1.5.2 Micrografting of *in vitro* regenerated shoots

Micrografting technique was developed in 1980s in prunes tree plants and consists of the placement of meristem tip or shoots tip explant onto a decapitated rootstock that has been grown aseptically from seed in plastic pots (Gebhardt and Goldbach 1988). The results of *in vitro* micrografting technique and the plant material derived from this technique can be acclimatized to outdoor conditions. Recently, attempts of many researchers to overcome rooting problem have been carried out on fruit trees and woody species using micrografting. Grafting is widely used to propagate several horticultural taxa (Hartmann et al. 1997) to overcome rooting problems and speed growth. Micrografting has specific utility in fruit tree production and protocols have been developed in many fruit crops including almond (Yildirim et al. 2013), cherry (Amiri 2006) and walnut (Wang et al. 2010). Thus micrografting technique can be utilized as an alternative approaches to increase the production of root in mungbean varieties.

Legumes are reported as highly recalcitrant plants towards *in vitro* regeneration and genetic transformation and highly genotype specific (Bajaj and Gosal 1981, Mroginski and Kartha 1984). Reports are also available regarding the problems towards *in vitro* rooting of legumes (Kar et al. 1996, Roy et al. 2001, Jayanand et al. 2003, Chakraborti et al. 2006 and Anwar et al. 2008) which is the main problem towards the genetic improvement of legumes through tissue culture based techniques. Scientist have developed a technique to transfer transgenes in plants through avoiding the *in vitro* regeneration step called 'in planta genetic transformation'. This method was first developed in Arabidopsis by Feldman and Marks (1987). In this study, efficiency of in planta genetic transformation technique was conducted to check whether it helps to avoid the trouble of the recalcitrant nature of mungbean towards *in vitro* regeneration.

1.5.3 Plant genetic transformation studies in mungbean

Availability of efficient *in vitro* regeneration system is a pre-requisite for effective genetic transformation in mungbean (Popelka et al. 2004, Eapen 2008). Mungbean has been regenerated via direct organogenesis (Mathews 1987, Gulati and Jaiwal 1994,

Tivarekar and Eapen 2001, Vijayan et al. 2006, Mahalakshmi et al. 2006, Mundhara and Rashid 2006, Amutha et al. 2006) and indirect organogenesis (Mendoza et al. 1992, Amutha et al. 2003). However, success in recovering transgenics using these regeneration systems has been limited. Jaiwal et al. (2001) reported recovery of transgenic plants using *nptII* gene as a plant selectable marker with a frequency of 0.9% whereas Mahalakshmi et al. (2006) generated transgenics using *hptII* gene at a frequency of 2%. Sonia et al. (2007) developed transgenic plants of mungbean with insecticidal α-amylase inhibitor for bruchid resistance and the *bar* gene for herbicide resistance with a frequency of 1.15%. No other agronomically desirable gene has been introduced into mungbean so far (Sahoo and Jaiwal 2008). An efficient selection system for the recovery of viable and fertile transgenic plants from transformed explants of *Vigna radiata* is required for development of transgenic plants (Sahoo et al. 2003). Methods to transfer genes are grouped in two broad categories: vector mediated and vector less or direct DNA transfer. The three methods, *Agrobacterium*-mediated (vector mediated), biolistic and electroporation (direct DNA transfer) have been used for genetic transformation of *Vigna* species. Among them, *Agrobacterium-tumefaciens* mediated genetic transformation is the preferred method of transformation.

1.5.4 *Agrobacterium*-mediated genetic transformation of mungbean

Agrobacterium-mediated genetic transformation is widely accepted technique for production of transgenic plants in mungbean as compared to direct gene transfer (Sahoo and Jaiwal 2008). This technique is applicable to nearly all types of explants ranging from individual cells, suspension cultured cells, thin cell layers, tissue slices and to whole organ sections. The system offers rapid and precise mode of DNA transfer, high transformation efficiency, low or single copy integration, easy to handle and the less expensive nature (Hiei et al. 1994, Veluthambi et al. 2003).

A quick and efficient method of transferring foreign gene with high transformation efficiency is inevitable for the development of transgenic plants (Eapen 2008). *Agrobacterium tumefaciens* is most used due to the development of highly virulent strains and binary vectors that are helpful for legume transformation. *Agrobacterium* Ti plasmid-based vector are simple, have high frequency of precise transfer and integrate transgenes with single copy insertion, show low incidence of transgene silencing and can transfer long stretches of DNA greater than 150 kb (Veluthambi et al. 2003). Direct organogenesis pathway from seedling explants, cotyledon, cotyledonary node and

primary leaf explants has been adapted for *Agrobacterium*–mediated transformation of *Vigna radiata*. The axillary meristems at the junction of the cotyledon and cotyledonary node explants contain cells most responsive for *in vitro* regeneration through multiple shoot induction and therefore, offers suitable target for gene delivery. For the first time Pal et al. (1991) recovered primary transformants of *vigna radiata* cvs. B1 and T4 from cotyledons using kanamycin selection pressure as a selection agent. On an average 4 - 5% of the shoots grown on the kanamycin selection were transformed as confirmed by dot blot assay and MUG assays. Though, large number of escapes on kanamycin selection, lack of evidence for stabilization and inheritance of transgenes ruled out the applicability of such transformation system. Phogat et al. (1999) selected transgenic calli on kanamycin (100 mg/l) selection medium from primary leaf explants co-cultured with *Agrobacterium tumefaciens* strain LBA4404 pKIWI105. The transformed calli were found resistant up to 750 mg/l of kanamycin, exhibited β-glucuronidase activity and showed integration of *nptII* gene by Southern analysis. However, plants could not be recovered from transgenic calli under practical culture conditions.

In *Vigna radiata*, about 80 -100% transformation efficiency of cotyledon and hypocotyls explants has been noticed with *Agrobacterium rhizogenes* strain LBA9402 (Jaiwal et al. 1998) but transformed tissue gave rise to roots only. Transgenic plants of *Vigna radiata* cv. K-851 were successfully produced from cotyledonary nodes co-cultured with *Agrobacterium tumefaciens* strain LBA4404 pTOK233 at an overall efficiency of 0.9% (Jaiwal et al. 2001). The plants selected on kanamycin containing medium were generated to maturity and stable *GUS* gene expression was detected in stamens, pollen grains and seeds of T_0 plants. Molecular analysis of T_0 transgenic plants stated the integration and expression of transgenes. Transgenic calli were invigorated from primary leaves and hypocotyls explants of *Vigna radiata* at a transformation frequency of up to 50% using *Agrobacterium-tumefaciens* strain EHA105 harbouring pBingusint (Jaiwal et al. 2001). Insertion of transgene was confirmed by southern hybridization analysis. However, in that study no viable shoots were regenerated from the callus. Mahalakshmi et al. (2006) recovered transgenic plants of *Vigna radiata* cv. K-851 from primary leaf explants of 10 days old *in vitro* germinated seedlings, co-cultured with *Agrobacterium-tumefaciens* strain C58 pCAMBIA1301 on hygromycin selection medium. Polymerase chain reaction analysis confirmed the stable integration and inheritance of *hpt* gene. Therefore, absence of plentiful regeneration from primary leaf explants and generation of

escapes on hygromycin containing medium may restrict the use of these explants for routine introduction of desirable genes to *Vigna radiata*. Fertile transgenic plants of *Vigna radiata* were generated from cotyledonary node explants co-cultured with *Agrobacterium-tumefaciens* strain EHA105 harboring a binary vector pKSB that contain bialaphos resistance (bar) gene and *Phaseolus vulgaris* α-amylase inhibitor-1 (αAI-1) gene (Sonia et al. 2007). Transformed shoots were regenerated and rooted when cultured on phosphinothricin (PPT) containing regeneration medium. The transformation frequency was enhanced when the explants were pre-cultured and wounding in the presence of acetosyringone and PPT based selection medium. Expression of the bar gene in primary transformants were confirmed by polymerase chain analysis and PPT leaf paint assay respectively. Integration of the *αAI-1* gene was confirmed by Southern blot hybridization technique. Transgenic plants were recovered after 8-10 weeks of co-cultivation with *Agrobacterium* with a frequency of 1.51%. Double cotyledonary node (DCN) explants were inoculated with *Agrobacterium tumefaciens* strain LBA 4404 harboring a binary vector pCAMBIA2301 containing *nptII* gene as selectable marker, β-glucuronidase (*GUS gene*) as a reporter gene and *annexin1bj* gene. Transient and constitutive GUS expression was noticed in DCN explants and different part of tissues of T_0 and T_1 transgenic plants. After root formation T_0 and T_1 shoots confirming PCR positive for *nptII* and *annexin 1bj* genes. The seeds were collected from the regenerated plants after maturity. Integration of *annexin* gene into the greengram genome was confirmed by Southern blotting technique (Yadav et al. 2012).

1.5.5 Optimization of transformation efficiency in mungbean

Although *in vitro* regeneration and genetic transformation are genotype and explant dependent, optimization of various parameters that affect *Agrobacterium*-mediated genetic transformation need to be optimized to develop transgenic plants with more accuracy and reproducibility (Somers et al. 2003, Sathyanarayana et al. 2012). The various approaches, such as mechanical wounding of explants before *Agrobacterium* inoculation, use of acetosyringone in bacterial inoculation and co-cultivation medium, lower pH (lower than 5.8 - 6.0) low temperature (< 25° C) during infection and co-culture, concentration of bacteria and duration of co-cultivation have been used to enhance transformation efficiency in *Vigna* species.

1.5.6 *GUS* and kanamycin used as marker genes

The β-glucuronidase (uidA/gusA) gene is widely engaged as reporter gene for transient and stable transformation studies in legumes including *Vigna* species (Sahoo et al. 2003). Although GUS assay is destructive in nature localized *GUS* activity in entire tissues can be visualized as blue spots within the tissue. Selectable marker genes are helpful in selection and maintenance of transformed tissue as they allow the transformed cells to proliferate and grow in the presence of a selective agent while the non-transformed cells either do not grow or multiply at a slow rate. Reporter genes or screenable marker gene code for the products that detoxify selective agents or catalyze specific chemical reactions whose products are detectable. The most commonly utilized selectable marker gene in legumes is neomycin phosphotransferase (*nptII*) which impart resistance to kanamycin or its analogues, geneticin by inactivating them through phosphorylation. It does not damage normal shoot regeneration and shoot vigor and helps in the early identification of green transformed shoots as the shoots emerging from non-transformed cells are bleached (albino). However, recovery of many escapes or chimeric shoots in the presence of kanamycin is the main problem. Kanamycin has been used for the selection of transformants in *V. radiata* (Jaiwal et al. 2001), *V. mungo* (Saini et al. 2003, Saini and Jaiwal 2005), *V. unguiculata* (Chaudhary et al. 2007) and *V. angularis* (Yamada et al. 2001). Geneticin in conjunction with *nptII* has been found to be more effective in selection of transformed shoots in cowpea (Solleti et al. 2008). The hygromycin phosphotransferase (*hpt)* gene which confers resistance to hygromycin has also been used in *Vigna radiata* (Mahalakshmi et al. 2006) and *V. angularis* (Shemy et al. 2002) for the recovery of transformants with minimum escapes. The efficiency of transformation has been improved by replacing antibiotic resistance genes with genes for herbicide tolerance, as selectable marker. Bialaphos resistance gene (bar) which acetylates and detoxify phosphinothricin has been efficiently used for selection of transformants in *V. radiata* (Sonia et al. 2007), *V. mungo* (Muruganantham et al. 2007) *V. unguiculata* (Popelka et al. 2006) and *V. angularis* (Khalafalla et al. 2005).

1.5.7 Strategies for the development of yellow mosaic virus resistance transgenic plants

As the major constraint affecting the yield of mungbean is yellow mosaic disease caused by mungbean yellow mosaic virus (MYMV) (Maiti et al. 2010, Singh et al. 2013b) and none of the known varieties of mungbean is fully resistant to MYMV. Therefore, plant genetic engineering based on "pathogen-derived resistance" is an alternative and promising approach to develop MYMV resistance which involves introduction of pathogen genes in a dysfunctional form or their over expression or expression at inappropriate stage during viral replication cycle, which could disrupt infection by the invading pathogen. Pathogen-derived resistance is generally mediated by RNAi which leads to identification of post-transcriptional gene silencing in plants.

Studies have been done to develop coat protein mediated virus resistant transgenic papaya (Tennant et al. 1994), tomato (Kunik et al. 1994) and tobacco (Beachy 1999). But limited studies have been carried out for the development MYMV resistant mungbean lines following an antiviral strategy to control yellow mosaic disease of mungbean. Therefore, an antiviral strategy could be adopted through development of transgenic plants expressing the viral coat protein gene (*CP*) and a silencing suppressor (*AC2*) gene in an anti-sense orientation to control MYMV caused yellow mosaic disease. Due to being in anti-sense orientation, the produced mRNAs will be complimentary to the mRNAs of *CP* gene and *AC2* gene of the MYMV. As a result, a heteroduplex will be formed and the translation of these two viral genes will be hindered. That is, the virus will no longer be able to produce coat protein and will also fail to silence the host defense mechanism. In a nutshell, through developing such a transgenic plant, it will be possible to eliminate the infection of MYMV as well as the formation of new MYMV particles in plants.

Thus it may be mentioned here that yellow mosaic disease is a major problem affecting the yield of mungbean and RNAi has been proved to be an efficient and promising technology against viruses in recent years. Therefore, the present investigation was undertaken to develop MYMV resistance in mungbean by using antisense RNAi technology via *Agrobacterium*-mediated genetic transformation of a newly developed antiviral gene construct (pBI121CP-AC2) targeting mungbean yellow mosaic virus (MYMV). This gene construct was developed in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. Before transferring the

gene construct in the plant of interest, i.e. mungbean, primarily it was inserted into a model plant *Nicotiana tabacum* through genetic transformation to evaluate its transforming efficiency.

Considering the importance of developing YMV resistance in mungbean, the present study was undertaken with the following objectives.

The objectives of the present investigation were:

1. Isolation and cloning of mungbean yellow mosaic virus (MYMV) coat protein gene (*CP*) and silencing suppressor gene *(AC2*) in pBI121 backbone vector of *Agrobacterium* in an antisense (as) orientation to generate antiviral pBI121CP-AC2 vector construct.
2. Establishment of suitable *in vitro* regeneration as well as *Agrobacterium*-mediated genetic transformation protocols for developing transformed mungbean lines using marker gene (pBI121GUS-NPTII).
3. Transformation of suitable explants of local cultivars of mungbean using newly developed pBI121CP-AC2 vector construct for developing transgenic mungbean lines conferring mungbean yellow mosaic virus (MYMV) resistance by expressing MYMV coat protein (*CP*) and *AC2* gene in an anti-sense orientation.

2. MATERIALS

2.1 Plant materials

Two varieties of mungbean [*Vigna radiata* (L.) Wilczek] namely, BARI mung-3 and Binamoog-5 were used in the present investigation. Yellow mosaic virus infected leaf samples of mungbean obtained from different fields as well as tobacco (*Nicotiana tabacum* L.) seeds were used as materials during this study.

2.1.1 Virus infected leaf samples for isolation of genomic DNA

Mungbean yellow mosaic virus (MYMV) infected leaf samples (Fig. 10 a, b, c, d) were collected from different experimental fields of Sher-e-Bangla Agricultural University (SAU), Bangladesh Agriculture Research Institute (BARI), Gazipur and Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. The yellow mosaic virus infected leaf samples were used as plant material for isolation of good quality of DNA.

2.1.2 Tobacco Plant materials used for *Agrobacterium*-mediated genetic transformation

In the present investigation Petit Havana variety of tobacco (*Nicotiana tabacum* L.) was used as a model plant for *Agrobacterium*-mediated genetic transformation.

2.1.2.1 Source

Seeds of tobacco (*Nicotiana tabacum* L. cv. Petit Havana) variety were collected from ICGEB (International Centre for Genetic Engineering and Biotechnology), New Delhi, India and maintained in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. Seeds of this tobacco variety are shown in Fig. 11 a.

Important characteristics of this variety are described below:

2.1.2.2 Petit Havana variety

It is a robust annual little branched herb up to 2.5 m high with large green leaves the leaves are large and oval, with rounded or pointed ends. The leaves vary in length from 50 to 60 cm and are about half as wide as they are long. Many flowered inflorescences are terminal in position, flowers are tube-shaped, and the tube is 5 - 6 cm long and 5 mm in diameter.

Flowers are white-pink in color. All parts of this plant are sticky, covered with short viscid-glandular hairs. The seeds are numerous, very small and black in color.

2.1.2.3 Explants

In the present investigation the leaf disc without midrib were used as explant. These explants were collected from the leaves of 25 - 30 days old *in vitro* grown seedlings of the above mentioned tobacco variety (Fig. 11 b).

2.1.3 Plant materials for *in vitro* regeneration and genetic transformation of mungbean

For the current experiment, two locally grown mungbean [*Vigna radiata* (L.) Wilczek] varieties namely, BARI mung-3 and Binamoog-5 were used. The seeds of these varieties are presented in Fig. 11c.

2.1.3.1 Source

Seeds of the variety Binamoog-5 were collected from Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh and seeds of the variety BARI mung-3 were collected from Pulse Research Centre, Bangladesh Agriculture Research Institute (BARI), Gazipur. Later on, the seeds were maintained in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka.

2.1.3.2 Description of the plant materials

Important characteristics and information about two varieties of mungbean used in this study are described below:

(i) Binamoog-5: A late winter-summer mungbean variety released in 1998. It matures between 70 - 80 days. Seed is green shiny and bigger than Binamoog-1. Almost all the pods mature at the same time. Plants are short and tolerant to *Cercospora* leaf spot and mungbean yellow mosaic virus (MYMV) diseases. Maximum seed yield production is 2.0 t/ha (average 1.5 t/ha).

(ii) BARI mung-3: It is a cross material derived from Sonamung (Bangladeshi local cultivar) and BARI mung-2 (released variety from Pulse Research Centre (PRC), BARI in 1987). It is a photosensitive and high-yielding variety with low disease incidence. It has tolerance to MYMV. This variety is erect and trifoliate. Leaves are moderately pubescent and the terminal leaf is ovate. Petiole length

is intermediate and greenish purple. Racemes are situated above the canopy. The corolla of the flower is yellow with a light green calyx. Mature plants obtain a height of 50 - 55 cm long. Flowering occurs in 30 - 34 days, and physiological maturity is reached in 60 - 65 days after emergence, which is 10 days earlier than the other local cultivar. The number of pods per plant varies from 8 to 12. After maturity, pods turn black and seeds are drum-shaped and greenish brown color. Seed weight is 29 g/1000 seed. The average yield of this variety is 1.3 t/ha.

2.1.3.3 Explants

During this experiment, two types of explants were used, such as cotyledon attached decapitated embryo (CADE) and cotyledonary node (CN). The cotyledon attached decapitated embryo (CADE) explants were prepared from overnight soaked seeds in autoclaved distilled water in dark (Fig. 12 a, b, c, d). Cotyledonary node (CN) explants were derived from 3 days old aseptically germinated seeds (Fig. 13 a, b, c, d) of the above-mentioned varieties.

2.2 *Agrobacterium* strain and vector constructs

Two different genetically engineered constructs were used in transformation experiments. These were:

a) Construct I (pBI121GUS-NPTII)

Agrobacterium tumefaciens strain LBA4404 contains plasmid pBI121GUS-NPTII (Fig. 14 a) (binary vector). This binary vector has two genes within the right border (RB) and left border (LB) region of the gene construct (Fig.14 b):

(i) The *udiA* gene (Jefferson et al. 1987) encoding *GUS* gene (β-glucuronidase), driven by CaMV 35S promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation. The *nptII* gene (Herrera et al. 1983) encoding neomycin phosphotransferase II conferring kanamycin resistance, driven by NOS promoter and NOS terminator.

The reporter gene *GUS* was used to assess the efficiency of transformation. The *GUS* gene 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 as a selectable marker gene.

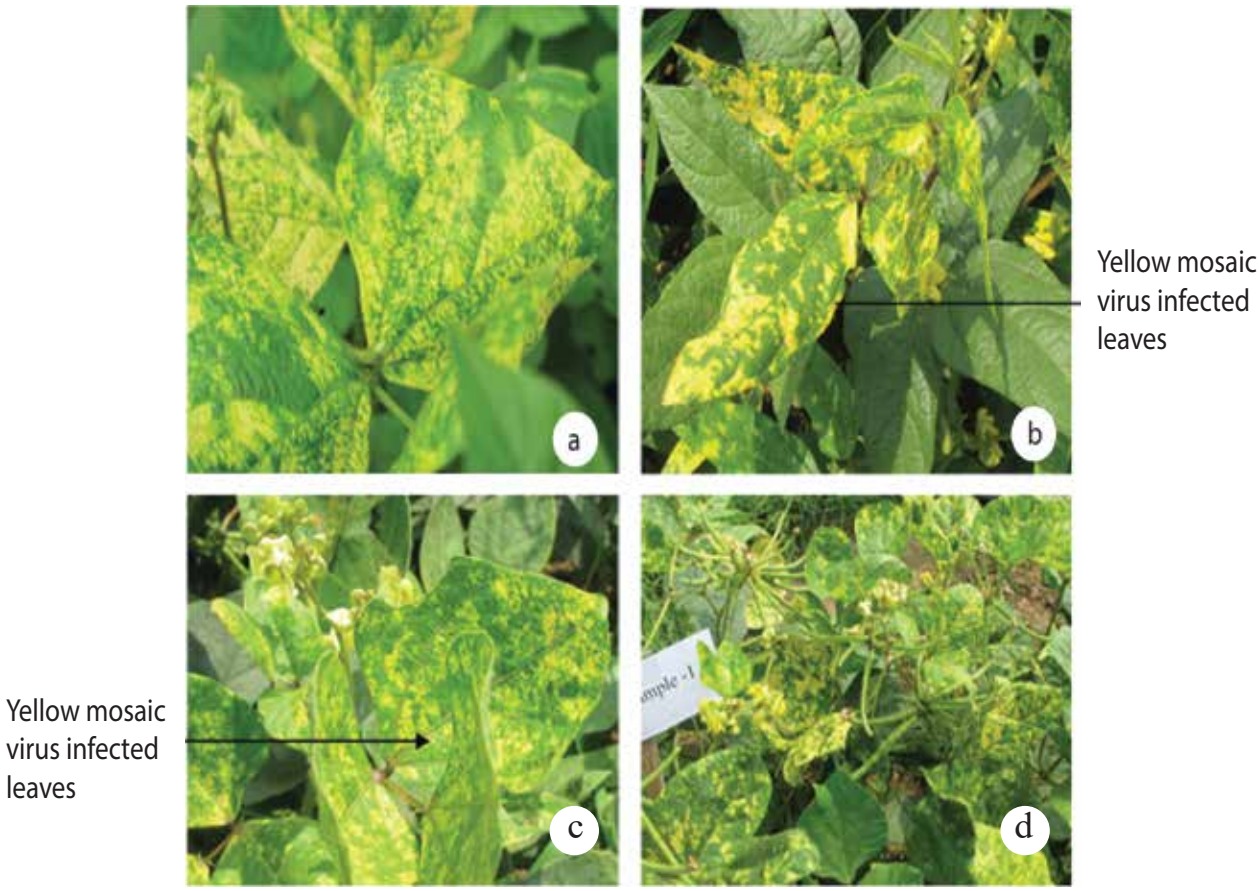


Fig. 10 (a-d): Mungbean yellow mosaic virus (MYMV) infected leaf samples were collected from different regions of Bangladesh. (a) MYMV infected leaf samples were collected from Sher-e-Bangla Agricultural University (SAU); (b) Bangladesh Agriculture Research Institute (BARI), Gazipur; (c-d) Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh.

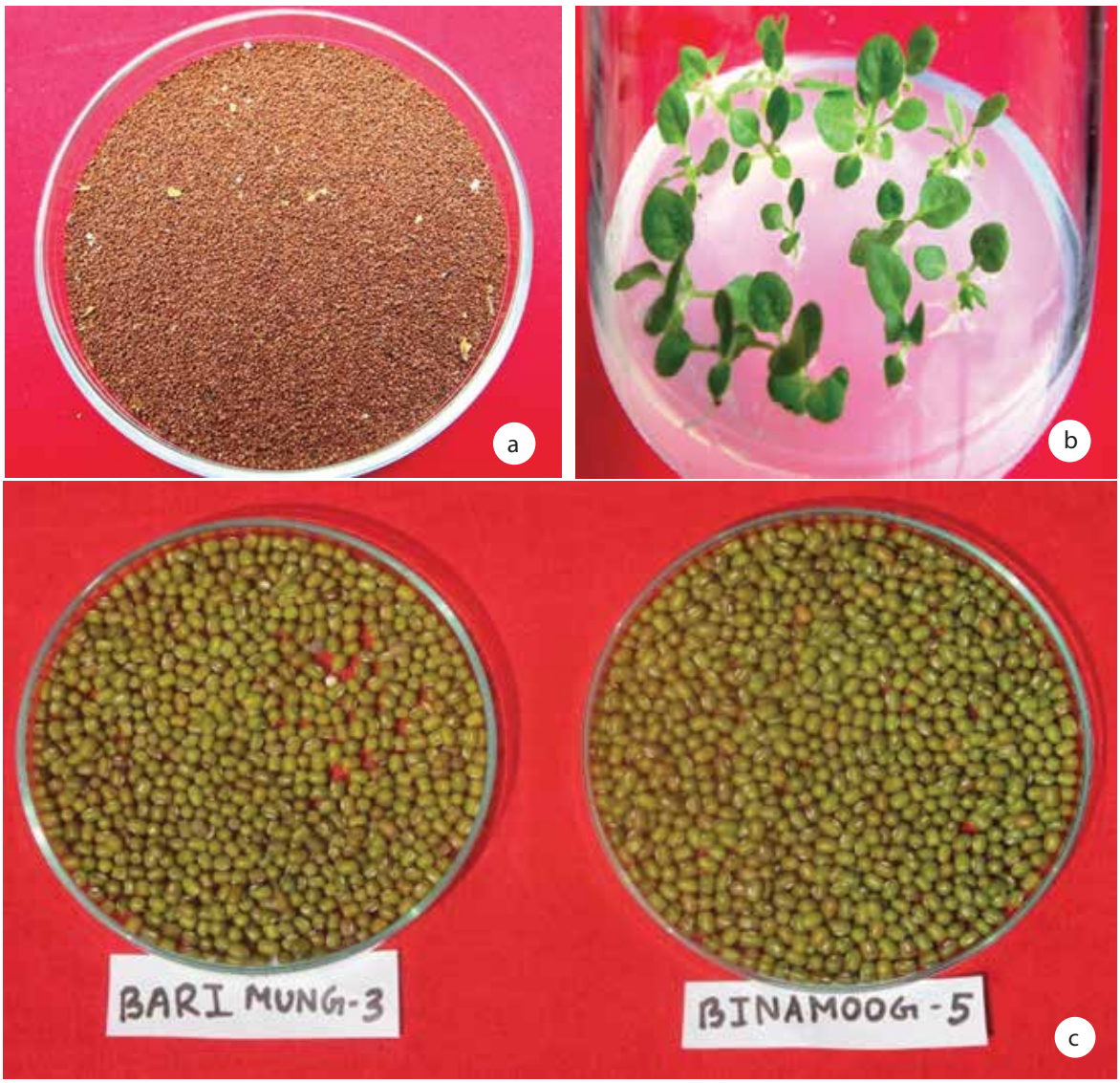


Fig. 11: Seeds of tobacco and mungbean plants. (a) Seeds of tobacco; (b) Seedling of Petit havana variety of tobacco and (c) Seeds of BARI mung-3 and Binamoog-5 varieties.

Fig. 12 (a-d): CADE explants preparation from two mungbean varieties. (a-b) Overnight soaked seeds of BARI mung-3 and Binamoog-5 varieties respectively; (c-d) Cotyledon attached decapitated embryo explants of the two varieties of mungbean.

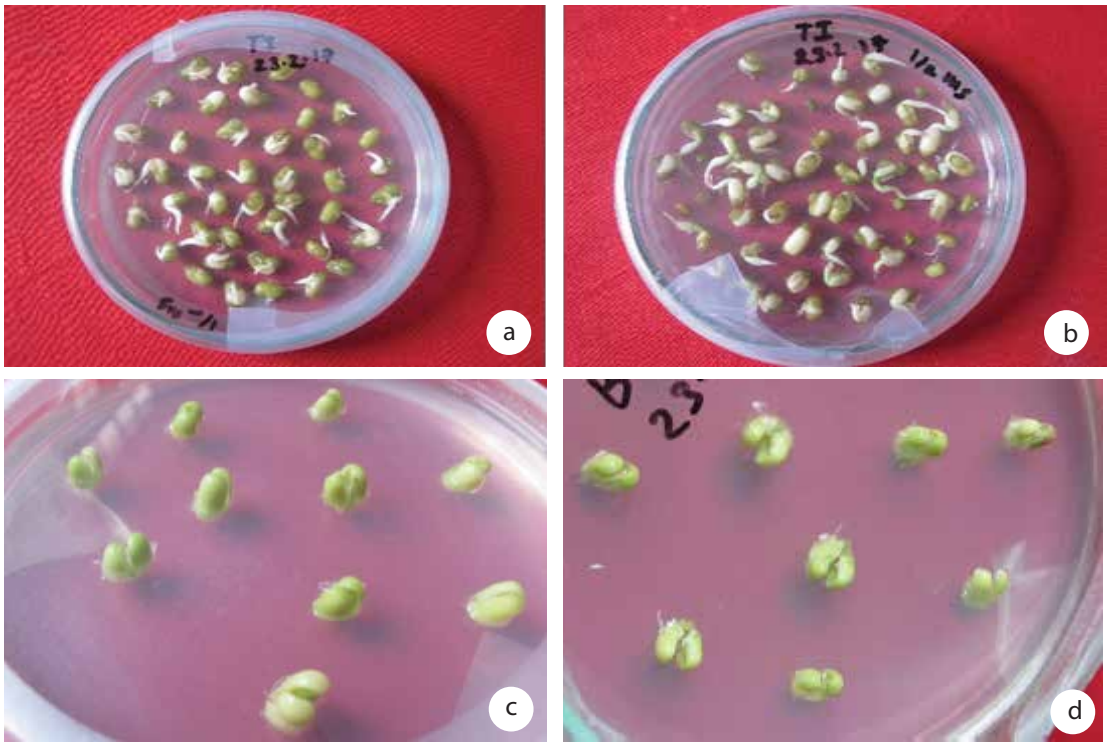


Fig. 13 : CN explants preparation from two mungbean varieties. (a-b) 3 days old in vitro germinated seedlings of BARI mung-3 and Binamoog-5 varieties; (c-d) Cotyledonary node explants of the two varieties of mungbean.

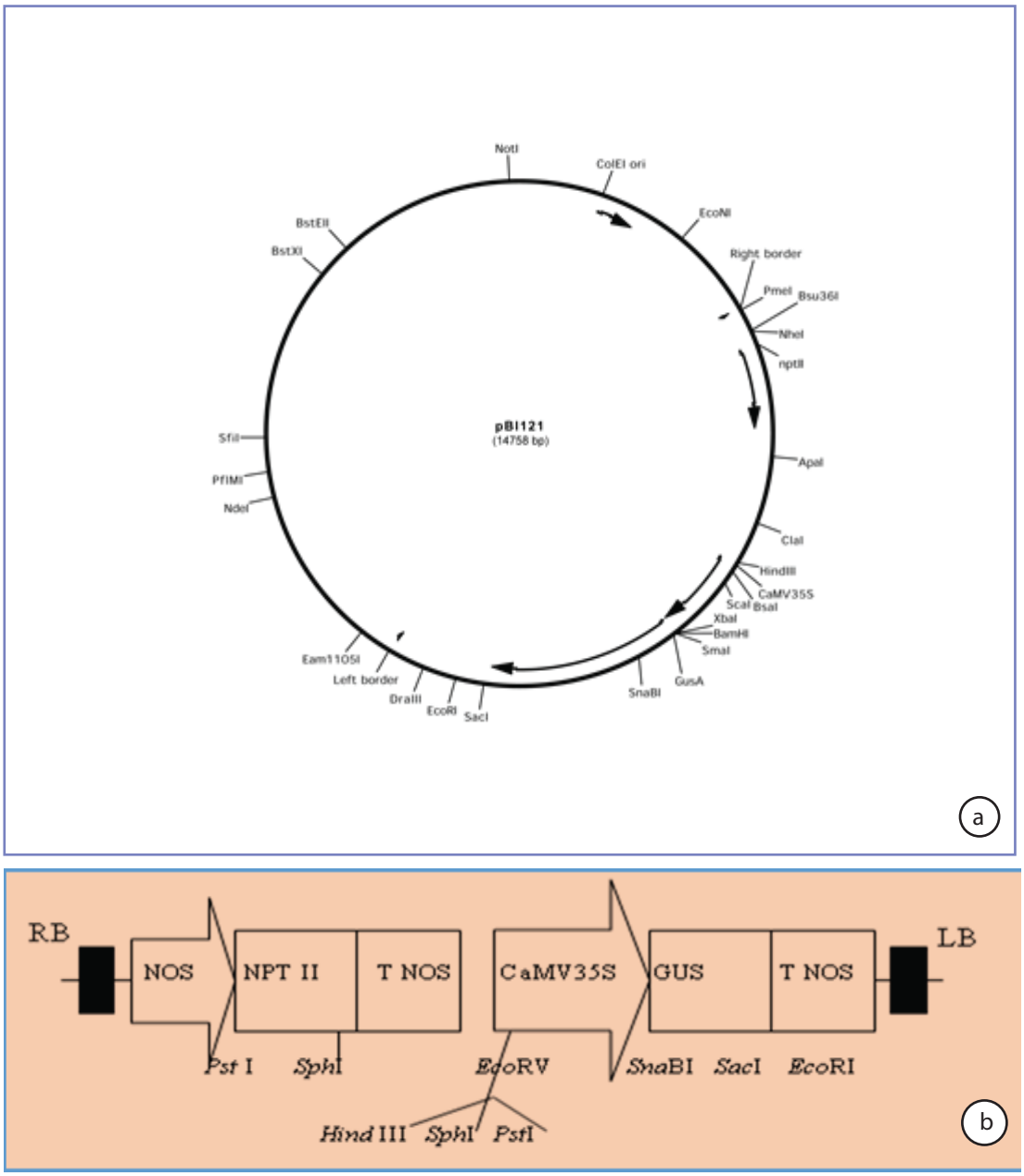


Fig. 14: Diagrammatic representation of the construct I (pBI121GUS-NPTII). (a) Circular map of the pBI121GUS-NPTII; (b) T-DNA region of the pBI121GUS-NPTII gene construct.

b). Construct II (pBI121CP-AC2)

Agrobacterium tumefaciens strain LBA4404 contains plasmid pBI121CP-AC2. This binary vector contains *nptII* and *CP-AC2* genes within the right border (RB) and left border (LB) region of the construct (Fig. 15). This strain LBA4404 was kindly provided by plant transformation group, ICGEB New Delhi, India. The *CP-AC2* gene was cloned into pBI121 plasmid vector instead of *GUS* gene and driven by CaMV 35S promoter. The vector construct was developed in the Plant Breeding and Biotechnology laboratory, Department of Botany, University of Dhaka. The construct was further transformed into *Agrobacterium tumefaciens* strain LBA4404.

2.3 Chemicals required

The following chemicals were used in the present study -

2.3.1 Chemicals used for isolation of plant genomic DNA

All the solutions were made with deionised, sterile distilled water.

(a) CTAB-buffer:

3 %	CTAB
1.4 M	NaCl
0.2 %	ß-Mercaptoethanol
20 mM	EDTA
100 mM	Tris-HCl pH 8.0
0.5 %	PVP (soluble)

Add CTAB and ß-Mercaptoethanol after autoclaving

(b) 24:1 CI Mix

24ml	Chloroform
1ml	Isoamylalcohol

(c) Wash buffer (WB)

76 %	Ethanol Abs.
10 mM	Ammonium acetate

(d) RNase A: 10µg/µl Stock sol. in ddH_2O

(e) 7.5 M NH_4-Acetate

(f) 0.5 M EDTA (pH 8)

(g) TE-buffer + RNase A

10 mM Tris-HCl, pH 8.0

1 mM EDTA

10 µg/ml RNaseA.

The volume was adjusted to 100 ml and stored at room temperature.

(h). 70% Ethanol

(i). Isopropanol

(j). Liquid nitrogen

2.3.2 Chemicals needed for agarose gel electrophoresis

(a) Electrophoresis buffer (50× TAE) 500ml

Tris base	121 g
Acetic acid	26.8 ml
0.5 M EDTA, pH 8.0	50 ml

(b) Ethidium-bromide

10 mg/ml stock solution, store at 4°C.

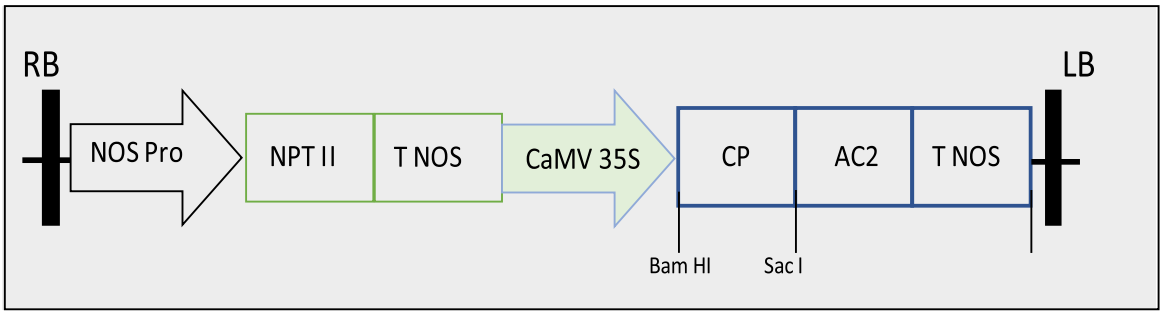


Fig. 15 : Diagrammatic representation of T- DNA region between left (LB) and right (RB) border of the construct II (pBI121CP-AC2).

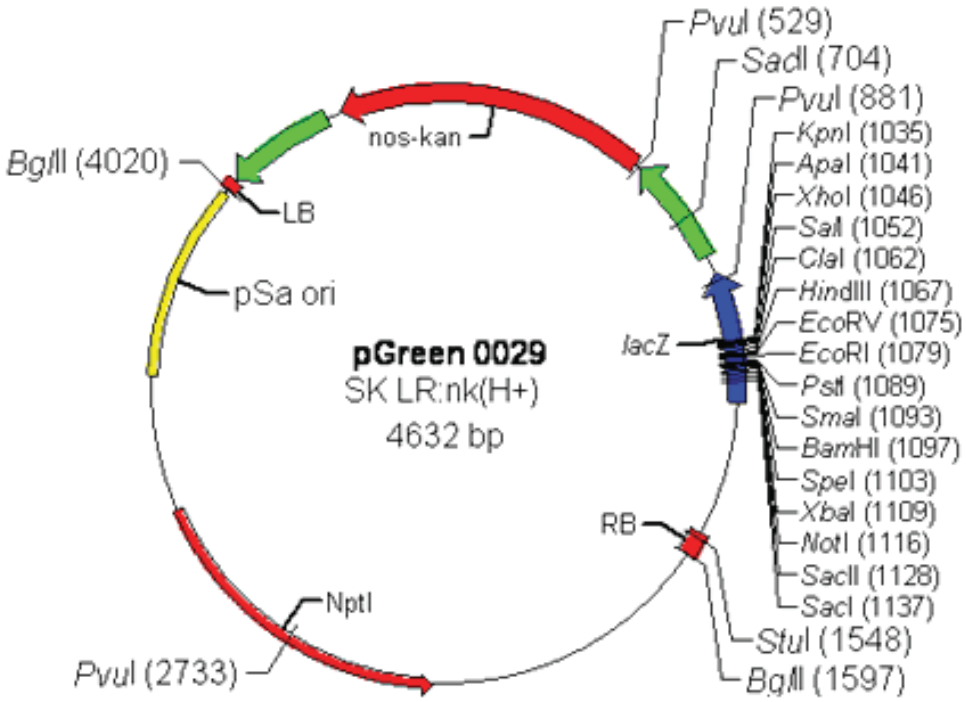


Fig. 16: Diagrammatic representation of the cloning vector pGeen0029.

2.3.3 Following primers (with their sequence information) were used for various PCR analysis during this study.

Primer name	Sequences of the primer
MYMV CP-F	5´- ACACGAGCTCCTCTACCCCGATATCGAATG -3´
MYMV CP-R	5´- ACACGGATCCGTTGCATACACAGGATTTG -3´
MYMVAC2-F	5´- ACACGGATCCTCATCCCAGAAGTCGCC -3´
MYMV AC2 -R	5´- ACACGGATCCATGCGGAATTCTACACCC -3´
pGreen-F	5´- CTCTTCGCTATTACGCCAGC -3´
pGreen-R	5´- GTGAGCGGATAACAATTTCAC -3´
pBI121-F	5´-CATTTGGAGAGAACACGGGG -3´
pBI121-R	5´-CAAGACCGGCAACAGGATTC -3´
Pic A-F	5´- ATG CGC ATG AGG CTC GTC TTC GAG -3´
Pic A-R	5´- GAC GCA ACG CAT CCT CGA TCA GCT -3´
Kan -F	5´- ATAAACCCAGCGAACCATTTGAGGT -3´
Kan -R	5´- TACGCAGCGGTATTTTTCGATCAGT -3´
GUS -F	5´- CCT GTA GAA ACC CCA ACC CG-3´
GUS -R	5´- TGG CTG TGA CGC ACA GTT CA -3´

2.3.4 Required materials for rolling circle amplification (RCA) of viral genome

1. Mungbean yellow mosaic virus (MYMV) infected plant genomic DNA
2. Templiphi^{100} rolling circle amplification kit (RCA kit)

2.3.4.1 Components of the Templiphi^{100} amplification kit (GE Healthcare Life Sciences, UK)

1. Sample buffer (white cap)
2. Reaction buffer (blue cap)
3. Enzyme mix (yellow cap)
4. Positive control DNA (pUC19, 2 ng/µl)

2.3.4.2 Following materials were used for cloning of full-length viral genome

2.3.4.2.1 Material for RCA digestion

1. Product of RCA reaction
2. 10× Fast digest green buffer
3. Fast digest Bam HI or Pst I
4. De-ionized, sterile H_2O

2.3.4.2.2 Required materials for restriction digestion of the pGreen0029 cloning vector

1. pGreen0029 cloning vector (Fig. 16)
2. Fast digest Bam HI and Pst I restriction enzyme
3. 10× Fast digest green buffer
4. Deionized distilled water

2.3.4.2.3 Chemicals required for dephosphorylation of vector

1. Fast alkaline phosphatase
2. 10× Fast green buffer
3. Linearized vector DNA
4. Deionized distilled water

2.3.4.2.4 Chemicals for ligation reaction

1. Linearized vector DNA
2. Insert DNA (Linearized RCA product)
3. Ligase enzyme
4. 10× buffer
5. Deionized H_2O

2.3.4.2.5 Preparation of competent cell

1. *E. coli* Bacterial strain Top10
2. TFB I and TFB II solution
3. YT medium
4. 87 % glycerine

2.3.4.2.6 Chemicals for transformation of ligation products

1. *E. coli* Top10 competent cell
2. Water bath maintaining 42°C
3. LBA medium
4. 37°C incubator

2.3.5 Antibiotics used for various purposes

Antibiotics	Molecular weight	Source	Solvent
Combactam	582.6	Pfitzer, USA	ddH_2O
Ticarcillin	428.4	DUCHEFA, Netherlands	ddH_2O
Cefotaxime	455.47	ALKEM, India	ddH_2O
Kanamycin	484.49	DUCHEFA, Netherlands	ddH_2O
Streptomycin	1457.4	DUCHEFA, Netherlands	ddH_2O
Rifampicin	822.94	DUCHEFA, Netherlands	ddH_2O

2.3.6 Required chemicals for Favor prep plasmid DNA extraction mini kit (Thermo Fisher scientific)

1. FAPD1 buffer
2. FAPD2 buffer
3. FAPD3 buffer
4. Wash buffer
5. W1 buffer
6. Elution buffer
7. RNase A (50mg/ml)
8. FAPD column 100
9. Collection tube 100

2.3.7 Required chemicals for PCR of RCA product

1. RCA product
2. Gene specific primers:
3. 10× PCR reaction buffer
4. 25 mM $MgCl_2$ in water for PCR
5. 10 mM dNTP mix (2.5mM of each dNTP)
6. Taq DNA polymerase
7. Sterile dH_2O

All the chemicals used for the preparation of these reagents were of molecular biology grade and water used was ultra-pure.

2.3.7.1 Purification of PCR product from gel by Gene Jet PCR purification kit (Thermo Fisher scientific)

1. Binding buffer
2. Wash buffer
3. Elution buffer
4. 3M sodium acetate (pH 5.2)
5. Column tube

2.3.7.2 Sequencing of PCR product from cloned sample

1. Gene specific primers
2. Purified PCR product
3. Automated sequencer machine

2.3.7.3 Tools for bioinformatics analysis

1. Bio-edit version 7.0 software
2. NCBI- BLAST databases

2.3.8 Following chemicals were used for cloning of *CP* and *AC2* gene into pBI121 cloning vector

2.3.8.1 Amplification of *CP* and *AC2* gene

1. RCA product isolated from MYMV infected genomic DNA
2. Gene specific primer
3. PCR chemicals

2.3.8.2 Cloning vector

- pBI121

2.3.8.3 Chemicals used for the restriction digestion of the vector

1. Vector DNA pBI121
2. Fast digest Bam HI and Sac I restriction enzymes
3. 10 × Fast digest green buffer
4. De-ionized water

2.3.8.4 Chemicals used for ligation reaction

1. Linearized vector DNA
2. Insert DNA (Purified *CP* and *AC2* gene)
3. Ligase enzyme
4. 10× buffer
5. Deionized H_2O

2.3.9 Chemicals required for transformation of pBI121CP-AC2 gene construct into *Agrobacterium tumefaciens* (LBA4404) competent cells

1. *Agrobacterium* strain LBA4404
2. Electroporator machine
3. Electro-competent agro cell
4. Cuvette

2.3.10 Chemicals used for tissue and cell culture

Components of tissue culture medium

Materials	Molecular weight	Source
MS Basal Salt Mixture	476	DUCHEFA, Netherlands
MSB_5 ready mix	484.47	DUCHEFA, Netherlands
Agar		DUCHEFA, Netherlands
Sucrose	180	SIGMA, USA
Gelrite		SIGMA, USA

2.3.11 Plant growth hormones (PGR) and additives

PGR	Molecular weight (MW)	Source	Solvent used
BAP	225.3	DUCHEFA, Netherlands	1[N] NaOH
Kinetin	215.2	DUCHEFA, Netherlands	1[N] NaOH
IAA	175.18	DUCHEFA, Netherlands	1[N] NaOH
NAA	186.2	DUCHEFA, Netherlands	1[N] NaOH
IBA	203.2	DUCHEFA, Netherlands	1[N] NaOH

2.3.12 GUS-assay buffer

(i) 100 mM sodium phosphate buffer (pH 7.0)

(ii) 0.5 mM potassium ferrocyanide

(iii) 10 mM EDTA

(iv) 1 mM (0.5 mg/ml) X-GLUC (dissolved in DMSO before adding it to GUS buffer)

2.3.13 DNA markers

DNA marker	Concentration	Company
Gene Ruler^{TM} 100 bp DNA ladder	0.5 mg/ml	MBI Fermentas
Gene Ruler^{TM} 1 kbp DNA ladder	0.5 mg/ml	MBI Fermentas

2.3.14 Solvent, sterilizers and others with their sources

Compound	Company
Dimethyl sulfoxide (DMSO)	SERVA, Germany
KOH	Carl Roth, Germany
NaOH	Carl Roth, Germany

3. METHODS

Various methods used in the present investigation have been presented under the following heads:

3.1 Primer designing

The primer design is an important step to get an optimum PCR products. It is necessary to design primers that are complementary to the template region of DNA. Short primers are mainly used for amplifying a small, simple fragment of DNA. Thus, proper primer design is necessary for successful DNA amplification. A set of gene specific primer was designed to amplify the MYMV coat protein gene (*CP*) and silencing suppressor gene (*AC2*) from the infected leaf samples.

3.1.1 Designing of coat protein gene (*CP)* primer

Coat protein gene sequences from different strains of MYMV was collected from the NCBI database. Using Bio edit software all the coat protein sequences were aligned to identify a common region for designing a set of *CP* gene specific primers (Fig. 17). For cloning purpose, Sac I restriction site was added to the 5′ end of forward primer and Bam HI restriction site was added to the 5′ end of reverse primer.

Forward primer (MYMV CP F): 5′ACACGAGCTCCTCTACCCCGATATCGAATG 3′

Reverse primer (MYMV CP R): 5′ ACACGGATCCGTTGCATACACAGGATTTG 3′

3.1.2 Designing of *AC2* gene specific primer

AC2 gene sequences from different strains of MYMV was collected from the NCBI database. Using Bio edit software all the *AC2* gene sequences were aligned to identify a common region for designing a set of *AC2* gene specific primers (Fig. 18). Bam HI restriction site was added with forward and reverse primer to amplify *AC2* gene.

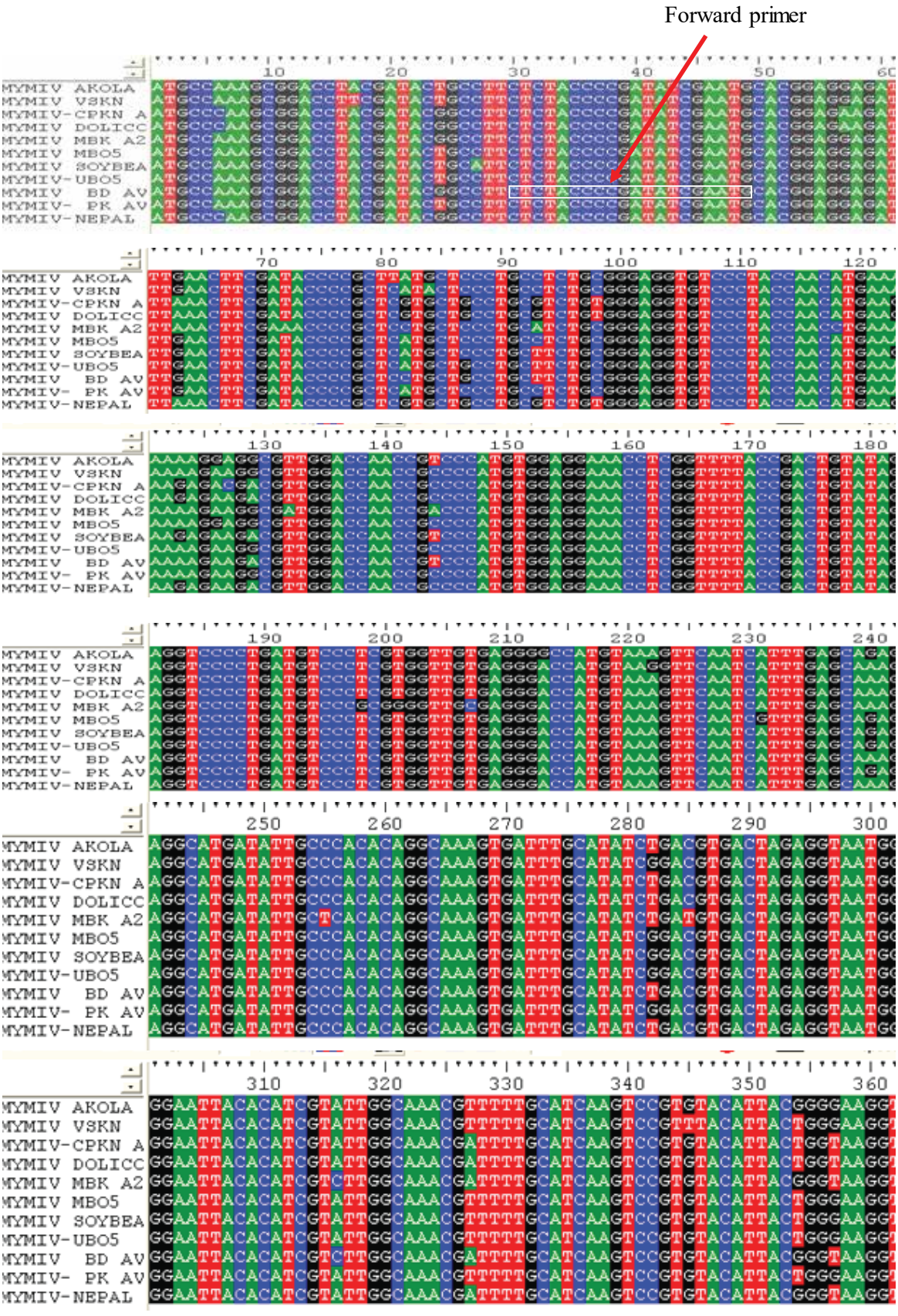
Forward primer (MYMV AC2 F): 5′ACACGGATCCTCATCCCAGAAGTCGCC 3′

Reverse Primer (MYMV AC2 R): 5′ ACACGGATCCATGCGGAATTCTACACCC 3′

3.2 Isolation of plant genomic DNA

The following chemicals and protocols were used for isolation of good quality of DNA

3.2.1 Chemicals used for isolation of plant genomic DNA


Forward primer
MYMIV AKOLA
MYMIV VSKN
MYMIV-CPKN A
MYMIV DOLICC
MYMIV MBK A2
MYMIV MBO5
MYMIV SOYBEA
MYMIV-UBO5
MYMIV BD AV
MYMIV- PK AV
MYMIV-NEPAL

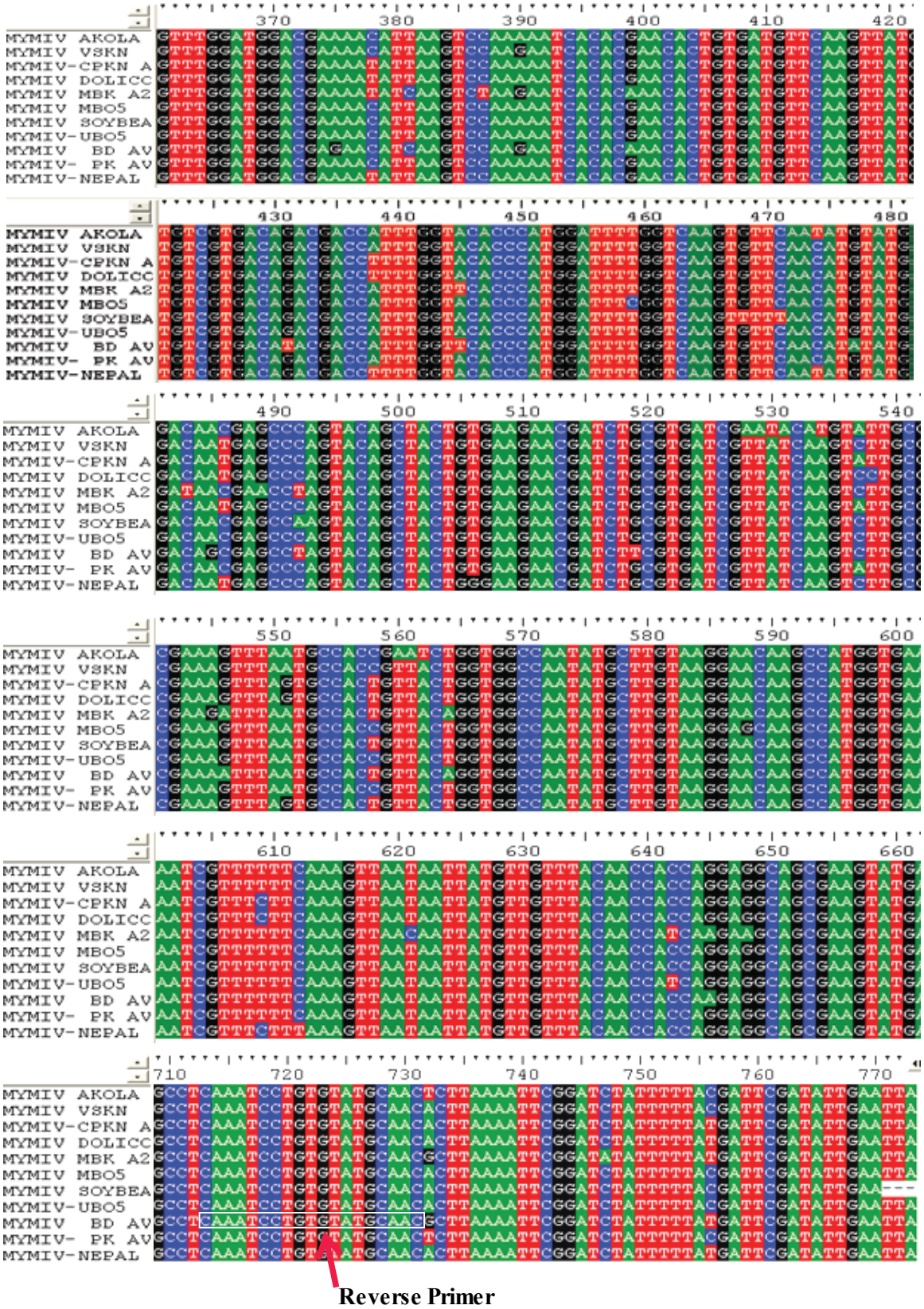


Fig. 17: Multiple alignment of coat protein gene sequences of different strains of MYMV.

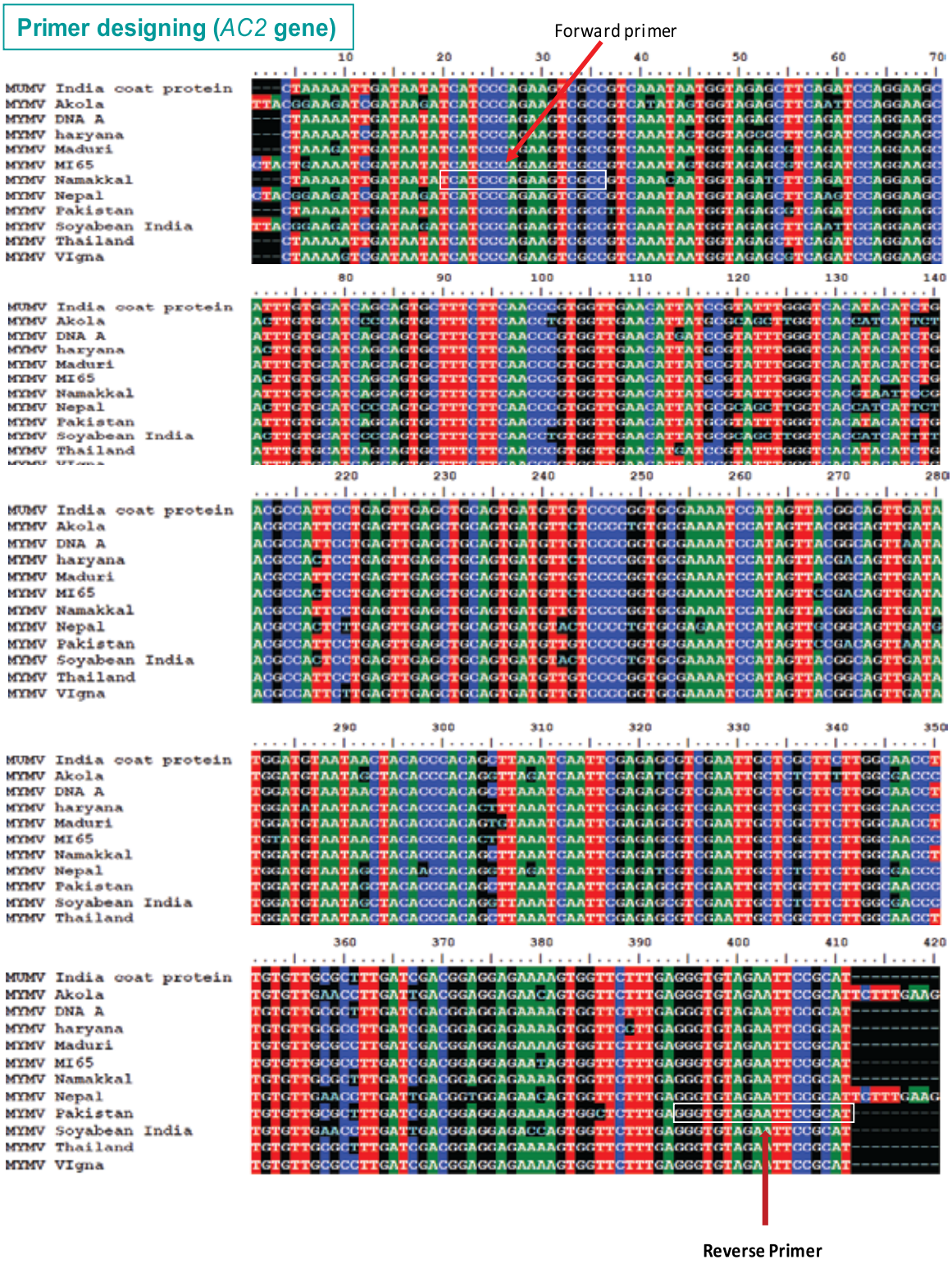


Fig. 18: Multiple alignment of *AC2* gene sequences of different strains of MYMV.

3.2.1.1 Preparation of 1 M Tris HCl stock solution pH 8.0 (100 ml)

12.14 gm of Tris base was dissolved in 80 ml of double distilled water. The pH of this solution was adjusted to 8.0. The final volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it sterilized by autoclaving and stored at 4ºC.

3.2.1.2 Preparation of 0.5 M stock solution EDTA pH 8.0 (100 ml)

18.61 gm of EDTA was added to 80 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 gm of NaOH pellets were added to adjust the final pH to 8.0. Final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.2.1.3 Preparation of 5 M NaCl stock solution (100 ml)

29.22 gm of sodium chloride (NaCl) was dissolved in 80 ml of distilled water. Then it was final volume to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.2.1.4 β-Mercaptoethanol

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

3.2.1.5 Preparation of Ribonuclease A stock solution

10 mg RNase A was dissolved in 1 ml of deionized distilled water and was stored in -20º C for long time used.

3.2.1.6 Preparation of Chloroform:Isoamyl alcohol (24:1) (100 ml)

24 ml of Chloroform and 1 ml of Isoamyl alcohol were mixed properly by vortex mixture. Chloroform: Isoamyl alcohol mixture is caustic and produces fumes. So, it was used only in a fume hood wearing gloves and eye protection.

3.2.1.7 Preparation of 70% Ethanol (100 ml)

For 100 ml 70 % ethanol preparation 30 ml double distilled water was added in 70 ml absolute ethanol.

3.2.1.8 Preparation of TE buffer pH 8.0 (100 ml)

From the stock solution (1M Tris-HCl) 1 ml 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 water. The stock solution was sterilized by autoclaving and stored at 4ºC.

3.2.1.9 Preparation of 7.5 M Ammonium acetate pH 5.2 (100 ml)

57.81 g of ammonium acetate was dissolved in 70 ml of distilled H_2O and was adjusted to a volume of 100 ml with the addition of distilled H_2O and was sterilized by autoclaving.

3.2.2 Preparation for Extraction Buffer

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

Name of the chemical	Molecular Weight	Stock con.	Reference con. /working con.	Working Volume	
				100 ml	1000 ml
CTAB			3%	3 g	30 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (pH 8)	372.24	0.5 M	20 mM	4 ml	40 ml
Trisbase (pH 8)	121.1	01 M	100 mM	10 ml	100 ml
β-Mercapto ethanol		14.4 M	100 mM	700 µl	7 ml

The following steps were performed for the preparation of Extraction Buffer (100 ml):

(i) 10 ml of 1 M Tris-HCl (autoclaved, pH 8.0) was taken in a 250 ml conical flask.

(ii) 28 ml of 5 M NaCl (autoclaved) was added to the conical flask.

(iii) 4 ml of 0.5 M EDTA (autoclaved, pH 8.0) was added in this solution.

(iv) Then 0.5 gm PVP and 2/3 gm CTAB was added freshly and kept it at 60ºC preheated water bath to dissolve the PVP.

(v) 200 µl β-mercaptoethanol was added prior to use the buffer and mixed it properly by shaking.

Freshly prepared extraction buffer solution was used. The whole task was performed in the fume hood.

3.2.3 Protocol used for isolation of genomic DNA

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

Step-by-step CTAB method protocol

1. Harvested 200 mg leaf material and placed them in liquid nitrogen.
2. Pulverized the plant material in the tube and placed them back in liquid nitrogen (heat up the water bath to 60°C).

3. Added 800 µl CTAB buffer, preheated in water bath at 60 °C (and mixed at once on a "wirlmix") and incubated for 30 min at 60 °C.
4. Added 800 µl CI mix and mixed gently to avoid shearing of genomic DNA.
5. Centrifuged at room temperature for 10 min at 12,000 rpm.
6. Transferred aqueous phase into a fresh tube; Step 5 and 6 may be repeated in order to obtain a clear sample.
7. For precipitation, added 2/3 volume isopropanol (550 µl), pre cooled at -20°C, mixed gently and monitored precipitation of DNA.
8. Pelleted the DNA by centrifugation for 10 min 13000 rpm.
9. Discarded supernatant and washed the pellet in 100 µl WB.
10. The washing buffer was carefully removed and re-suspended the pellet in 100 µl TE buffer solution supplemented with RNase A (final concentration 10 mg/ml) and incubated for 30 min at 37 °C.
11. Added 100 µl 7.5M NH_4-Acetate and 750 µl absolute ethanol abs. and mixed gently
12. Centrifuged at 13,000 rpm in lab centrifuge for 10 min at room temperature
13. Discarded supernatant completely, dried the pellet and re-suspended in 100 µl water and allow DNA to dissolve overnight at 4 °C.

3.2.4 Estimation of quality and quantity of isolated DNA samples

Prior to PCR amplification, it is the condition precedent to observe the quality and quantity of isolated genomic DNA. Different DNA extraction methods produces DNA of widely different purity. It is necessary to optimize the amount of DNA to achieve reproducibility and strong signal in PCR assay. Excessive genomic DNA may result smears lack of clearly defined bands in the gel. On the other hand, too little DNA will give non reproducible band patterns (Williams et al. 1990). Measurement of isolated DNA concentration can be done by comparing DNA with the standard DNA on agarose gel electrophoresis or by estimating the absorbance of DNA by spectrophotometer at 260 nm. Both the methods were carried out in this investigation.

3.2.5 Measurement of DNA concentration and quality by agarose gel electrophoresis

The concentration and quality of DNA measured by agarose gel electrophoresis in the following way

3.2.5.1 Preparation of stock solutions used for agarose gel electrophoresis

For conducting the gel electrophoresis, the following stock solutions and other solutions were prepared.

3.2.5.1.1 Preparation of 50× TAE Buffer solution for 1000 ml (p^H 8.3)

242 gm Tris base (MW=121.14) was dissolved in 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (p^H 8.0) was added in it. They were mixed well. The p^H of the solution was adjusted by mixing concentrated HCl at pH 8.3. The final volume of the solution was adjusted to 1000 ml.

3.2.5.1.2 Preparation of ethidium bromide solution

For 1ml solution, 10 mg of ethidium bromide was added to 1 ml of sterile de-ionized distilled water. It was then mixed by hand shaking. The solution was then transferred to a dark bottle and stored at room temperature. Stock solution of 10 mg/ml can be purchased directly from companies.

3.2.5.2 Protocol for agarose gel electrophoresis:

i. The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described by Sharp et al. (1973).
ii. 1.0 gm of agarose was heated to melt into 100 ml of TAE buffer; ethidium bromide was added as 0.5 μg/ml final concentration and poured into gel tray fixed with appropriate combs.
iii. After the gel was solidified it was placed into gel running kit containing 1× TAE buffer.
iv. Plant genomic DNA solutions were loaded with 6× gel loading dye and electrophoresis was continued until DNA fragments were separated well.

3.2.5.3 Documentation of the DNA sample

i. After gel electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed in gel documentation system (CSL-MDOCUV254/365 1D, Cleaver scientific Ltd., USA) for checking the DNA bands.
ii. The DNA was observed as bands and photographed using gel documentation system.

3.3 Rolling circle amplification (RCA)

The rolling circle amplification (RCA) was performed for the yellow mosaic virus infected leaf (both infected and non-infected) samples using the TempliPhi 100 amplification kit (GE Healthcare Life Sciences, UK) following the manufacturer's instruction:

Kit component	Reaction volume	Storage temperature
Sample buffer (white cap)	1 × 0.5 ml	-70 ˚C or -20 ˚C
Reaction buffer (blue cap)	1 × 0.5 ml	70 ˚C or -20 ˚C
Enzyme mix (yellow cap)	1 × 20 µl	-70 ˚C
Positive control DNA -20 ˚C (pUC19, 2 ng/µl)	1 × 50 µl	-70 ˚C or -20 ˚C

3.3.1 Protocol for rolling circle DNA amplification

1. The sample buffer (5 µl aliquots) was transferred to an eppendorf tube.
2. 1 µl sample DNA was added to the dispensed sample buffer.
3. The samples were denatured by heating at 95˚C for 3 min and then cooled down to room temperature or 4˚C.
4. By this time TempliPhi premix was prepared in a separate tube by combining 5 µl of reaction buffer and 0.2 µl enzyme mix for each TempliPhi reaction. Master mixture was prepared for multiple samples.
5. 5 µl of the TempliPhi premix was transferred to the cooled, denatured sample and incubated at 30˚C for 18 hrs.
6. Heat inactivated the enzyme by incubating at 65˚C for 10 min and then cooled to 4˚C.

3.3.2 Digestion of RCA product

The following Protocol used for fast digestion of different DNA samples:

1. The following reaction components were used in the digestion of RCA product:

Component	Plasmid DNA	PCR/ RCA product	Genomic DNA
Water	15 µl	16 µl	30 µl
10X Fast digest green buffer	2 µl	3 µl	5 µl
DNA	2 µl (up to 1 µg)	10 µl (~0.2 µg)	10 µl (5 µg)
Fast digest enzyme	1 µl	1 µl	5 µl
Total volume	20 µl	30 µl	50 µl

Components were mixed gently and spinned down.

2. Incubated at 37°C in a heat block or water thermostat for 30 min.
3. The enzyme was inactivated by heating for 5 min at 80°C.

3.3.3 Electrophoresis of the digested RCA products and documentation

The amplified products were separated electrophoretically on 0.8 % agarose gel. The gel was prepared using 0.8 gm agarose powder containing 0.5 µg/ml ethidium bromide and 100 ml 1×TAE buffer. Agarose gel electrophoresis was conducted in 1x TAE buffer at 90 volts and 300 mA for 1 hours. One molecular weight marker 1.0 kb plus DNA ladder was electrophoresed alongside the RCA digested reaction samples. DNA bands were observed on UV-trans illuminator and photographed by a gel documentation system (CSL-microdoc system, cleaver scientific Ltd.).

3.3.4 Purification of PCR/RCA product from agarose gel by gel extraction Kit

DNA samples were resolved by electrophoresis on 1.0 % agarose gel. The desired fragment was identified using standard molecular weight marker (1.0 kb ladder) and purified using Gene JET PCR purification kit (Thermo Fisher Scientific). The overall protocol of gel extraction as prescribed by manufacturer is as follows-

a) The agarose gel containing the DNA band was excised and weight of the band was measured.
b) An equal volume of binding buffer was added to this band. For 100 mg gel 100 µl binding buffer was used.
c) Then this was put into pre-warmed 65°C water bath until the gel was completely dissolved.
d) The color of the solution was checked. A yellow color indicates an optimal pH for DNA binding. If the color appeared as orange or violet, then 10 µl of Sodium Acetate pH 5.2 solutions was added and mixed well. Then the color became yellow.

e) The samples were then transferred in column tube, centrifuged at 13,000 rpm for 1 min. at room temperature and the flow through was discarded. The capacity of the column was 800 µl at a time.
f) Then 700 µl wash buffer was added and centrifuged at 13000 rpm for 1 min and again centrifuged for 1 min.
g) The flow through was discarded.
h) Then 50 µl elution buffer was added and it was kept for 2 minutes before centrifugation.
i) The column was centrifuged at 13,000 rpm for 1 minute.
j) The soup was taken in an Eppendorf tube and stored in -20°C.

3.4 Cloning of RCA product into pGreen vector

The following protocols were used for the cloning of RCA product into pGreen vector

3.4.1 Preparation of vector for cloning purpose

To clone MYMV genome, pGreen vector was prepared by digestion of the same enzyme by the following protocol

3.4.1.1 Digestion of vector DNA

Vector DNA was digested with fast digest Bam HI and Pst I restriction enzyme as the RCA product was digested with the same enzyme.

3.4.1.2 Dephosphorylation of linearized vector DNA

To dephosphorylate the linear vector DNA, following reaction mixture was prepared using fast alkaline phosphatase (Fast AP)

1.

Fast digested vector DNA	1 µg
10× reaction buffer for AP (alkaline phosphatase)	2 µl
Water, nuclease-free	17 µl
Total volume	20 µl

2. The reaction was mixed thoroughly, spinned briefly and incubated 10 min at 37ºC
3. The reaction was stopped by heating for 5 min at 75ºC.

3.4.1.3 Calculation of required amount of insert and vector DNA

$$\text{Insert mass in ng} = 6 \times \frac{\text{Insert length in bp}}{\text{Vector length in bp}} \times \text{Vector mass in ng}$$

Using the above formula the insert to vector molar ratio of about 3:1 was calculated for ligation reactions.

3.4.1.4 Preparation of ligation mixture for 2 reactions

Protocol for room temperature ligation reaction, the following components were combined to execute the ligation reaction:

1	Linear vector	20-100ng
2	Insert DNA	3:1 molar ratio over vector
3	10× T4 DNA ligase buffer	2 µl
4	T4 DNA ligase	1 µl
5	Water, nuclease-free	to 20 µl
	Total volume	20 µl

The above mixture was prepared and incubated at 22°C for 30 minute.

3.4.2 Preparation of competent cells

Various components were required for the preparation of competent cells:

3.4.2.1 Preparation of Luria Broth Medium (LB)

To prepare Luria broth medium, the following components and concentrations were used

Ingredients	250 ml	500 ml	1000ml
Bactotryptone	2.5gm	5 .0 gm	10 gm
Yeast extract	1.25 gm	2.5 gm	5.0 gm
Nacl	2.5 gm	5.0 gm	10 gm

(i) The above components were mixed properly and the whole mixture was then made up to final volume 1litre with further addition of distilled water.

(ii) pH of the medium was adjusted to 7.2 with a digital pH meter (TOA, Japan) with the help of 1N NaOH and 1N HCl that was required. Before that, the pH meter was calibrated with two buffer solution having pH 4.01 and 6.86, respectively.

(iii) For solidification 14 gm (at 14%) of bacto agar was added to the desired medium.

3.4.2.2 Preparation of 2× YT Medium

To prepare YT medium the following components and concentrations were used.

Ingredients	250 ml	500 ml	1000ml
Bactotryptone	4.0 gm	8 .0 gm	16 gm
Yeast extract	2.5 gm	5.0 gm	10 gm
Nacl	1.25 gm	2.5 gm	5.0 gm

I. The above components were mixed properly and the whole mixture was then made up to final volume of 1 liter with further addition of distilled water.
II. pH of the medium was adjusted to 7.2 with a digital pH meter (TOA, Japan) with the help of 1N NaOH and 1N HCL, that was required. Before that, the pH meter was calibrated with two buffer solution having pH 4.01 and 6.86 respectively.

3.4.2.3 Preparation of TFB I solution (200 ml)

The following components and concentrations were used.

Ingredients	200 ml
30 mM $KC_2H_3O_2$	0.549 gm
100 mM RbCl	2.418 gm
10 mM $CaCl_2.2H_2O$	0.294 gm
50 mM $MnCl_2.4H_2O$	1.979 gm
15% v/v glycerol	30 ml 100%

1. The above components were mixed properly and the whole mixture was then made up to a final volume of 1 liter with further addition of distilled water.
2. pH was adjusted to 5.8 with a digital pH meter (TOA, Japan) with the help of 0.2M CH_3COOH
3. It was than sterilized with filter paper and stored at 4°c.

3.4.2.4 Preparation of TFB II solution (200 ml)

The following components and concentrations were used

Ingredients	200 ml
10 mM MOPS	0.419 gm
10 mM RbCl	0.242 gm

75 mM $CaCl_2.2H_2O$	0.294 gm
50 mM $MnCl_2.4H_2O$	1.979 gm
15% v/v glycerol	30 ml 100%

1. The above components were mixed properly and the whole mixture was then made up to a final volume of 1 liter with further addition of distilled water.
2. pH was adjusted to 6.6 with a digital pH meter (TOA, Japan) with the help of 5M KOH and stored at 4°c.

3.4.2.5 Protocol for competent cell preparation

a) Streaked out cell on LB plate.
b) 20 ml 2× YT medium was inoculated with one colony and incubated at 37°C with shaking overnight.
c) Next day, sub cultured 1ml in 200 ml 2× YT medium (pre-warmed).
d) Incubated at 37°C with vigorous shaking until 0.480 (or slightly less) O.D (measured at 600 nm).
e) Transferred culture at sterile centrifuge tube and kept in ice for 5 min.
f) Centrifuged at 3000 rpm for 10 min at 4°C and discarded the supernatant. Then re-suspended the cells in 80 ml ice cold TFB I solution and kept in ice for 10 min.
g) Centrifuged as above and re-suspended the cells in a total of 8 ml ice cold TFB II solution and kept in ice for 15 minutes or longer.
h) Using pre-chilled pipette tips aliquot 200µl cells into eppendorf tube and snap freeze in liquid nitrogen.
i) Stored the competent cells at -80°C for longer used.

3.4.3 Transformation protocol of ligation product

a) Slowly thawed the competent cell in ice for 5-10 min.
b) The ligation mixture was added to the competent cells; also maintained a control transformation with known plasmid DNA.
c) Gently mixed and incubated for 30 minutes in ice.
d) The cells were then subjected to heat shock at 42°C for 60 sec and chilled immediately on ice for 2 min.
e) The cells were allowed to recover by adding 400µl of 2× YT medium to the tube and incubated at 37°C for 1 hrs. with slow shaking.

f) Different volumes of transformed competent cells were plated on LB plates containing desired antibiotics and incubated overnight at 37°C.

3.4.4 Selection of ligation product

The following components were required for the selection of ligation product

3.4.4.1 Preparation of kanamycin solution

For the preparation of antibiotic kanamycin solution 1 gm of antibiotic was dissolved in 10 ml of deionized water. Dissolved by vortexing and after filter sterilization stored at -20 °C freezer for further use. Antibiotic kanamycin was used 50 mg/l in final concentration.

3.4.4.2 Preparation of 1M IPTG Solution

a. 2.38 gm of IPTG powder was added in 8 ml distilled water and dissolved properly.
b. Brought to a final volume of 10 ml with molecular biology grade water.
c. Filter sterilize with 0.22 µm syringe filter store at -20ºC.

3.4.4.3 Preparation of X-Gal stock solution

a. 100 mg of X-Gal was dissolved into 5 ml of di-methyl formamide.
b. Divide into 1 ml aliquots.
c. Aliquoted solution was warped in aluminum foil.
d. Stored at -20°C.

3.4.4.4 Protocol for the blue white colony screening

a. 40 µl of the X-Gal Solution (20 mg/ml) was added in the agar containing bacterial plate.
b. 20 µl of 100 mM IPTG solution was also spread evenly on the plate with a sterile glass rod and the plate was air dried in 37°C incubator.
c. Then bacterial culture was evenly spread on the plate. Incubated overnight at 37°C.

3.4.5 Plasmid DNA isolation protocol by using Favor Prep plasmid DNA extraction mini kit (Thermo Fisher Scientific)

The following protocol is used for the isolation of plasmid DNA

a. The overnight grown bacterial culture (1-5 ml) was transferred to eppendorf tube.

b. Centrifuge the bacterial culture at 13000 rpm for 1-2 min and discarded the supernatant completely.
c. 250 µl of FAPD1 buffer was added to the pellet and re-suspended the cells completely by pipetting.
d. 250 µl of FAPD2 buffer was added and gently inverted the tube 5 times to lyse the cells and incubated at room temperature for 2 min.
e. 350 µl of FAPD3 buffer was added and inverted the tube 5 times immediately but gently.
f. Centrifuged for 10 min at 13000 rpm. During centrifuging, a FAPD column was placed in a collection tube.
g. The supernatant was transferred carefully to FAPD column. Centrifuged for 1 min then discarded the flow-through.
h. 400 µl of W1 buffer was added to FAPD column. Centrifuged for 1 min then discarded the flow-through.
i. 750 µl of wash buffer was added to FAPD column. Centrifuged for 1 min then discarded the flow-through.
j. Centrifuged for an additional 5 min to dry the column.
k. FAPD column was placed into a new 1.5 ml micro centrifuge tube.
l. 50 µl ~ 100 µl of elution buffer or ddH2O was added to the membrane center of FAPD column. The column was kept for 1 min.
m. Centrifuged for 1 min to elute plasmid DNA.
n. Stored the plasmid DNA at 4°C or -20°C.

3.4.6 Colony PCR of cloned DNA sample

The following protocols were used for colony PCR of the positive clone samples

3.4.6.1 Protocol of colony PCR

a) 50 µl sterile water was taken into an eppendorf tube.
b) One single bacterial colony was transformed into the eppendorf tube using a loop and re-suspended it.
c) Re-suspended bacterial cells were then boiled at 95ºC in a water bath for 5 minute.
d) Centrifuged at 13000 rpm for 2 min at 4°C and the supernatant was used as template in PCR reaction.
e) PCR reaction mixture was prepared and 1µl of supernatant was used for each sample.
f) Then PCR reaction was run following particular reaction of respective gene and primers.

3.4.6.2 Preparation of the master mixture for PCR reaction

Master mixture was prepared by mixing all of the PCR components e.g. reaction buffer, dNTPs, forward primer, reverse primer, Taq DNA polymerase etc. In each reaction, the volume of PCR buffer was used $\frac{1}{10}$ volume of the total reaction volume which was 25µl. After proper mixing and momentary spin of the master mixture, it was transferred to different PCR tubes. The final volume was made 25µl by adding varying amounts of sterilized ultra-pure water. Then template DNA was added into the master mixture. DNA polymerase was added just before the start of the reaction. Finally, the tubes were subjected to short spin and transferred to thermo cycler for the amplification of the DNA (eppendorf master cycler gradient).

3.4.6.3 Thermal cycling profile used in PCR reaction

The working concentration of the template DNA was 100 ng for each sample. The PCR condition was used for RCA product amplification as described below:

3.4.6.4 The PCR condition for cloned RCA product into pGreen vector

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	5 min	1
Denaturation	94°C	1 min	30
Annealing	58°C	1 min	
Elongation	72°C	3 min	
Final elongation	72°C	5 min	1

3.4.7 Sequencing of the purified PCR product (2.7 Kb) from RCA cloned plasmid

The purified PCR product from RCA clone plasmid were sent for automated sequencing.

3.4.8 Analysis of the sequence result (2.7Kb)

Sequence result of the PCR product were analyzed using the bioedit software version 7.0. Database searches with geminivirus sequences were carried out by NCBI-BLAST search program (http://blast.ncbi.nlm.nih.gov.).

3.5 Construction of antiviral vector (pBI121CP-AC2) by cloning *CP* and *AC2* gene in antisense orientation

The following components were used for the construction of antiviral vector construct:

3.5.1 Polymerase chain reaction for *CP* and *AC2* gene

a. For amplification of the *CP* and *AC2* gene the RCA product isolated from infected leaf samples were subjected to polymerase chain reaction (PCR) using gene specific primers.
b. Master mixture was prepared by mixing all of the PCR components. The volume of the PCR reaction was set to 25 µl.

3.5.1.1 PCR condition for amplification of *CP* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	4 min	1
Denaturation	94°C	1 min	30
Annealing	59°C	1 min	
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1

3.5.1.2 PCR condition for amplification of *AC2* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	5 min	1
Denaturation	94°C	1 min	30
Annealing	50°C	30 sec	
Elongation	72°C	30 sec	
Final elongation	72°C	5 min	1

3.5.2 Sequencing of *CP* and *AC2* Gene

PCR amplified products were purified through PCR extraction kit and the purified product were sequenced through automated sequencer in Centre for Advanced Research in Sciences, University of Dhaka. The sequence result obtained through automated sequencing were analysed through BLAST search in order to confirm that it was from MYMV *CP* and *AC2* gene.

3.5.3 Cloning of *CP* gene into pBI121 vector in antisense orientation

The binary vector pBI121 has been widely used in plant transformation. The total size of pBI121 is 12.8 kb according to its construction map (Jefferson et al. 1987). The T-DNA region (6193bp) contains the right border, expression cassettes for a neomycin phosphotransferase II (*NPTII*) selection marker and ß–glucuronidase (*GUS*) reporter gene and the left border. The non T DNA region (8565 bp) was constructed according to the Bin 19 vector. PCR amplified MYMV coat protein product was used for cloning into pBI121 vector in place of *GUS* gene. PCR amplified products and cloning vector pBI121 both were digested with restriction enzymes Bam HI and sac I in a double digestion reaction. Digested products were resolved on 0.8% agarose gel and the bands corresponding to the gene and vector (*GUS* less pBI121) were purified using gene Jet gel extraction kit. Then purified digested vector and insert were subjected to ligate in a ligation reaction set at 16ºC for 18 hrs. Ligation products were transformed in *E.coli* Top10 competent cells. Cloned samples was confirmed through colony PCR and other molecular processes such as restriction digestion, automated sequencing etc.

3.5.4 Cloning of *AC2* gene into pBI121CP vector in antisense orientation

The pBI121CP clone plasmid were digested with Bam HI restriction enzyme and the AC2 insert were digested with the same restriction enzyme. Then the *AC2* gene was sub-cloned into pBI121CP cloned plasmid by using the cloning procedure. After cloning, cloned samples were confirmed through colony PCR and other molecular processes such as restriction digestion, automated sequencing etc.

3.5.5 Polymerase chain reaction for *CP-AC2* gene to confirm antisense orientation

a. The CP-AC2 cloned plasmid was subjected to polymerase chain reaction (PCR) for amplification of the CP-AC2 gene by using CP forward and AC2 reverse gene specific primers.
b. Master mixture was prepared by mixing all of the PCR components. The volume of the PCR reaction was set to 25 µl.

3.5.5.1 The PCR condition for amplification of *CP-AC2* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	5 min	1
Denaturation	94°C	1 min	30
Annealing	54°C	1 min	
Elongation	72ºC	1 min	
Final elongation	72°C	5 min	1

3.5.6 Preparation of *Agrobacterium* culture medium

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

3.5.6.1 Preparation of YMB (Yeast Extract Mannitol Broth) medium

For the growth of *Agrobacterium tumefaciens* strain 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 of the medium was adjusted at 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 further used. Filter sterilized antibiotic kanamycin (50 mg/l), streptomycin (25 mg/l) and rifampicin (25 mg/l) solution was added to the autoclaved liquid medium (working) prior to bacterial inoculation and to the autoclaved maintenance medium when the medium was

cooled down enough. The medium was then poured into petri plates. After solidification, the media were ready for bacterial culture. These culture plates were stored at 4˚C for further use.

3.5.6.2 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 for YEP media was same as has been described in case of YMB media.

3.5.7 Preparation of *Agrobacterium* competent cells (LBA4404) and transformation of CP-AC2 clone plasmid into *Agro*- competent cell

The following protocols were used for transformation of clone plasmid into *Agro*-competent cells

3.5.7.1 Preparation of *Agrobacterium* competent cells (LBA 4404)

The protocol used for *Agrobacterium* competent cells preparation are given below-

a. *Agrobacterium* strain LBA4404 harboring only the LBA4404 helper plasmid (no binary plasmid) was grown in YMB medium supplemented with 25 mg/l streptomycin and rifampicin.
b. The bacterial culture grown until mid-log phase ($A_{600} \cong 0.5$) are chilled on ice for 10 min before harvesting at 3000 rpm for 20 minutes at 4ºC.
c. Pelleted bacteria at 4°C for 10 min at 4400 rpm, re-suspend in 25 ml ice-cold 10% glycerol, step 2 was repeated.
d. Pelleted bacteria at 4°C for 10 min at 4400 rpm, re-suspend in 2.5 ml ice-cold 10% glycerol, step 3 was repeated.
e. Pelleted bacteria at 4°C for 10 min at 4400 rpm, re-suspend in 1 ml ice-cold 10% glycerol, step 4 was repeated.
f. Split aliquots of 100 µl in 2 ml eppendorf tubes and put immediately in liquid nitrogen, stored at -80°C for long time used.

3.5.7.2 Transformation of CP-AC2 clone plasmid by electroporation method

Cleaned and dried electroporation cuvettes thoroughly on the cuvette washer. Chilled on ice and allowed to air dry. Put the thawed electro competent cells into ice. Our cells were generally of 100 µl aliquots. Turned on electroporator and set voltage of 2500 V for 2 mm cuvettes. For plasmid transformation, 1 µg of binary plasmid DNA was added to the *Agrobacterium* competent cells to mix gently incubated on ice for 10 min. Added cell/DNA mixture to the electroporation cuvette. Then the cuvette were placed in the electroporator machine. Double pressed the "Pulse" button on the electroporator to shock cells. Removed cuvette from the chamber and immediately add 800 microliter ice cold YEP liquid medium. Incubated tube in 28°C shaker for at least 2.5hr to permit expression of antibiotic resistance gene.The transformation mix was plated on LB agar medium containing the requisite antibiotics and incubated at 28°C for 48 h to allow for the colonies to appear. Colonies were checked for the presence of the binary vector by colony PCR and the positive colonies were used for plant transformation.

3.6 Protocol for *in vitro* regeneration of tobacco and mungbean plants

The following methods were used for preparation of various chemicals and *in vitro* regeneration of tobacco and mungbean plants.

3.6.1 Preparation of stock solutions for MS medium

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

3.6.1.1 Preparation Stock solution A (Macro nutrients) for MS medium

This stock solution was prepared to such an extent that its strength was higher than 40 times of the final strength of the medium in 1.0 L distilled water. For this reason, 40 times weight of different salts required for 1.0 L of medium was measured correctly and dissolved sequentially one by one in a 1litre volumetric flask with 750 ml of distilled water. The final volume of the solution was made up to 1.0 L in addition of distilled water. The solution was filtered through Whatman filter paper to get off all the solid contaminants like dust, cotton etc. Then the solution was poured into a lucid plastic container. After labeling, the solution was stored in a refrigerator at 4°C for several weeks.

3.6.1.2 Preparation of stock solution B (Micro nutrients) for MS medium

For the requisite of the medium two separate stock solutions were prepared.

3.6.1.3 Stock solution B_1 (all micro-nutrients except iron source)

This part of the stock solution was made with all the micro-nutrients except $FeSO_4.7H_20$ and Na_2-EDTA. This was prepared 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.

3.6.1.3 Stock solution B_2 (Iron chelate solution)

The second solution was prepared 100 times of the final strength of $FeSO_4.7H_20$ and Na_2-EDTA in 500 ml distilled water in a conical flask and warmed slowly at low temperature until the salts were dissolved completely. Finally, the solution was filtered and stored in a refrigerator at 4°C for few weeks.

3.6.1.4 Preparation of stock solution C (organic constituents) for MS medium

It was also prepared 100 times of the final strength of medium in 500 ml of distilled water as mentioned earlier. This solution was also filtered and stored at 4°C for future use.

3.6.1.5 Stock solution D (B_5 vitamin) for MSB_5 medium

For the preparation of B_5 vitamin following components were added in distilled water.

Components	Amount needed for 1000 ml medium
Myo inositol	10.0 gm
Thiamin	1.0 gm
Nicotinic Acid	0.1 gm
Pyridoxin HCl	0.1 gm

This was made 100 times the final strength of necessary components in 500 ml of distilled water. The solution was filtered and stored at 4˚C.

3.6.1.6 Preparation of MS/ MSB_5 medium

To prepare 1 litre culture medium following steps were carried out successively-

a. For the preparation of MS/ MSB_5 medium 30 g of sucrose was dissolved in 500 ml of distilled water in a litre of volumetric flask.
b. 25 ml of stock solution A, 10 ml of stock solution B and 10 ml of stock solution C were added to this 500 ml distilled water and mixed well. Stock solution D was added instead of stock solution C for the preparation of MSB_5 medium.

c. 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.
d. Required amount of hormonal supplements were taken from the stock solution of hormone and added to the medium either individually or in combinations and were mixed thoroughly. Since each of the hormonal stock solution contained 20 mg of the chemical in 20 ml of solution, the addition of 10 ml of any hormonal stock solution will make 1 litre of medium resulted in 1mg/l concentrations of that hormonal supplement.
e. The whole mixture was then volume up to 1litre with distilled water.
f. pH of the medium was adjusted to 5.8 with a digital pH meter (TOA, Japan) with the help of 1[N] NaOH and 1[N] HCl. Before that, the pH meter was calibrated with two buffer solution having pH 4.01 and 6.86 respectively.
g. For solidifying 8.0 g (at 0.8%) of Phytoagar (Duchefa, Netherlands) or 2.0 g (at o.2%) of phytagel (Sigma, USA) was added to the medium. To dissolve solidifying agent, the whole mixture was heated in a microwave oven (National, Japan).
h. To make liquid medium the last step (g) of media preparation was omitted.

3.6.1.7 Preparation of MSB_5 medium for plants using MSB_5 powder

Occasionally commercially available dry powdered MSB_5 medium (Duchefa Biocheme, Netharlands) containing all the constituents of MSB_5 medium (inorganic salts, vitamins and amino acids) except sucrose and agar were used to prepare medium for *in vitro* regeneration of plants. Appropriate amount of media powder is dissolved in 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 agar solidified media, 0.8% agar was added.

3.6.1.8 Stock solutions for growth regulators

The growth regulators are naturally produced 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. 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. Kinetin

The growth regulators, their solvents, and molecular weight are listed below (Sigma Plant cell culture catalogue, 1992)

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
2,4-D	Ethanol	221.04

To prepare any of the above mentioned hormonal stock solution, 10 mM stock solutions of each hormones were prepared. For this purpose, certain amount of the hormone was weighted and dissolved in required amount of appropriate solvent and then the final volume of the solution was made up to 50 ml by addition of distilled water. The solution was then filtered and poured into clean plastic container/another 50 ml in sterilized falcon tube and stored in a refrigerator at 4ºC for future use.

3.6.2 Surface sterilization, seed germination and seedling development

The seeds of mungbean [*Vigna radiata* (L.) Wilczek] were first surface sterilized with a detergent and few drops of tween-20 under running tap water until the detergent washed out completely. The floating seeds were discarded. The seeds were then treated with 70% alcohol for 1 minute followed by washing with distilled water for three to four times. Seeds were then treated with 0.1% $HgCl_2$ solution for ten to fifteen minutes inside the laminar flow cabinet with constant agitation. Then the seeds were washed five times with sterilized distilled water. After that the surface sterilized seeds were inoculated on the seed germination media. Three types of germination medium were used for the germination of

seeds. 0.8% (w/v) agar solidified 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 in mungbean seeds. In case of tobacco, to reduce the level of surface organisms the seeds were first washed with running tap water for 3 - 5 times. The floating seeds were discarded. The seeds were then dipped in 70% alcohol for 30 seconds to 1 minute followed by washing with distilled water inside the laminar flow cabinet. The surface sterilized seeds were then inoculated into conical flask, containing half strength of MS medium with 3% sucrose were used for seed germination and seedling development. The cultured seeds were kept in dark condition till the germination took place and then transferred to 16 hour light condition at 25 ± 2°C in growth room. Generally, 7-15 days were required for germination of seeds.

3.6.3 Explants preparation

In mungbean, cotyledonary node (CN), cotyledon attached decapitated embryo (CADE) explants were used in this investigation. Cotyledonary nodes were excised from aseptically grown three days old seedlings. For the culture of cotyledon attached decapitated embryo surface sterilized seeds were grown on distilled water overnight and explants were excised after 12 hours. The explants were then cultured on MSB_5 media supplemented with different concentrations and combinations of BAP, Kn, for *in vitro* regeneration of shoots. In case of tobacco, the leaf segment explants were excised from 25 - 30 days old *in vitro* germinated seedling.

3.6.4 Shoot regeneration and elongation

The explants were cultured on MS/MSB_5 media containing hormonal supplements, singly or in combinations for shoot regeneration. 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. The culture tube and the conical flasks containing explants 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. For shoot initiation and development, MSB_5 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) were used. In case of tobacco, MS medium supplemented with 4.4 µM BAP and 0.57 µM NAA were used for initiation and development of shoots.

3.6.5 Sub-culture

After two weeks, regenerated shoots were transferred to fresh medium. The regenerated shoots were sub-cultured regularly, at an interval of 15 days for maintenance and were routinely examined.

3.6.6 Rooting

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. In mungbean, 2 - 4 cm long regenerated shoots were excised and transferred to test tubes (25× 150 mm) containing freshly prepared MSB_5 medium as well as half strength of MSB_5 medium with different combinations and concentrations of IBA (10 - 25 mg/l). in case of tobacco, about 0.5 to 1.0 cm long shoots were separated and cultured on freshly prepared rooting medium containing half strengths of MS media without hormonal supplement.

3.6.7 Hardening of plantlets and transfer to earthen pots

The plantlets having sufficient roots were taken out from the culture vessels. Then the roots were washed by running tap water. Subsequently the plantlets were transplanted to small plastic pots containing sterilized soil at the same time pots were covered with transparent porous 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 environment for 2 -7 hours daily and replaced in growth room for another week. Three weeks after transplantation, when the regenerated plants were fully established in the small plastic pots, then they were transferred to larger earthen pots. The plants were hardening in the culture room for 1to 2 weeks and then maintained in net house for flower and pod formation.

3.6.8 Protocol for micrografting in mungbean plants

Seeds of locally grown mungbean variety BARI mung -3 were washed in tap water and then washed with 70% ethanol for 1 minute. The seeds were then surface sterilized with

0.1% $HgCl_2$ (W/V) for 10 min followed by 4-5 times repeated washing with distilled water. The surface sterilize seeds were aseptically germinated on half strength MSB_5 medium with 0.8 % agar in Petri plates for germination at 25 °C in the dark. The cotyledonary node (CN) explants were prepared from 3 days old *in vitro* germinated seedlings by cutting 4 mm of epicotyl and 4 mm of hypocotyl, retaining both the cotyledons. Explants were cultured for 30 days of sub-culturing at 15 days interval on the above mentioned medium. All the cultures were maintained at a temperature of 25 ± 1°C under a 16/8 hr (light/dark) photoperiod provided by cool white fluorescent light (3000 lux).

For micrografting *in vitro* regenerated shoots were used as scions. Rootstocks were prepared from 7 or 14 days old *in vitro* raised seedlings. After that, the shoot tip, axillary buds and cotyledons were removed with a surgery blade. After removing the shoot, the top cut surface was split longitudinally up to a depth of 50 mm. Shoots of 28 days with 1.5-3 cm length were selected as scions. A "V" shaped cut was made at the basal portion of the scion. Grafting was done by inserting the scion gently within the vertical split of the rootstock so that at least a part of the cambium layers of the scion and rootstock became aligned. After insertion of the scion, the grafted region was surrounded by masking tape then it was covered with a transparent polythene bag and subjected to hardening. During the first 2 weeks, the bags were gradually punctured to allow air exchange for plant acclimatization. After acclimatization the successful grafted plants were transferred in earthen pots in outdoor until the development of flowers and pods. After 30-40 days survival rate of the grafted plants was evaluated. Anatomical study of the grafted region was performed under stereo- microscope observation.

3.6.9 Protocol for in planta genetic transformation

The seeds were first surface sterilized to obtain axenic culture. Then the seeds were soaked in sterile distilled water in dark for overnight in order to germinate the seeds. To prepare half seed explant, the germinated seeds were then de-coated and the cotyledons were removed carefully so that, the embryo remained attached with one cotyledon. For injuring the whole embryo 0.5 ml insulin disposable syringe was used. A few pricks (2-3) were done at the embryonal axis of the explant. The overnight cultured *Agrobacterium* in YMB media was pellet downed by centrifugation at 5000 rpm for 10 mins. Then the pellet was re-suspended in Winan's AB medium containing required antibiotics as mentioned earlier, followed by culture in an orbital shaker for 18h. For the induction of *vir* gene activity, wounded tobacco leaf extract was added to the media 5h before the infection.

The half seed explants were injured at the embryonal axis point with a sterile 0.5 ml insulin disposable syringe and incubated in bacterial suspension in AB medium for different incubation periods. Before incubation, the bacterial suspension containing the half seed explants were subjected for vacuum infiltration for different periods of time. After incubation, the explants were soaked dried on filter paper and then inoculated to ½ MS media, devoid of any kind of hormone. After the formation of well-developed root system, the plantlets were transferred to plastic pots containing soil and hardened as mentioned before. After successful hardening the plants were transferred to larger earthen pots containing soil for further growth and development.

3.7 *Agrobacterium*- mediated genetic transformation protocol

Following chemicals and protocols were used for *Agrobacterium*-mediated genetic transformation:

3.7.1 *Agrobacterium* culture

3.7.1.1 Media used for *Agrobacterium* culture

YEP (Yeast Extract Peptone), YMB (Yeast extract Mannitol Broth) and LB (Luria Broth medium) with appropriate antibiotics were used to grow genetically engineered *Agrobacterium tumefaciens*.

3.7.1.2 *Agrobacterium* culture

Two types of culture media (LB, YMB) were used for bacterial culture: solid media were used for maintaining *Agrobacterium* stock and liquid media were used for infection of explants. For maintenance of the bacterial culture, one single colony from previously maintained stocks was streaked into freshly prepared petri dish containing YMB or LB medium having appropriate antibiotics. The petri dish was enclosed with parafilm and kept incubator at 37°C temperature for at least 48 hours for optimum growth. Then the bacterial plate was observed for bacterial growth and kept it at refrigerator in 4°C temperature. The bacterial culture was sub-cultured every week in freshly prepared media to maintain the stock. For infection of explants, the 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, LB or YMB) with requisite antibiotic. This liquid bacterial culture was allowed to grow overnight at 28°C on a rotary shaker to get optimum population for infection and co-cultivation of explants.

3.7.1.3 Preparation of stock solutions of antibiotics

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

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

For the preparation of kanamycin, ticarcillin and combactam stock solutions, 1 gm of each antibiotic was separately dissolved in 10 ml of deionized water. After micro filter sterilization, these solutions are stored in 1.5 ml eppendorf tubes at - 20ºC in the dark.

3.7.2 Infection and incubation of explants

The overnight grown *Agrobacterium* culture was centrifuged for 10 minutes at 5000 rpm and the pellet was dissolved in liquid MS medium (pH 5.8) to make the *Agrobacterium* suspension. This *Agrobacterium* suspension was used for infection and incubation of explants. Previously, "Optical Density" (O.D.) of the bacterial suspension was checked at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). After that the explants were dipped in bacterial suspension for different incubation periods before transferring them to co-cultivation medium.

3.7.2.1 Preparation of acetosyringone stock solution

Molecular weight of acetosyringone is 196.19 g in order to prepare a stock solution 20 ml of 100 mM acetosyringone in DMSO, 0.39238 g of acetosyringone powder was dissolved in 20 ml DMSO and mixed well with a vortex machine. Then it was filter sterilized and aliquots were made. Aliquots were stored at -20°C for further use.

3.7.2.2 Preparation of co-culture medium

Co-cultivation of the infected explants with *Agrobacterium* solution was carried out on agar solidified shoot regeneration medium. Therefore, the preparation of co-cultivation medium is same as shoot regeneration medium.

3.7.2.3 Co-culture

After infection and incubation, the explants were co-cultured in suitable co-culture medium. The co-cultivation medium contained MSB_5 supplemented with 100 µM acetosyringone with pH 5.8 were prepared. 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 2-4 days for co-cultivation. Co-cultured petri dishes containing explants were placed under fluorescent illumination with 16/8 hours light/dark cycle at 25 ± 2°C. The intensity of light was maintained at 1500 lux.

3.7.3 Shoot regeneration

After co-cultivation the explants were washed with distilled water four times until no opaque suspension was seen, then they were washed for 10 minutes with distilled water containing 300 mg/l ticarcillin. After that, the explants were gently soaked with a sterile Whatman filter paper and transferred to regeneration medium with 100 mg/l ticarcillin or 100 mg/l carbenicillin. After 2 weeks, the regenerated shoots were sub-cultured in regeneration medium containing selection agent.

3.7.4 Selection medium and selection of putative transformed shoots

Different concentrations of kanamycin were added to the regeneration media for the selection procedure. Along with selection agent antibiotics such as ticarcillin and combactam were used to check the overgrowth of bacteria. Regeneration medium with appropriate antibiotics was prepared for the selection of putative transformed shoots. 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. When the regenerated shoots were 1-2 cm long then the regenerated shoots were sub-cultured in selection medium containing 50 mg/l kanamycin and 100 mg/l ticarcillin. Regenerated shoots were sub-cultured regularly with an interval of 15-21 days and the concentration of selection agents was gradually increased up to 200 mg/l kanamycin on selection medium. Shoots surviving on selection medium were sub-cultured on rooting media. As control, non-infected explants were cultured in normal regeneration medium. 7 - 10 days old shoots were sub-cultured in selection medium to detect the effect of selection agents on these control shoots. These controls were maintained with each set of transformation experiments to perform various comparative studies.

3.7.5 GUS (β-glucuronidase) histochemical assay

Agrobacterium strain LBA4404/pBI121GUS-NPTII used in the present study contains *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) and gives an insoluble indigo blue colour. In this way, it permits transformed tissues to be screened histochemically.

3.7.5.1 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
Mannitol	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 100 ml)

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 adjusted to 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 in a pyrex tube. The final volume was done with 50 mM solution of Na_3PO_4. This was stored at -20˚ C.

3.7.5.2 GUS (β-glucuronidase) histochemical assay of transient expression in 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 shoots were immersed in X-gluc (5- Bromo-4- Chloro-3- indolyl-β-D glucuronide) solution. For this purpose, explants were dipped in histochemical reagent, i.e. GUS buffer followed by several washes (3 times) and were incubated at 37˚C overnight. A characteristic indigo blue color could be the expression of *GUS* (β-glucuronidase) gene in

the plant tissue. In each experiment non-transformed explants were used as control. After X-gluc treatment, explants were transferred to 70% alcohol for decolourization decolorized explants were observed under stereomicroscope (Olympus, Japan). For confirmation slides were prepared with transformed tissue and were observed under microscope. Following each subculture some randomly selected plant parts (leaves, shoots) were also subjected to *GUS* histochemical assay according to the above mentioned method. Plant parts of the transformed plantlets were also examined through this method.

3.7.5.3 Anatomical study

Anatomical studies were carried out following maceration of the plant tissue to observe the expression of *GUS* gene within the explants tissue as well as with the other parts of putatively transformed plantlets.

3.7.5.4 Maceration of plant tissues

Explants showing GUS expression following X-gluc treatment were transferred to 70% alcohol for decolorization. After decolorization, blue colour containing explants were transferred to 1N NaOH into fresh glass vessels. These glass vessels were incubated for 20 mins at 60°C for adequate softening of the tissue. After cooling these materials were taken on a clean and oil free slide having a drop of 50% aqueous solution of glycerol (v/v) and clean cover slip was placed on each material and gentle pressure was given on the cover slip with a needle to spread the tissue uniformly. These slides were observed under microscope (Nikon Eclipse, Japan).

3.7.6 Observance of pollen viability and pollen tube growth

To investigate pollen grain viability, *in vitro* flowers were excised and removed the sepals and petals. After that, anthers were transferred to a slide and piled in a solution containing 0.01% (w/v) fluorescin diacetate (FDA) and 20% (w/v) sucrose followed by incubation at room temperature for 30 min (Heslop 1970). Maintaining this incubation period, debris was removed and pollen grains were observed under a fluorescence microscope. To observe the growth of the pollen tubes, the self-pollinated pistils from *in vitro* grown flowers were collected, fixed and softened in a mixture of acetic acid : alcohol (1 : 3, v/v) at 60º C for 10 minutes and thereafter stained with 0.1% (w/v) decolorized aniline blue solution. After staining, pistils were placed in a drop of 50% (v/v) aqueous solution of glycerol prior to microscopic observation. Stained pistils and pollen grains thus observed under a fluorescence microscope fitted with an incident UV illumination system (Nikon, Microphot; excitation 450 - 490 nm) according to Kho and Baer (1968).

3.7.7 Molecular analysis of putative transgenic plants

To extract genomic DNA, *in vitro* regenerated putative transgenic shoots and control shoots were collected. The materials were washed in distilled water and dried on fresh tissue paper to remove any components of medium nutrients. Genomic DNA was isolated according to previously mentioned method and PCR was carried out. PCR positive plants were further transplanted to the earthen pot for successful growth and development.

3.7.7.1 Thermal cycling profile used in PCR

The working concentration of the template DNA was 100 ng for each sample. The PCR condition was used for gene amplification are described below: The PCR condition of *CP-AC2* gene are mentioned earlier.

The PCR condition for *GUS* and *npt II* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1 min	35
Annealing	54°C	1 min	
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1

3.7.7.2 Visualization of PCR Product

After a short spin the PCR products were loaded in wells of 1.0-1.2% agarose gel containing ethidium bromide (0.05µl/ml) solution. Electrophoresis was accomplished at 90 volts and the PCR products were visualized under UV transilluminator and photographs were taken for documentation by gel documentation system (Bio.Sci. Tech.Gelsccan, 6.0, Professional, Germany).

3.7.8 Transplantation and acclimatization of plantlets

The transgenic shoots were rooted in root induction medium as well as transplanted into plastic pots having sterilized soil. The roots were tenderly washed to remove agar attached with the roots. Then the plastic pots were covered with transparent porous polythene bags. Inner side of these bags was moistened with water to prevent desiccation. The plants were acclimatized in the culture room for 1 to 2 weeks and subsequently maintained in net house for biosafety regelation until flower and pod formation.

4. RESULTS

The present investigation was carried out to create yellow mosaic virus resistance in mungbean (*Vigna radiata* (L.) Wilczek) through RNAi mediated gene silencing mechanism. For this purpose, to carry out plant genetic transformation, an antiviral gene construct was developed using mungbean yellow mosaic virus coat protein (*CP*) and silencing suppressor (*AC2)* genes in an antisense orientation into pBI121 backbone vector. During this study two different genetically engineered gene construct were used, namely, pBI121GUS-NPTII (considered as construct I) and pBI121CP-AC2 (considered as construct II). Construct I was used as marker gene for the development of a suitable transformation protocol and construct II was used for the integration of desired gene into mungbean varieties. Two locally grown of mungbean [*Vigna radiata* (L.) Wilczek] varieties, namely, BARI mung-3 and Binamoog-5 were utilized for this investigation. This study was carried out in three phases. In the first phase, antiviral gene construct (pBI121CP-AC2) was developed through cloning of *CP* and *AC2* genes in an antisense (as) orientation. In the second phase, a suitable *in vitro* regeneration and *Agrobacterium*-mediated genetic transformation protocol for mungbean were developed. Further the optimization of *Agrobacterium*-mediated genetic transformation was carried out using the marker gene construct (Construct I) since it contained two marker gene, such as *GUS* (β-glucoronidase) and *nptII* (neomycin phosphotransferase). In the third phase, experiments were conducted to develop yellow mosaic virus resistant mungbean line by the integration of the *CP* and *AC2* genes into the plant genome by using antiviral gene construct II (pBI121CP-AC2). The results of the present investigation obtained from various experiments are presented in the following sections.

4.1 Construction of the antiviral vector

For the integration of antiviral gene into the local varieties of mungbean, *Agrobacterium*-mediated genetic transformation was carried out by developing a new gene construct since no antiviral construct was available for genetic transformation. For this purpose, the antiviral vector (pBI121CP-AC2) was produced through the isolation and cloning of various antiviral components of the mungbean yellow mosaic virus (MYMV). The results for the development of an antiviral gene construct has been described below-

4.1.1 Confirmation of mungbean yellow mosaic virus (MYMV) disease infection

Yellow mosaic disease (YMD) was initially diagnosed by the presence of whitefly and yellow mosaic symptom. PCR based detection of partial *CP* gene (encoding coat protein) was carried out to confirm the presence of yellow mosaic virus infecting mungbean. For this reason, yellow mosaic virus infected leaf samples were collected from different region of Bangladesh and used as a plant material for isolation of genomic DNA because the genomic DNA samples may contain the viral genome. Using modified CTAB methods good quality genomic DNA were isolated (Fig. 19) and was used for PCR based confirmation using CP specific primer as well as rolling circle amplification (RCA).

4.1.1.1 Detection of yellow mosaic virus infection at molecular level

To confirm the presence of mungbean yellow mosaic virus (MYMV) in the infected plants amplification of *CP* and *AC2* gene was carried out.

4.1.1.2 Amplification of *CP* gene using MYMV CP forward and reverse Primer

In this study, coat protein (*CP*) gene specific primers were designed (section 3.1.1) to isolate MYMV *CP* gene through polymerase chain reaction (PCR). During primer design, Sac I restriction recognition site was attached with forward primer and Bam HI restriction recognition site was attached with the reverse primers in order to facilitate the cloning of *CP* gene in antisense orientation into pBI121 vector to obtain pBI121CP plasmid. Isolated DNA samples were subjected to polymerase chain reaction (PCR) using MYMV CP forward and MYMV CP reverse primers. Two PCR amplified bands (~750 bp size) were visualized after agarose gel electrophoresis (Fig. 20 a, b). For further confirmation, PCR amplified DNA was sequenced through automated sequencer and the DNA sequence was subjected to BLAST search to confirm the presence of MYMV coat protein (*CP*) gene. The sequence result of *CP* gene generated through automated sequencing were as follows:

CCAGAGGCTGTTGTAAGTTACGAACCCCGCTCCTGCTGACAGGACCTGCGGGAGCTG
CCCTACCAACATGAAAAGAAGACGCTTGGACCAACCGTCCCATGTGGAGGAAACCTC
GGTTTTACCGACTGTATAGGTCCCCTGATGTCCCTCGTGGTTGTGAGGGACCATGTAA
AGTTCAATCATTTGAGCAAAGGCATGATATTGCCCACACAGGCAAAGTGATTTGCAT
ATCTGACGTGACTAGAGGTAATGGAATTACACATCGTCTTGGCAAACGATTTTGCATC
AAGTCCGTGTACATAACGGGTAAGGTTTGGATGGACGAGAACATCAAGTCCAAGAAT
CACACAAACACTGTGATGTTCAAGTTATGTCGTGACAGACGACCATTTGGTTCACCCA
TGGATTTTGGTCAAGTGTTTAACATGTATGACAACGAGCCTAGTACAGCTACTGTGAA
GAACGATCTGCGTGATCGTTATCAAGTCTTGCGAAGATTTAATGCCACTGTTACAGGT
GGCCAATATGCTTGTAAGGAACAAGCCATGGTGAATCGTTTTTTCAAAGTTAATAATT
ATGTTGTTTACAACCACCAAGAGGCAGCGAAGTATGAGAACCATACTGAGAACGCAT
TATTATTGTATATGGCATGTACTCATGCCTCAAATCCTGTGTATGCAACGAATCCCGT
TGT

4.1.1.3 Sequence analysis and comparison with selected geminiviruses *CP* gene

Partial sequences of the two isolates obtained through automated sequencing were analyzed using database searches to find similarity with other geminivirus sequences. The database searches were carried out by NCBI-BLAST program (http://blast.ncbi.nlm.nih.gov). BLAST search results showed that the nucleotide sequences of the two isolates had sequence similarities and showed identities with coat protein (*CP*) gene of the different strains of MYMV. However, both had highest sequence similarity and exhibited identity with MYMIV-Bangladesh strain (Accession number- AF314145.1). From the BLAST analysis it was revealed that these two isolates showed more than 90% sequence identity with coat protein gene of other geminiviruses (Table 1) indicating that the yellow mosaic symptomatic leaf sample were infected with MYMV.

Table 1. Percent identities (nucleotide) of amplified MYMV *CP* gene with different begomoviruses reported worldwide.

Accession	Description	Max score	Total score	Query score (%)	E value	Max identification (%)
AF31414.1	Mungbean yellow mosaic India virus [Bangladesh] DNA-A complete sequence	1138	1138	87	0.0	**98**
JN181006.1	Mungbean yellow mosaic India virus isolate Vizianag-aram pre coat protein (AV2) gene, partial cds; and coat protein (AV1) gene, complete cds	1103	1103	86	0.0	97
AJ416349.1	Mungbean yellow mosaic India virus [Soybean TN] complete genome	1079	1079	88	0.0	96
AJ315667.1	Soybean yellow mosaic virus partial av1 gene for coat protein	1079	1079	88	0.0	96
JN368438.1	Mungbean yellow mosaic India virus Indonesia isolate Brebes 3 segment DNA-A, complete sequence	1077	1077	87	0.0	97

4.1.1.4 Amplification of *AC2* gene using MYMV AC2 forward and reverse primer

From the isolated DNA samples MYMV *AC2* gene was amplified through PCR using MYMV AC2 forward and MYMV AC2 reverse primers designed during this study. Amplification of 450 bp *AC2* gene was detected following gel electrophoresis (Fig. 21). The PCR product were purified and subjected to automated sequencing for further confirmation. The sequenced *AC2* gene was analyzed through BLAST search and confirmed the presence of MYMV *AC2* gene. The analysis showed that isolated *AC2* gene has more than 98% sequence identity with *AC2* gene of MYMV-BD strain (Table 2) that confirm these samples were infected by MYMV.

Table. 2 Percent identities (nucleotide) of MYMV *AC2* gene of selected geminiviruses reported worldwide.

Accession	Description	Max score	Total Score	Query score %	E value	Max identification %
AF31414.1	Mungbean yellow mosaic India virus [Bangladesh] DNA-A complete sequence	601	601	79	0.0	**98**
AY937195.1	Mungbean yellow mosaic India virus clone MBK-A25 segment DNA-A complete genome	606	606	79	0.0	98
AY 937197.1	Mungbean yellow mosaic India virus clone CP 1.8 segment DNA-A , partial sequence	606	606	79	0.0	98
JN368438.1	Mungbean yellow mosaic India virus Indonesia isolate Brebes 3 segment DNA-A, complete sequence	595	595	79	0.0	97
JN368439.1	Mungbean yellow mosaic India virus indonesia isolate Brebes 4 segment DNA-A, complete sequence	595	595	79	0.0	97

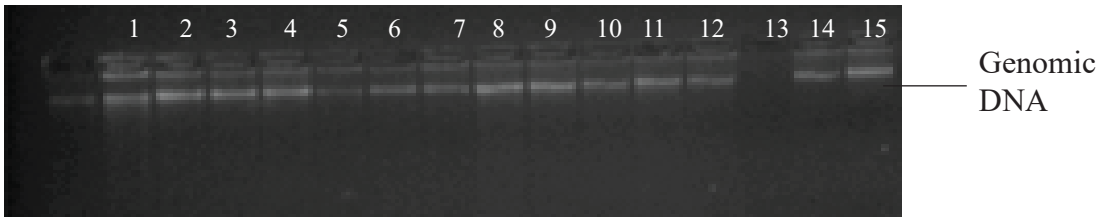


Fig. 19: Electrophoretic separation of genomic DNA isolated from MYMV infected leaf samples by CTAB method. Lanes 1-12 and lanes 14-15 showing the isolated genomic DNA of mungbean infected plants.

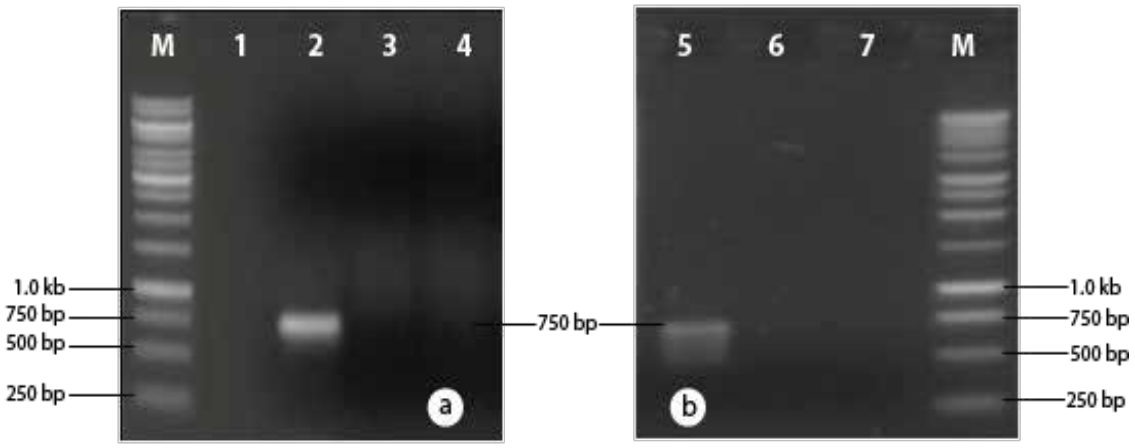


Fig. 20: Amplification of MYMV *CP* gene through PCR using *CP* specific primer (a-b). Lane M refers for 1.0 kb DNA marker; lane 2 & 5 showed amplified CP band which is identical to the 750 bp band. No signal from negative control (lane 1) & water conttrol (lane 7).

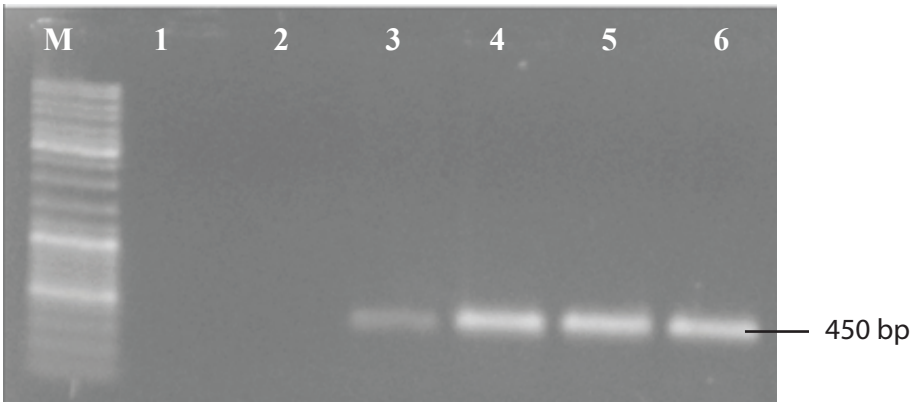


Fig. 21: Amplification of MYMV *AC2* gene through PCR using *AC2* gene specific primer. Lane M refers for 1.0 kb DNA marker; lane 3-6 showed amplified AC2 band which is identical to the 450 bp band.No signal from negative control (lane 1) & water conttrol (lane 2).

The sequence result of *AC2* gene generated through automated sequencing were as follows:

TATGCTGACTTCAGTCCAGGAGCACTTGTGCATCCCCAGTGCTTTCTTCAACCC
GTGGTTGAACATTATGCGCAGTTTGGTGACAACCATTCTGGACCCTGCTTGAAT
TGGACTGTGGTGCATGATCTTGAAAGAGAGGGGATTTTGCACCTCCCAGATAT
AGACGCCACTCCTGAGTTGAGCTGCAGTGATGCTCTCCCCTGTGCGAAAATCC
ATAGTTACGGCAGTTGATATGGATGTAATAGCTACACCCACAGGTCAGGTCAAT
TCGAGATCGTCGAATTGCTTTCTTCTTGGCGACCCTGTGTTGAACCTTGATTGA
CGGAGGAGAACAATGGTTCCTTGAGGGTGTAGAATTCCGCAGGATCCGTGTA

4.1.2 Amplification of mungbean yellow mosaic virus full genome and sequence characterization

Mungbean yellow mosaic virus is a bipartite begomovirus that containing DNA-A and DNA-B. In this part of this investigation characterization of full (DNA-A and DNA-B) genome was carried out. For this purpose, rolling circle amplification and cloning of full genome was undertaken. The results of this part of the investigation has been presented under the following heads.

4.1.2.1 Rolling circle amplification (RCA) of the isolated DNA samples

Total DNA was isolated from the yellow mosaic symptomatic leaves to diagnose the presence of a suspected causal agent from *begomovirus* genera. In order to confirm the presence of viral genomic DNA in the infected leaf samples, rolling circle amplification (RCA) experiment was performed with the isolated DNA, using the TempliPhi™ 100 amplification kit (GE Healthcare). Restriction digestion of 2 µl of RCA product was performed with various different restriction enzymes to check the presence of full length viral genomic DNA components (~2.7kb) of either DNA A or DNA B. RCA product (1µg) was subjected to restriction digestion with Bam HI and Pst I enzymes respectively obtained ~2.7kb band to identify the unique sites for cloning of viral genome (Fig. 22 a, b). The presence of the begomovirus within the DNA of the infected mungbean leaves can be detected through the amplification of the rolling circular DNA having a band of ~2.7kb.

4.1.2.2 Cloning of RCA product ~2.7kb into pGreen vector

Mungbean yellow mosaic virus is a bipartite begomovirus which contains single strand genomic DNA referred to as DNA-A and DNA-B. In order to amplify the MYMV viral genome from the DNA of the infected mungbean leaf samples TempliPhi™ DNA amplification kit based rolling circle amplification (RCA) experiment was performed. The RCA products produced in the reactions were linearized by restriction digestion with fast digest Bam HI and Pst I restriction enzymes (Thermo Scientific) separately. After gel

electrophoresis the presence of ~2.7 kb band was observed in case of DNA samples infected with MYMV (Fig. 22 a, b). The RCA amplified ~2.7 kb band was gel purified by using gene jet gel purification Kit (Thermo Scientific) for cloning into pGreen0029 vector.

4.1.2.3 Vector preparation, ligation, transformation and screening for positive clones

The vector was digested with fast digest Bam HI and Pst I (Thermo Scientific) restriction enzymes separately, which produced a ~4.5 kb band obtain through gel electrophoresis and the linear vector DNA was dephosphorylated by alkaline phosphatase enzyme (Fast AP, Thermo Scientific) to prevent self-ligation (Fig. 23). The purified RCA products were used as insert for ligation reaction. The ligation reaction was set by mixing the vector and insert (1:3) ratio in an eppendorf tube along with buffer and ligase enzyme and incubated at 22ºC for 30 minutes. The *E. coli* top10 competent cells were transformed with ligation products. After transformation the *E. coli* cells were plated on kanamycin containing LB agar medium to select the bacterial cells as single colony transformed with the ligation products. For cloning confirmation, the colonies were further subjected to colony PCR using pGreen forward and reverse primer. Following colony PCR, a 3 kb size DNA band was obtained in agarose gel electrophoresis confirming the cloning of the insert into the pGreen0029 vector (Fig. 24 a, b). For further confirmation of cloning restriction digestion of the isolated plasmid DNA was performed from the colony PCR positive clone using fast digest Bam HI and Pst I restriction enzymes separately and observed 2.7 kb bands (Fig. 24 c, d). The 2.7 kb DNA was sequenced for further confirmation of either the MYMV DNA-A or DNA-B genome.

4.1.2.4 Sequencing of the cloned product and sequence analysis through NCBI-BLAST Search

Amplified PCR product 2.7 kb were separated on 1% agarose gel and purified from the gel using gel extraction kit (Qiagen, USA) in following the manufacturer's protocol. The 2.7 kb cloned RCA product were sequenced through automated sequencing. After sequencing nucleotide similarity searches were performed by BLAST search at NCBI database. Complete nucleotide sequences of the full-length genomes were aligned, and percentage of pair-wise identity matrix was generated in Bioedit program.

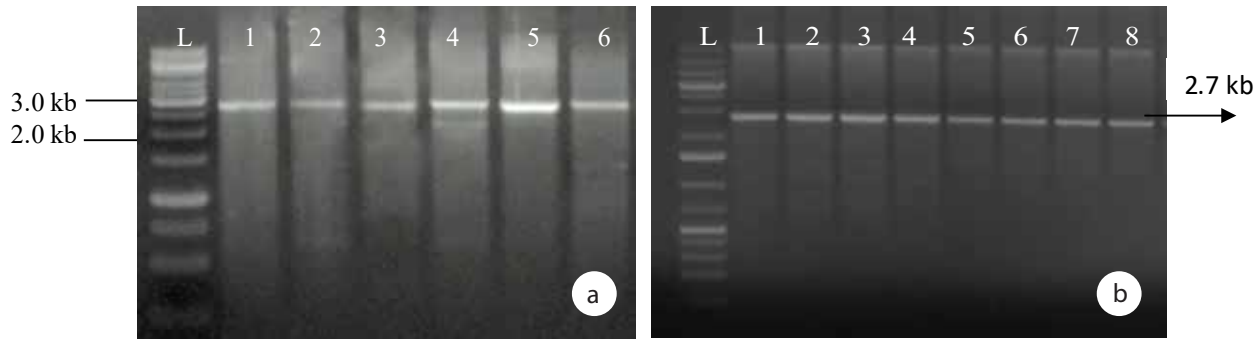


Fig. 22(a-b) : Gel electrophoresis of the RCA products digested with Bam HI and Pst I restriction enzymes. (a) Restriction digestion using fast digest BamHI restriction enzyme to identify viral genome where lane 1-6 showing positive bands at 2.7 kb; (b) Restriction digestion using fast digest PstI restriction enzyme to identify viral genome where lane 1-8 showing positive bands at 2.7 kb.

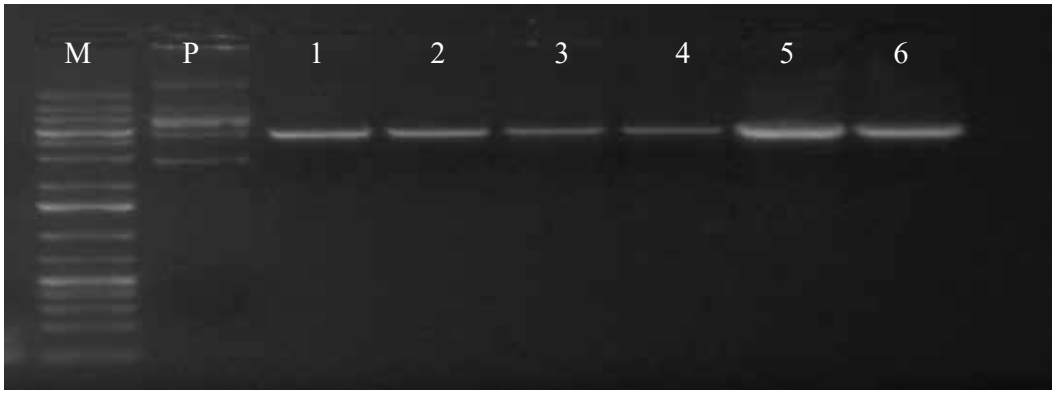


Fig. 23: Electrophoretic seperation of pGreen vector DNA undigested (lane P) as well as digested with fast digest Bam HI (lane 1-3) and Pst I (lane 4-6) restriction enzymes respectively.

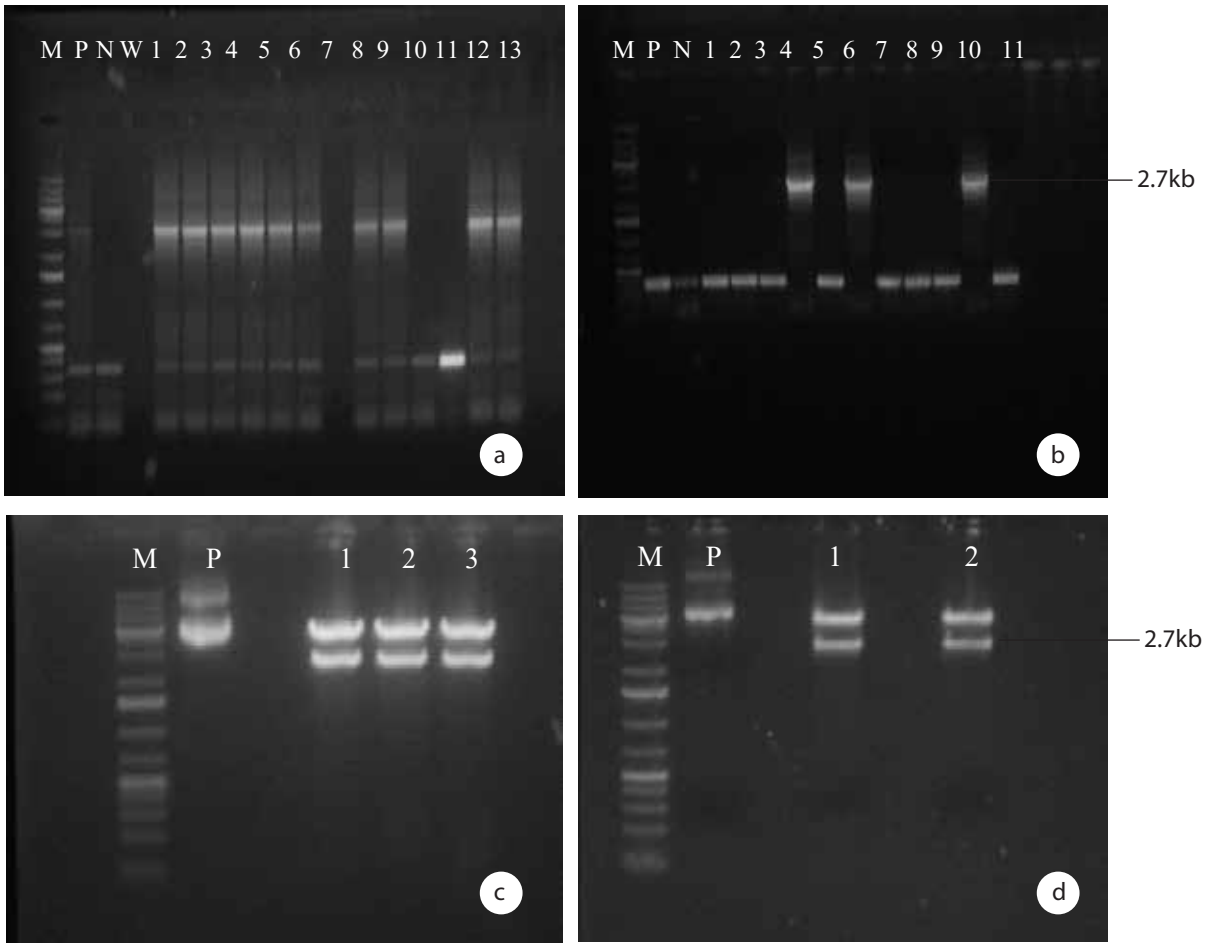


Fig. 24 (a-d): Colony PCR and restriction digestion confirmation of MYMV RCA clone plasmid. (a) Colony PCR for Pst I restriction digestion. Lanes 1-6 , 8, 9 and 11-12 showed positive bands at 2.7 kb; (b) Colony PCR for Bam HI restriction digestion. Lanes 4,6,10 showing positive bands at 2.7 Kb; (c) The RCA clone plasmid digested with PstI restriction enzyme showing 2.7 kb DNA fragment along with vector bands. Lane M =1 kb DNA ladder; Lane P - undigested plasmid, Lane 1-3 showing digested three cloned plasmid. (d) The RCA cloned plasmid digestion with restriction enzyme Bam HI showing release of 2.7 kb DNA fragment along with vector bands. Lane M =1 kb DNA ladder; Lane P - undigested plasmid, Lane 1-2 showing digested two cloned plasmid.

Sequence results obtained through Bam HI restriction digestion

CATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCG
CGGTGGCGGCCGCTCTAGAACTAGTGGATCCAATGATGCCTCTGGCAATTTATGCAAATGTCT
AAGTGGATAATCTGCGTCTGAAGCGCTGGTGTTGTCTAGAATACTGGGCCTGTCGTTGTTCGTG
TACCGCATGGAGCTGCTGCGCCCAATAGATGACCTTGTAGCCCAAGTCTCACCTGGCTGTAGC
ATAATGGGCCTATGCCCACTTATGGGATATGGACCCTGATTGGGTCCTGGAAAAAGCATACGC
CTAATGGGCTTGGGCTTCTCAACAGACCAAAAGTCCACACATTCTTCTGTGTAGTCCTTAGACA
ATATGTTTATTGTCGGGGGTTTGAATCTAATGTCTGTTGAGTGTTTTGCGGAAGACAACTTCAA
CTTGGCCTTTATCTGGGCAAATGTTGTTCCGTCGATCACATTGGAGTCCTCGACTTTGTAGACT
ATCTCCCATGGTGTTTCATCCTTCAGAGAGAAGAAGGATGATGAGAAGTAATGGAGATCGACG
TTACATGCAATTGGGAAAGTGAAAGCAGCTTGTGCTGCTTGTTCATAGCTTAGCCTTGTGTCAC
GAATTGTGACTATGACCGTTCCCTTGGCATTGAACGGAACTTGGTTTCTGTATTCAATCACAGC
ATGGTCTACCTTCATGCATCTGCCCATAATCTGAACAGTTTTCTGTTCCAGATAAGAAGGAAAT
TGTAACTTGATGGGCATCTCGTTGTTTGTTAGTCTGTATTCGCAACTCTTTGTTTCCACATACTT
GTTATTAACAACTGCTCCTGAATAATTCTCCATTATTCAGAAATTGTGTTGCGAATTAATCCCT
GAAAAGGAACACACACCAGGAAATTATTCTATTAATTTCAATAGGCCGCGCAGCGGCTATGA
GGTGAGTTTAGATTTATAATCATAATCTAAACCAAAGAGGGATAAAAAAACCTAATACCGGA
ATTCTTGTAATTATCTCAGAAAATTAGAGCACTGGATGTGCACAGAGGAAGTAACAAATATTT
AATTAAATATTTGTTGTTGTGATATTTCTAAGTGATTCACTCAATCGATTTAAATAAAAGAGAA
ACATGCGCAGGATTGCGTCCTGGATATTAAATAGGGGAGTATTTAATATCCCCAAATGGATAT
ATCTGGTGCCTCCAACGTTTCGGTGTCTAAGCGCATGTTTCTAGAGAGAGAAGCATAGCAAAA
CGCAAGACGCTAGGCAAAAGAGCGTGTCGTTTCATATCGGTGTATACACCGATTACTTCTCTC
TCTATATATCGGTGTATTGGTGTACTATATATAGTAAAGTTACTAGGGGCTCTCAGCTATAATA
TTACCTGAGAGCCCCGCGACCGGTGTATTGGTATTAGAGCACGTGGGTGGTCCCTCTATTACG
TGGTGCTCTCTGGAGTCTCGCTCGGAGCTTGTTTATTGAACGACTACTTGGAGTAACAGGTAAC
CGGATAGGTGACCGTTCGTACATGGACAAATTCGTCTTTTCCTCAAAAAGACCGCTATTGCCA
TTTGGTGTACAACTTTTTATGCCTATGGCGCCTAAACCCCTCGGGGTATTTGTGTCATTTCTCTG
AAATGACTTTAATTTGAAATCGTTTTTATTTTATAAAAACGATCTTCGTTCAATTTGTCCACACT
ATGCTATACGCACTAAGTCGTTCGTTTATTTTAAGTTAAACATTTCAATATTTTTATCTGGATAT
AAATCTAAGACATGAACGTGAGTTTTCAACCATGAAATGTTTAACCGCAATTATCGAACTCCT
CTTAAATTACGTCATAGTAATTTTGGGTTCAGATGGCAGCCTATGACCCCGTCAAGAGGACGT
TTACGTCTTAATAAGCCTAGTGCTTCACGTAAATTGTCATATGACCGCGTGGAACGGGAAATG
CGTACCAATTCCATTGTTGAGGTTCAACATGGAAGCCATATGTCCCTTGAGAAGAACACGGAC
GTGTCTTCATTTGTGCAATACCCTGTTCGTGGAATCAACGGGGATGGACGTTGTAGGGATTAC
ATCAAGTTGCTCAAACTTGATGTCTCTGGTGTGATAAACATTAAGTCTTCGAATGGAGACCAA
GACATGGAACCAGGTGACAAGTTAAGTGGCCTATTTATCCTGACTGTCTTGTTAGACAAGAAA
CCCTATCTTCCAGAAGGTGTGAACAAGTTACCCTCCTTTGCGGAGTTATTTGGACCTTATTCTG
CTGCATATGCGAATATGCACCTCTTGGATTCTCAGAAGCCACGCTTCAAGGTCCTTGGGACAA
TAAAGAAGTTCGTGAATTGCACACCAGGGACACTATATGGCCCTCTGAAATTAAATATGCCGT
TATCACGGCGAAAGTGTCCTTTGTGGACTACGTTCAAGGACCCTGATCAGGGTAACTGTGGTG

GAAATTATAAGAACATTTCTAAAAATGCTATTGTATTGAGCTATGCATTTATATCAATGCATAG
CCTAATTGTGGAACCATACTTTCAATTCGAATTGAAATATGTGGGATAAAATAAATAAGAAGT
TATTTATTTTCATTGTGTTACAACGCTTTGTTCACATTAGACCGTTGACTTATAAGACATTTGCT
AATAGTCGTCTCTATAATGTCCTCTATTTCTCTCTTGCTCATTGCGTTAGAATGAGACTGAGAT
ACTGAATCTCCAGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGAC
CTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGC
CGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCTGCAGCA

Table 3. Percent identities of the nucleotides of Bam HI digested sample with different begomoviruses reported worldwide.

Accession	Description	Max score	Total Score	Query score %	E value	Max identification %
KU950431.1	Mungbean yellow mosaic India virus segment DNA B, complete sequence	2396	4689	91	0.0	**99**
KF947526.1	Mungbean yellow mosaic virus isolate LBG623-EcoRI segment DNA-B, complete sequence	2891	4400	90	0.0	97
KP319016.1	Mungbean yellow mosaic virus strain LjKu01, complete sequence	2235	4373	91	0.0	97
KF928962.1	Mungbean yellow mosaic virus isolate LBG623 segment DNA-B,complete sequence	4396	4396	91	0.0	96

Sequence results obtained through Pst I restriction digestion

TGCTAGGAGTGCTGCAGCGATTAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACG
TTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGG
TACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCA
GCTCAACTCAGGAGTGGCGTCTATATCTGGGAGGTGCAAAATCCCCTCTCTTTCAAGA
TCATGCACCACAGTCCAATTCAAGCAGGGTCGAGAATGGTTGTCACCAAACTGCGCA
TAATGTTCAACCACGGGTTGAAGAAAGCACTGGGGATGCACAAGTGCTTCCTGGACT
TGAAGCTCTACCATTATATGAAGGCGACTTCTGGGATGATCTTATCGATCTTCCGTAA
CCAATTATTTAGGTTTCTAAATAATTTAGGGGTCATTAGCTTAGCAAATATTTTATATT
TCGCGTCGGATTTCCTGTATGTAAAACTCCACCTGGTGGAGGATGTAAGCTTTACGCA

TAATGTTCAATACAAACTTTATTAATTCAATATCGAATCATAAAAATAGATCCGAATT
TTAAGAGTTGCATACACAGGATTTGAGGCATGAGTACATGCCATATACAATAATAAT
GCGTTCTCAGTATGGTTCTCATACTTCGCTGCCTCTTGGTGGTTGTAAACAACATAAT
TATTAACTTTGAAAAAACGATTCACCATGGCTTGTTCCTTACAAGCATATTGGCCACC
AGTAACAGTGGCATTAAATCTTCGCAAGACTTGATAACGATCACGCAGATCGTTCTTC
ACAGTAGCTGTACTAGGCTCGTTGTCATACATGTTGAACACTTGACCAAAATCCATGG
GTGAACCAAATGGTCGTCTATCACGACATAACTTGAACATCACAGTGTTTGTGTGATT
CTTGGACTTGATGTTCTCGTCCATCCAAACCTTACCCGTAATGTAAACGGACTTGATG
CAAAATCGTTTGCCAAGACGATGTGTAATTCCATTACCTCCAAGTCACGTCAGAATAT
GCAAATCACTTTTGCCTGGGTTTGGGCAAAATCATGGCCTTTGGCCCAAAGGATTGG
AATTTTACATGGGTCCCTTCCAAACCCCCGAAGGGACACCTCGGGGGACCCTTATAC
AATCGGGGAAAAACCGAAGTGGCTATTTTTTCCCCTTTGTAACACACCCTTTTTACAC
CAAAAGATTTTCTTCCCCGCTTTTTTTGTGGGAAATCTCCCAAGAGAGGAGGGACCAC
CCTCTAATTCCCCATGTTTTTGTCTCCATTTTAAGAGGGGGCGCAACAAAAAGAAAAA
ACAAAAAGGTTTTTTTGGACCTTTTTTTCCCAGATGCAGATTGGGCCACTTTCCCTTCC
GAACCTTTCAGGCAGTTAAAAAGCTGCTCAGACGTTAAACCATACATGGAGAAAGAC
GGAGACGTCCTTGATCATGGAACTTTCCAAATCGATGGCCGATCAGCTCGAGGAGGT
AAACAATCTGCCAACGACGCATATGCCGAGGCACTCAACTGTGGATCAAAGTTGGAG
GCCCTCCTTATACTGAAAGAAAAGGCTCCTAAGGATTTTATTTTACAATTTCATAATT
TAAATTGTAATTTGTCTCGGATTTTCACAGAGCCTGTCCAGGCATATGAGTCGCCTTT
TACACTAGAGTCATTTAACAAGGTTCCGAGCTACATTTCTTCATGGGCTGAAAGAAAT
GTGAGAGAACCCGCTGCGCGGCCAGAAAGACCTATTAGTATTGTTATTGAGGGTGAT
AGTCGCACGGGTAAAACCATGTGGGCACGTGCCATAGGTCCTCATAATTATCTTTGC
GGCCATTTGGATCTAAACGACAAAACATACTCTAACGAGGCATGGTACAACGTCATC
GATGACGTTGATCCACATTATTTGAAACATTTCAAAGAATTCATGGGCGCGCAAAGA
GACTGGCAGTCTAACGTCAAGTACGGGAAGCCCACTAAAATTAAAGGTGGTATCCCC
ACCATCTTTCTGTGTAACCCTGGTCCCAAGTCCTCCTATAAAGAGTACTTGGCTGAAG
AGAACAATGCTGCGCTTAAACAGTGGGCTTCAAAGAATGCGGAGTTCTACACCCTCA
AGGAACCATTGTTCTCCTCCGTCAATCAAGGTTCAACACAGGGTCGCCAAGAAGAAA
GCAATTCGACGATCTCGAATTGACCTGACCTGTGGGTGTAGCTATTACATCCATATCA
ACTGCCGTAACTATGGATTTTCGCACAGGGGAGAGCATCACTGCAGCCCGGGGGATC
CACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTG
AGGGTTAATTGCGCGCTGGCGTAATCATGTCAAGCGTCGAC

Table 4. Percent identities of the nucleotides of Pst I digested sample with different begomoviruses reported worldwide.

Accession	Description	Max score	Total Score	Query score (%)	E value	Max identification (%)
KU950430.1	Mungbean yellow mosaic India virus segment DNA A, complete sequence	1611	3167	80	0.0	**95.83**
HF922628.1	Mungbean yellow mosaic India virus isolate Bengal, segment A, complete viral segment	1600	3095	80	0.0	95.63
AF314145.1	Mungbean yellow mosaic India virus-[Bangladesh] DNA-A, complete sequence	1589	3165	82	0.0	96.48

4.1.2.5 Sequence analysis of DNA-A and DNA-B

The nucleotide sequences of both strands of DNA A and DNA B components were determined. On the basis of the sequence, one clone was identified as DNA-A and two clones as DNA-B. The nucleotide length was determined to be 2746 bp for DNA-A and 2649 bp for DNA- B. The DNA-A sequence when analyzed in NCBI database exhibited 96.48 percent identity to an isolate of mungbean yellow mosaic India virus-[Bangladesh] DNA-A, complete sequence (Gene bank AF314145.1), whereas the clones of DNA-B showed 99 % identity (Tables 3 and 4). Viral sequences were aligned, ORFs were deciphered, the analysis of the sequences showed them to encode predicted open reading frames (ORFs) typical of the old world begomoviruses. The DNA-A encodes seven predicted ORFs, two in the virion-sense (AV1 encoding the CP and AV2 encoding a protein of unknown function) and four in the complementary-sense (AC1 encoding the replication initiation protein [Rep], AC2 encoding the transcriptional activator protein, AC3 encoding the replication enhancer protein and AC4 (a pathogenicity determinant). DNA B encodes two predicted open reading frames, one in each orientation; BV1 encoding the nuclear shuttle protein (NSP) and BC1 encoding the movement protein (MP). One clones of the potentially full-length DNA-A (produced using restriction enzymes Pst I and two clones of the potentially full length DNA B (produced by using Bam HI, respectively) were selected

for further analysis and sequencing. These DNA-A and DNA–B clones are helpful for developing infectious clone. The full-length DNA-B sequence is published at NCBI database (Accession number KY303697.1).

4.1.3 Cloning of *CP* and *AC2* gene in pBI121 vector in antisense orientation to generate antiviral pBI121CP-AC2 vector construct

A proper gene construct is crucial for the success of producing ideal transgenic line. A vector acts as a vehicle that transports the gene of interest into a target cell for replication and expression. Common vector consists of three components: an origin of replication, multi cloning site or recombination site, and selectable marker. The binary vector pBI121 has been widely used in plant transformation. The total size of pBI121 is 12.8kb according to its construction map (Jefferson et al. 1987). In this study, we have reconstructed the binary vector pBI121 where replacing *GUS* gene and cloning of *CP* and *AC2* gene in antisense orientation to generate a new antiviral gene construct using the following protocol.

4.1.3.1 Cloning of *CP* gene in pBI121 vector in antisense orientation

After confirmation through sequencing and sequence analysis of the MYMV coat protein (*CP*) gene were used for cloning into pBI121 vector in antisense orientation by replacing *GUS* gene. PCR amplified *CP* gene and pBI121 vector were digested with the Bam HI and Sac I restriction enzyme respectively. The digested vector produced two bands, one for *GUS* gene (~2.0 kb) and another for GUS less pBI121 vector (~12.7 kb) on the other hand CP (insert) produced single ~ 750 bp band visualized through agarose gel electrophoresis (Fig. 25 a, b). Digested vector and insert bands were purified using a gel purification kit. Following that a ligation reaction was set up in a eppendorf tube and during ligation a 1:3 ratios of vector and insert was used to obtain positive clones. After transformation of the ligation products into the *E. coli* Top10 competent cells the positive colony was selected under kanamycin selection in LB medium containing kanamycin as a selective agent. The positive colony was further confirmation through colony PCR and other molecular techniques such as restriction digestion, automated sequencing etc. Confirmation of CP clone in antisense orientation was done by PCR with the CP forward and reverse primers, restriction digestion with Bam HI and Sac I enzymes and automated sequenced of the *CP* gene (Fig. 26 a, b). Partially amplified MYMV-CP gene was cloned in pBI121 vector in antisense orientation in place of GUS gene by using Bam HI and Sac I restriction

recognition sites and produced pBI121CP vector construct (Fig. 27). The CP clone gene was subjected to BLAST search to confirm the cloned sequence which showing 98 % sequence similarity in mungbean yellow mosaic virus in Bangladesh strain.

The sequence result of *CP* clone gene generated through automated sequencing were

CCGGGGGAAGATAAGAACTTCGAATCCCCGCTCCTGCTGCCTGCTTCTGCGGGAGGT
GTCCCTACCAACATGAAAGGAAAAACTTCATCCGTCCCATGTGGAGGAAACTGGCAA
AAAAGTGAGTAGGTCCCCTGAGGGCCTCGTGGTTGTGAGGGACCGGGTAAAGTTCAA
TCATTTGATGCAAAGCCCTGATATTGCCCACACAGGCTTTTTTAATTTGCATATCTGA
CGTGACTAGGGGTAATGGAATTACACATCGTCTTGGCAAACAATTTTGCATCAAGTC
CGTGTACATAACGGGTAAGGTTTGGATGGACAAAAAAATCAAGTCCAAAAATCCCCC
AAACACCGGGATGTTCAAGTTATGTCGTGACAAACAACCTTTTGGTTCACCCTTGGAT
TTTGGTCAATTGTTTAACATGTATGAATATTTTTTCCTTTTTACACCTCCCGTGAAAAA
AGATCTGGCTGGAATCTTTATTCACCCCCTTAGAAAGGATTTAAGGGGGGGCGGTA

4.1.3.2 Sub-cloned of *AC2* gene into pBI121CP vector in antisense orientation

Geminivirus encoded AC2 acts as an RNA silencing suppressor and interferes with the accumulation of siRNA that's why *AC2* gene was sub-cloned into pBI121CP vector in antisense orientation to down regulate its expression. The pBI121CP vector were digested with the Bam HI restriction enzyme and produced linearized vector followed by dephosphorylation. The amplified *AC2* gene was digested with the same restriction enzyme. The ligation of vector and insert (1:3) was performed to produce pBI121CP-AC2 vector construct (Fig. 28). The positive colony was confirmed through colony PCR with AC2 forward and reverse primer (Fig. 29). Confirmation of *CP-AC2* gene in antisense orientation was done through PCR with the CP forward and AC2 reverse gene specific primers. Amplification of *CP-AC2* gene at 1.2 kb (Fig. 30) indicating that the *AC2* gene was cloned in antisense orientation to produce pBI121CP-AC2 vector construct. The *AC2* clone gene was further confirmed by automated sequencing. The sequenced results were subjected to BLAST search and it showed that 96.52% similarity in mungbean yellow mosaic India virus *AC2* gene product.

The sequence result of AC2 clone gene generated through automated sequencing were

TGATGTGCTCAGTCCAGGAGCACTTGTGCATCCCCAGTGCTTTCTTCAACCCGTGGTTGAACAT
TATGCGCAGTTTGGTGACAACCATTCTGGACCCTGCTTGAATTGGACTGTGGTGCATGATCTTG
AAAGAGAGGGGATTTTGCACCTCCCAGATATAGACGCCACTCCTGAGTTGAGCTGCAGTGATG
CTCTCCCCTGTGCGAAAATCCATAGTTACGGCAGTTGATATGGATGTAATAGCTACACCCACA
GGTCAGGTCAATTCGAGATCGTCGAATTGCTTTCTTCTTGGCGACCCTGTGTTGAACCTTGATT
GACGGAGGAGAACAATGGTTCCTTGAGGGTGTAGAATTCCGCATGGATCCTCTAGAGTCCCCC
GTGTTCTCTCCAAA

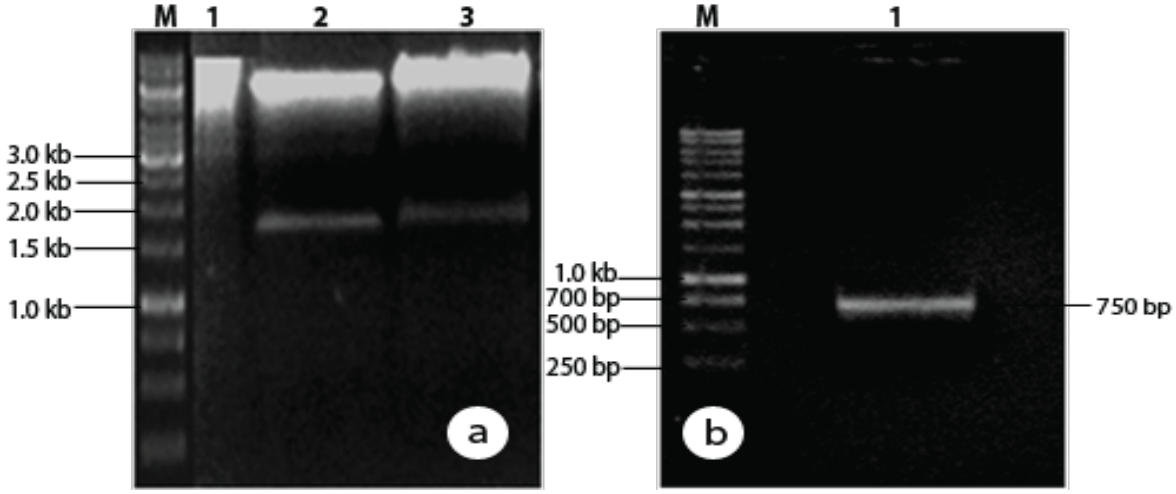


Fig. 25 (a-b): Restriction digestion of pBI121 vector and insert DNA (CP). (a) Digestion of pBI121 vector with BamHI and SacI restriction enzymes. Lane M = 1.0 kb DNA Ladder; lane 1- undigested vector DNA , lanes 2 & 3 - digested vector DNA which release *GUS* gene at 1.8 Kb. (b) Restriction digestion of insert (*CP* gene) with same restriction enzymes. Lane M = 1.0 kb DNA Ladder; lane 1- digested *CP* gene.

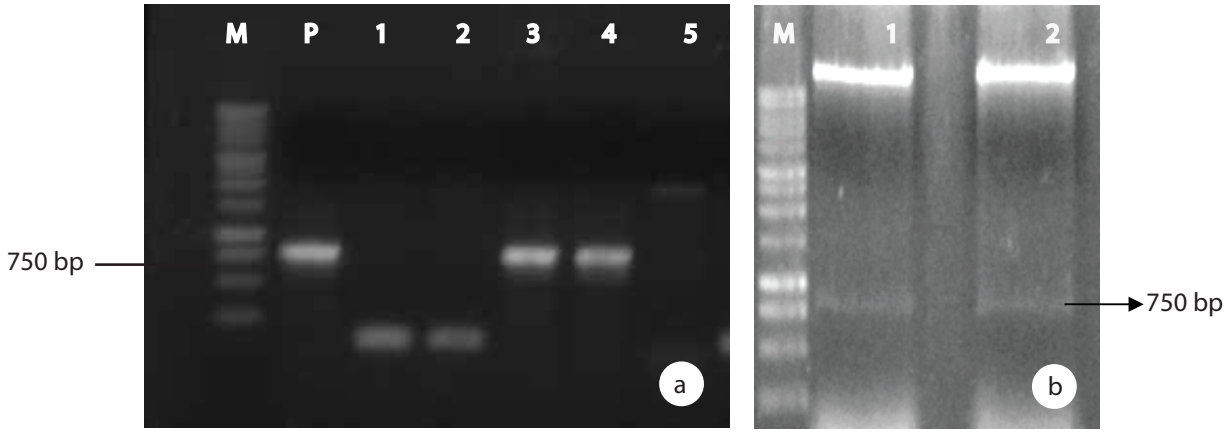


Fig. 26 (a-b): Cloning confirmation of *CP* gene by colony PCR and restriction digestion of CP cloned plasmid with Bam HI and Sac I restriction enzymes. (a) Colony PCR using CP forward and reverse primer; lane M = 1.0 kb DNA ladder; lane 3-4 showing amplified positive bands at 750 bp which is identical to the positive control (lane p), lane 2-negative control and lane 1 showing water control. (b) Restriction digestion of CP clone plasmid with Bam HI and Sac I restriction enzymes; Lane M= 1 Kb DNA ladder; lane 1-2 showing 750 bp bands after digestion.

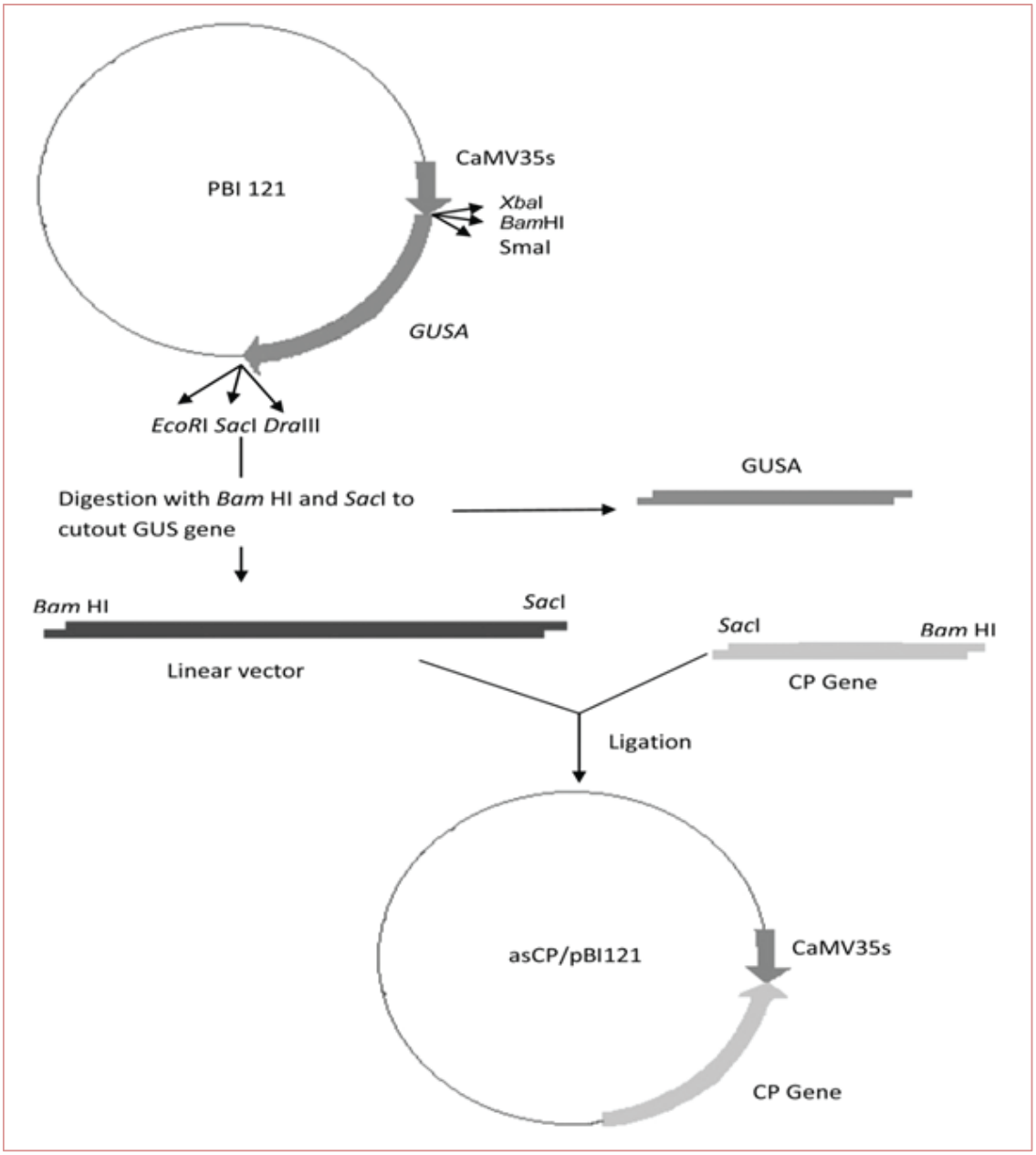


Fig. 27: Schematic presentation of construction of pBI121CP vector construct by ligation of *CP* gene in an antisence orientation under CaMV35s promoter . The linear vector was produced after cutting out of the *GUS* gene through digestion of pBI121 plasmid DNA with Bam HI and Sac I restriction enzyme. At the same time , PCR amplified *CP* gene was digested with the same restriction enzymes and ligated the *CP* gene to create pBI121CP vector construct.

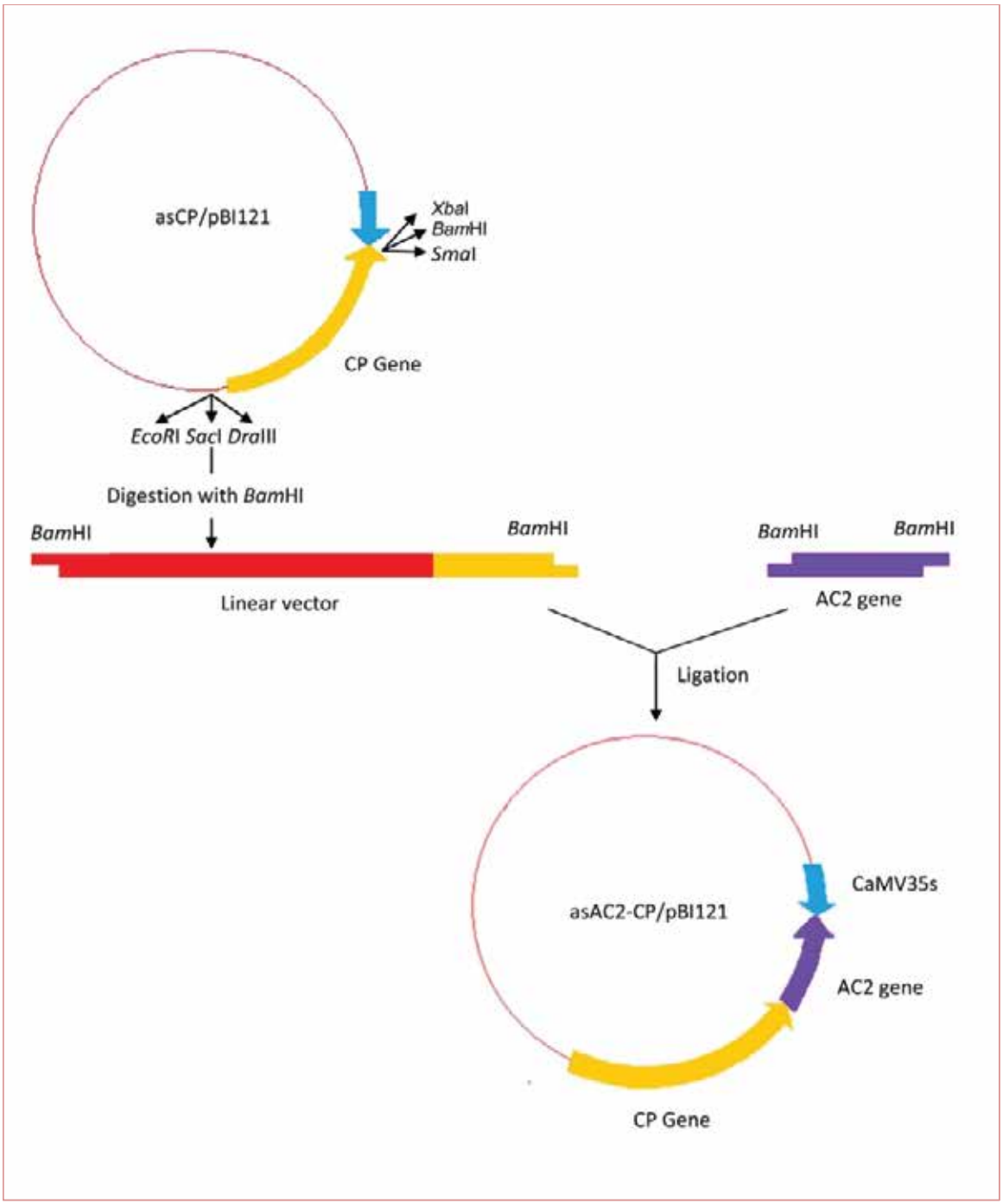


Fig. 28: Schematic presentation of construction of pBI121CP-AC2 vector construct by ligation of *AC2* gene in an antisense orientation under CaMV35S promoter; The linear vector was produced after digestion of pBI121/CP vector construct with Bam HI restriction enzyme and ligated with Bam HI digested PCR products of *AC2* gene to create antiviral vector construct pBI121CP-AC2.

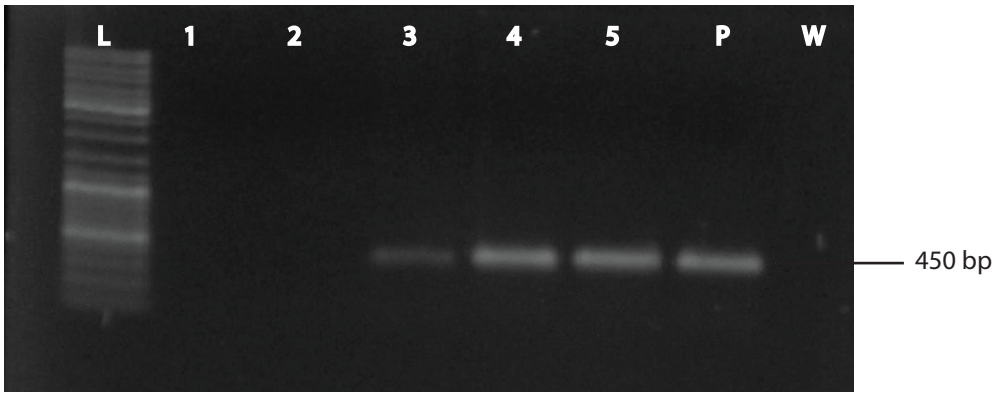


Fig. 29: Cloning confirmation of *AC2* gene by colony PCR of CP-AC2 cloned plasmid with AC2 forward and reverse primer; Lane M = 1.0 kb DNA ladder; lane 3-5 showing amplified *AC2* gene at 450 bp which is identical to the positive control, lane p-positive control and lane 2-negative control.

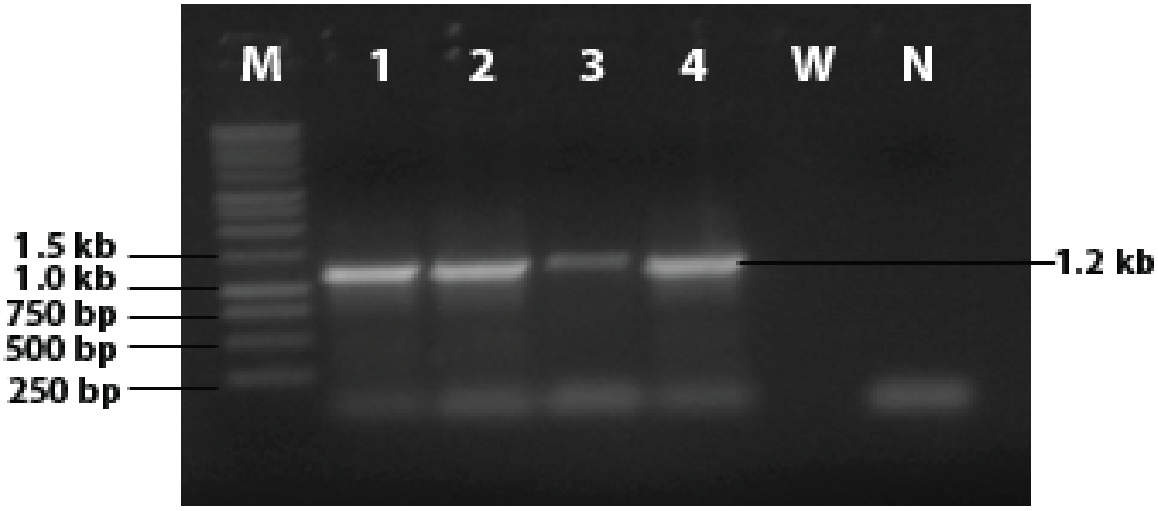


Fig 30: Confirmation of CP-AC2 clone in antisense orientation through PCR with CP forward and AC2 reverse Primer. Lane M = 1.0 kb DNA ladder; lane 1-3 showing amplified positive bands at 1200 bp which is identical to the positive control lane 4, lane W - water control and lane N showing negative control.

4.1.3.3 pBI121CP-AC2 vector construct was transformed into LBA4404 strain of *Agrobacterium*

LBA4404 are widely used *Agrobacterium-tumefaciens* strain for T-DNA transfer into plant cells. Therefore, pBI121CP-AC2 antiviral vector construct were transferred to LBA4404 strain by electroporation method. After transformation of the cloned antiviral vector construct into the LBA4404 electro-competent cells the positive colony was selected under rifampicin, streptomycin and kanamycin selection in the medium. The pBI121CP-AC2 clone plasmid were isolated from the positive colony and transformation was confirmed through PCR using *CP* and *AC2* gene specific forward and reverse primers respectively (Fig.31 a, b). Transformation of pBI121CP-AC2 plasmid into LBA4404 strain was further confirmed through restriction digestion with Bam HI and Sac I restriction enzyme and automated sequenced of the *CP-AC2* gene. After restriction digestion the pBI121 plasmid produce two bands whereas the clone CP-AC2 plasmid produced 3 bands one is vector back bone and other two bands are *CP* and *AC2* (Fig. 31 c, d) at 750 bp and 450 bp respectively. The sequenced results were subjected to NCBI-BLAST search and the cloned sequences showed 98.26 % (accession number ANA08966.1) and 97.56 % (accession number ANA08963.1) sequence similarity with mungbean yellow mosaic India virus *AC2* and *CP* gene respectively. The LBA4404 strain containing newly developed pBI121CP-AC2 antiviral vector construct was used for *Agrobacterium-tumefaciens* mediated genetic transformation during this investigation.

The sequence results of *CP-AC2* clone gene after transformation into LBA4404 strain generated through automated sequencing were-

TGTCTGATGTAGTTACGATACCCGCTTATGCTGCTGCTTCTGCGGGAGGTGTCGCTACCAACAT
GAAAAGAAGAAAGAAGAACAACCGTCCCATGTGGAGGAAACCTCGGTTTTACCGACTGTATA
GGTCCCCTGATGTCCCTCGTGGTTGTGAGGGACCATGTAAAGTTCAATCATTTGAGCAAAGGC
ATGATATTGCCCACACAGGCAAAGTGATTTGCATATCTGACGTGACTAGAGGTAATGGAATTA
CACATCGTCTTGGCAAACGATTTTGCATCAAGTCCGTGTACATAACGGGTAAGGTTTGGATGG
ACGAGAACATCAAGTCCAAGAATCACACAAACACTGTGATGTTCAAGTTATGTCGTGACAGAC
GACCATTTGGTTCACCCATGGATTTTGGTCAAGTGTTTAACATGTATGACAACGAGCCTAGTAC
AGCTACTGTGAAGAACGATCTGCGTGATCGTTATCAAGTCTTGCGAAGATTTAATGCCACTGT
TACAGGTGGCCAATATGCTTGTAAGGAACAAGCCATGGTGAATCGTTTTTTCAAAGTTAATAA
TTATGTTGTTTACAACCACCAAGAGGTAGCGAAGTATGAGAACCATACTGAGAACGCATTATT
ATTGTATATGGCATGTACTCATGCCTCAAATCCTGTGTATGCAACGGATTCCGGGTTTAAACTA
TGCTGACTTCAGTCCAGGAGCACTTGTGCATCCCCAGTGCTTTCTTCAACCCGTGGTTGAACAT
TATGCGCAGTTTGGTGACAACCATTCTGGACCCTGCTTGAATTGGACTGTGGTGCATGATCTTG
AAAGAGAGGGGATTTTGCACCTCCCAGATATAGACGCCACTCCTGAGTTGAGCTGCAGTGATG
CTCTCCCCTGTGCGAAAATCCATAGTTACGGCAGTTGATATGGATGTAATAGCTACACCCACA
GGTCAGGTCAATTCGAGATCGTCGAATTGCTTTCTTCTTGGCGACCCTGTGTTGAACCTTGATT
GACGGAGGAGAACAATGGTTCCTTGAGGGTGTAGAATTCCGCATGGAATCCGTGTA

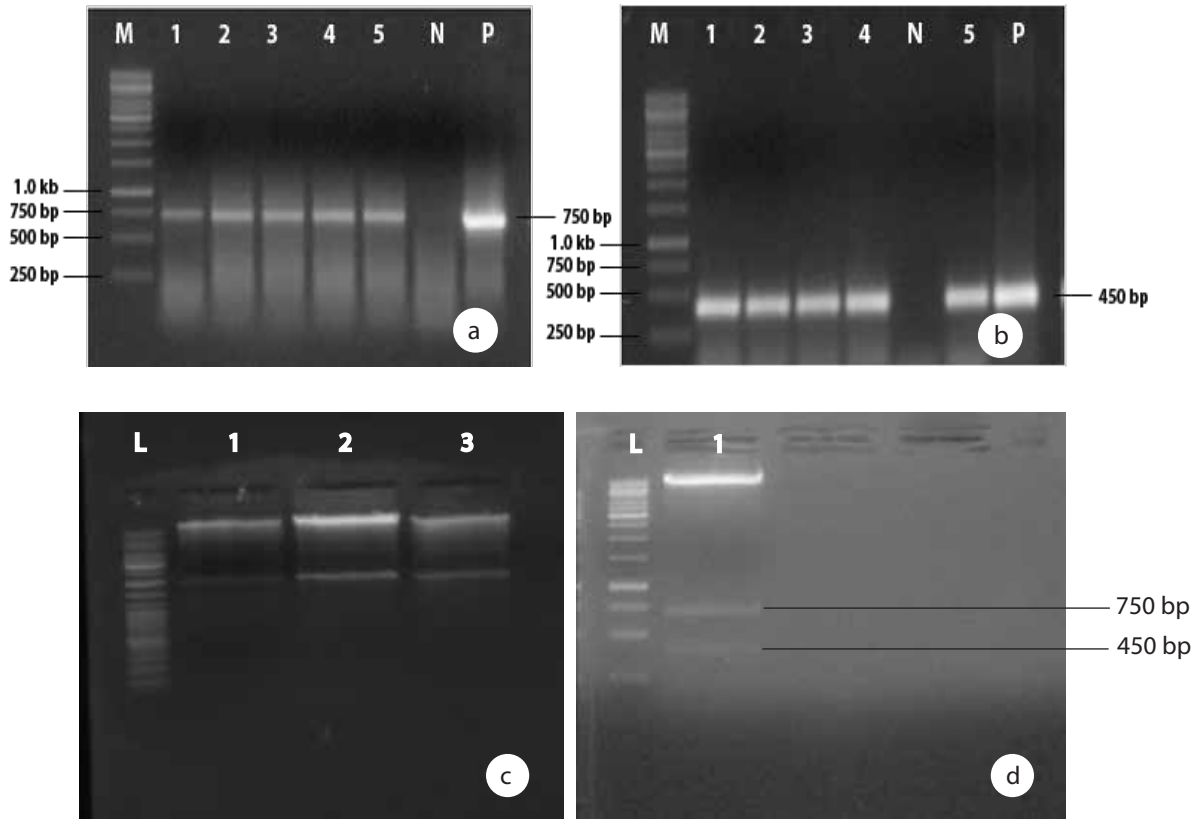


Fig. 31(a-d): Cloning confirmation of *CP-AC2* gene by colony PCR and restriction digestion of CP-AC2 cloned plasmid with Bam HI and Sac I restriction enzymes. (a) PCR amplification of MYMV *CP* gene with *CP* gene specific primer; lane M-1.0 kb DNA ladder, lane 1-5 showing amplified *CP* gene which is identical to the positive control lane P, no band was amplified in negative control lane N; (b) PCR amplification of MYMV *AC2* gene with *AC2* gene specific primer; Lane M-1.0 kb DNA ladder, lane 1-4 & 5 showing amplified *AC2* gene identical to the band positive control lane P, no band was amplified in negative control lane N; (c-d) Restriction digestion of pBI121 and cloned pBI121CP-AC2 plasmid with Bam HI and Sac I restriction enzyme; in case of fig. c only *GUS* gene were produced after restriction digestion but in fig. d after restriction digestion three bands were produced (lane1), two bands were identical to the *CP* and *AC2* gene.

4.2 Development of transgenic tobacco plants using the antiviral vector construct pBI121CP-AC2

As a model plant *Nicotiana tabacum* L. (variety; Petit Havana) were used for *in vitro* regeneration and *Agrobacterium*-mediated genetic transformation to examine the transforming ability of the newly developed construct pBI121CP-AC2. The following experiments were carried out to develop transgenic tobacco plants using this construct.

4.2.1 *In vitro* regeneration of tobacco plantlets from leaf disk explants

In vitro regeneration is an essential component for successful plant genetic transformation. In this experiment, leaf disk explants were collected from 20-25 days old *in vitro* grown seedlings (Fig. 32 a). MS medium supplemented with 4.44 μM of BAP and 0.54 μM NAA were used for callus induction and shoot formation. Callus formation was initiated within 12-15 days and numerous shoot primordia were found to develop from the callus tissue (Fig. 32 b). Shoot formation from such callus tissue was initiated within 14-18 days following callus development (Fig. 32 c). Regenerated shoots were transferred to half strength of MS medium without any hormonal supplement for their root formation. Effective healthy roots were formed from the regenerated shoots and the time required for root induction was 12-15 days after inoculation (Fig. 32 d).

4.2.2 Transplantation of rooted plantlets and development of viable seeds

Following enough development of roots, plantlets of tobacco were transplanted into small plastic pots containing autoclaved soil for acclimatization. The surviving plantlets are presented in Fig. 32 e. After one week of hardening these plantlets were transferred to larger earthen pots for further growth and establishment (Fig. 33 a). Successfully acclimatized plants were found to develop flower and many healthy viable seeds were obtained from the plants (Fig. 33 b, c).

4.2.3 *Agrobacterium* - mediated genetic transformation of tobacco plants

A series of experiments were conducted to develop a suitable protocol for *Agrobacterium*-mediated genetic transformation of the model plant *Nicotiana tabacum* with a newly developed antiviral construct (pBI121CP-AC2) against mungbean yellow mosaic virus. This experiment was carried out to understand the efficiency of this antiviral construct regarding plant transformation. Following successful development of transformation protocol, this gene construct was exploited in transforming mungbean plant. These newly

developed constructs contained *nptII* gene conferring kanamycin resistance, applicable for selection of transforments. Therefore, kanamycin was used as selectable agent during these experiments. For genetic transformation, leaf disc explants of tobacco were incubated for 20 minutes with *Agrobacterium* suspension. For infection of the explants the required optical density of the *Agrobacterium* suspension was 0.6 measured at 600 nm wavelength. A co-cultivation period of 72 hours was maintained for the infected explants. Following co-cultivation, explants were transferred to shoot regeneration on MS medium supplemented with 4.44 µM BAP and 0.54 µM NAA, along with 100 mg/l kanamycin as a selective agent.

4.2.4 Shoot formation and selection of putatively transformed shoots after infection with LBA4404 strain carrying pBI121CP-AC2 construct

After co-cultivation, the infected leaf disc explants were washed with 300 mg/l carbenicillin and transferred to shoot induction medium for regeneration of multiple shoots. Moreover, to control the overgrowth of *Agrobacterium*, 100 mg/l carbenicillin was added to the regeneration medium. After 12-15 days of co-cultivation callus formation was initiated and the fully developed callus were transferred to same medium for multiple shoot formation (Fig. 34 a, b). Multiple shoots were found to regenerate after 12-15 days of culture. The regenerated shoots were transferred to shoot induction medium containing 100 mg/l kanamycin concentration (Fig. 34 c). The selection of putatively transformed shoots was carried out using kanamycin since the pBI121CP-AC2 construct contained *nptII* gene conferring kanamycin resistance. All the negative control shoots were failed to survive at 100 mg/l kanamycin concentration and 100 mg/l kanamycin concentration was considered as an optimum kanamycin concentration for selection of putatively transformed shoots (Fig. 34 d). It was noticed that during this selection experiment the non-transformed shoots that became albino failed to survive at optimum kanamycin concentration. The putatively transformed shoots were survived in the selection medium containing 100 mg/l kanamycin concentration for 15 days (Fig. 34 e). These survived healthy green shoots were separated and considered as putatively transformed shoots.

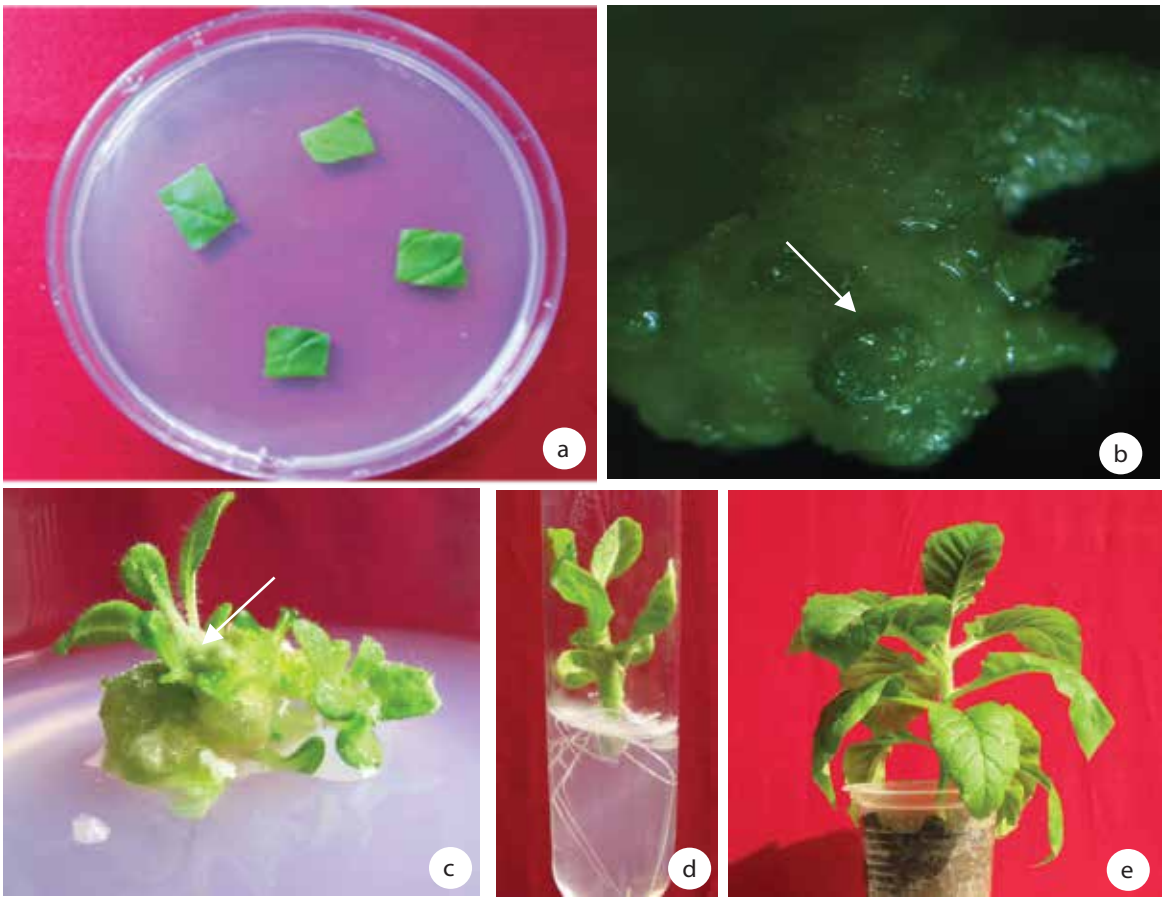


Fig. 32 (a-e): *In vitro* regeneration of tobacco plants from leaf disc explants. (a) Cut leaf explants from 25 days old *in vitro* germinated seedlings; (b) Initiation of callus (arrows) from leaf explants on MS medium supplemented with 4.44 µM BAP and 0.57 µM NAA; (c) Development of shoots from callus; (d) Initiation and elongation of roots on half strength of MS medium without any hormonal supplement; (e) Regenerated plantlets transferred to small plastic pots containing soil.

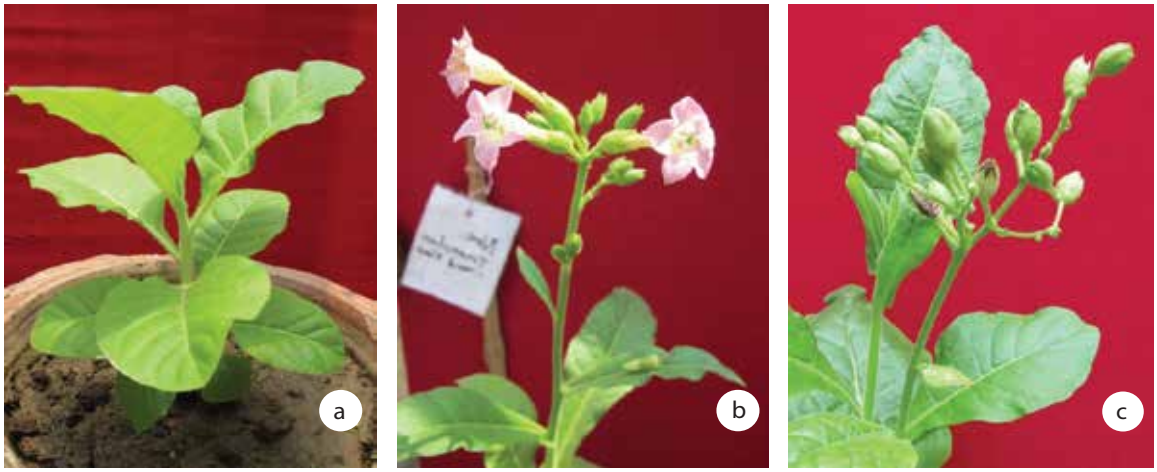


Fig. 33 (a-c): *In vitro* regenerated plants developed viable seeds. (a) *In vitro* regenerated plants were transferred into larger earthen pots; (b & c) Flower and seed formation after maturation from the regenerated plants.

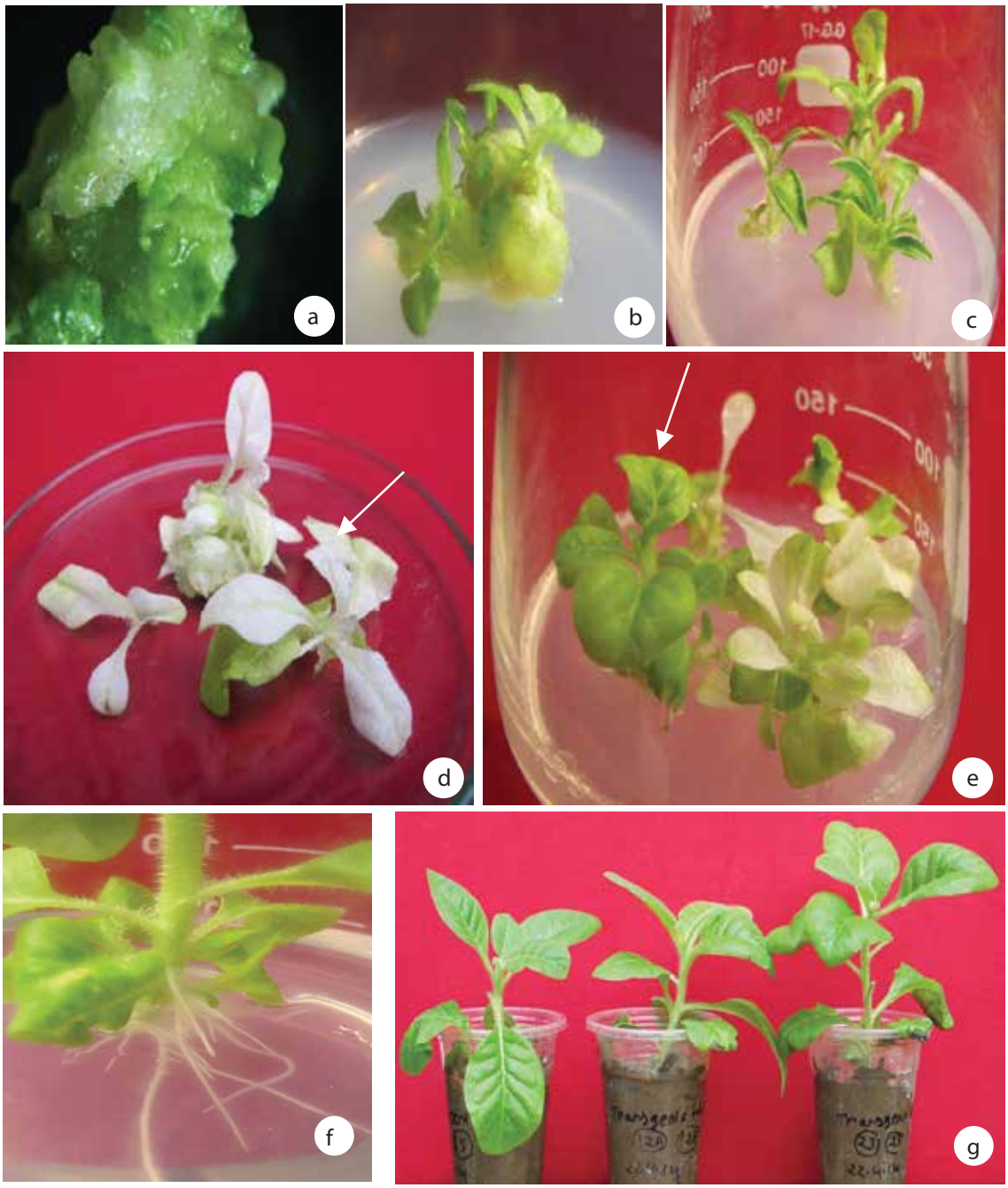


Fig. 34 (a-e): Different stages of development of putatively transformed plantlets from leaf disc explants of tobacco following transformation with *Agrobacterium* tumefaciens strain LBA4404 containing pBI121CP-AC2 gene construct. (a) initiation of callus from leaf disc explants on MS medium supplemented with 4.44 µM BAP and 0.57 µM NAA; (b) development of shoots from callus;(c) Regenerated shoots sub-cultured in selection medium containing 100 mg/l kanamycin concentration; (d) Control shoots became albino and failed to survive (arrows) on selection medium containing 100 mg/l kanamycin; (e) Putatively transformed shoots (arrow) remained green at selection pressure of 100 mg/l kanamycin concentration;(f) Formation of roots from the putatively transformed shoots on MS medium without any hormonal supplement; (g) Acclimatization of putatively transformed plantlets in a soil containing small plastic pots.

4.2.5 Root formation and hardening of putatively transformed plantlets

Putatively transformed shoots that survived in the selection media were transferred to MS medium without any hormonal supplement for root development. In most of the cases, about 15-18 days were necessary for the development of healthy roots in rooting medium (Fig. 34 f). These putatively transformed plantlets with adequate root system were transplanted to soil containing small plastic pot for proper hardening (Fig. 34 g).

4.2.6 Molecular confirmation of *CP-AC2* gene integration through PCR analysis

The genomic DNA was isolated from the putatively transformed shoots and also from non-transformed shoots. The DNA of the engineered *Agrobacterium* strain containing respective genes were used as positive control during the PCR experiments. The isolated genomic DNA were subjected to PCR for the amplification of *CP-AC2* gene by using CP forward and AC2 reverse primer. Amplification of *CP-AC2* gene was confirmed and the amplified DNA was analyzed through agarose gel electrophoresis. After gel electrophoresis, corresponding band (~1.2 kb bp) was observed (lane 1-10) which were identical to the DNA band of positive control. Therefore, ten PCR positive *Nicotiana tabacum* plants were identified as transformed plants (Fig. 35 a). This result indicated that the *CP-AC2* gene was successfully inserted into the genomic DNA of the transformed plant. On the other hand, non-transformed plant (lane N) and water control (Lane W) did not show any band. In separate PCR, Pic A primer was used to nullify the contamination of DNA samples with *Agrobacterium* genomic DNA (Fig. 35 b).

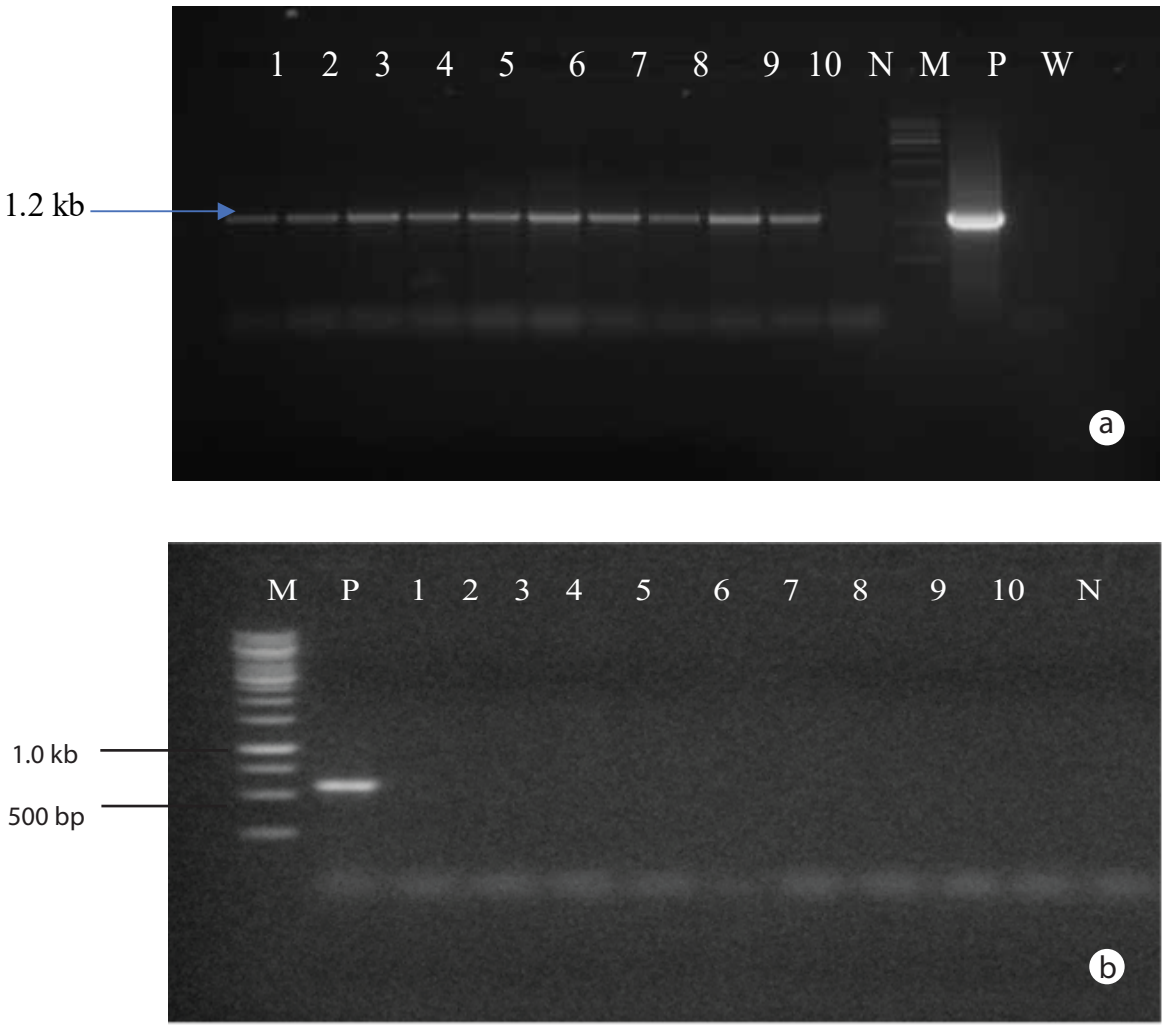


Fig. 35: PCR confirmation of the *CP-AC2* gene in ten To transgenic tobacco lines. (a) PCR amplification of *CP-AC2* gene with CP forward and AC2 reverse primer; Lane M refers to 1.0 Kb DNA ladder; lane 1-10 showing amplified positive bands at 1.2 kb which is identical to the positive control (lane-P), lane N-negative control and lane W showing water control. (b) PCR amplification of Pic A gene with pic A primer; lane M- refers to 1.0 kb ladder, lanes 1-10 genomic DNA of transformed shoots, note that no bands were amplified in genomic DNA lane 1-10 after PCR amplification, only one band was found to amplify in lane p (positive control), lane N showing negative control.

4.2.7 Test of pollen viability and development of mature T_0 seeds

PCR positive transgenic tobacco plants were transferred to earthen pots to grow till maturity. Within 45 - 50 days of culture flowers and pods formation were observed. Then pods became mature and able to produce transgenic T_0 seeds (Fig. 36 a, b, c, d). Apart from the molecular analysis, microscopic observation was carried out to test the pollen viability of the transgenic tobacco plants. Pollen viability is important to test the capability of the pollen grains to take part in effective pollination and fertilization. Therefore, viability of pollen grains of *in vivo* grown flowers was tested using fluorescein diacetate (FDA) under fluorescent microscope. The anthers of transgenic tobacco flowers contained many pollen grains. Viability of pollen grains from these flowers were determined based on size, shape and the degree of staining properties using aniline blue. Fluorescent microscopic observation was carried out using a fluorescent microscope (Nikon, Japan) and it was observed that about 95% of the pollen grains were viable (Fig. 37 a, b, c). Self-pollinated pistils were examined to monitor *in situ* growth of the pollen tubes within the stylar tissue. Healthy callose plugs formed within the pollen tubes (Fig. 37 d). The T_0 transgenic seeds were germinated in earthen pots and ultimately have got T_1 transgenic seeds (Fig. 38 a, b, c). Newly developed construct found to be effective in developing transgenic tobacco plants. Therefore, further experiments were conducted in locally grown mungbean varieties for genetic transformation.

Fig. 36 (a-d): Seed formation from transgenic tobacco plants. (a) Hardened transgenic tobacco plants were transferred to earthen pots; (b & c) Flower and seed formation after 50-60 days of culture; (d) Development of mature transgenic tobacco seeds.

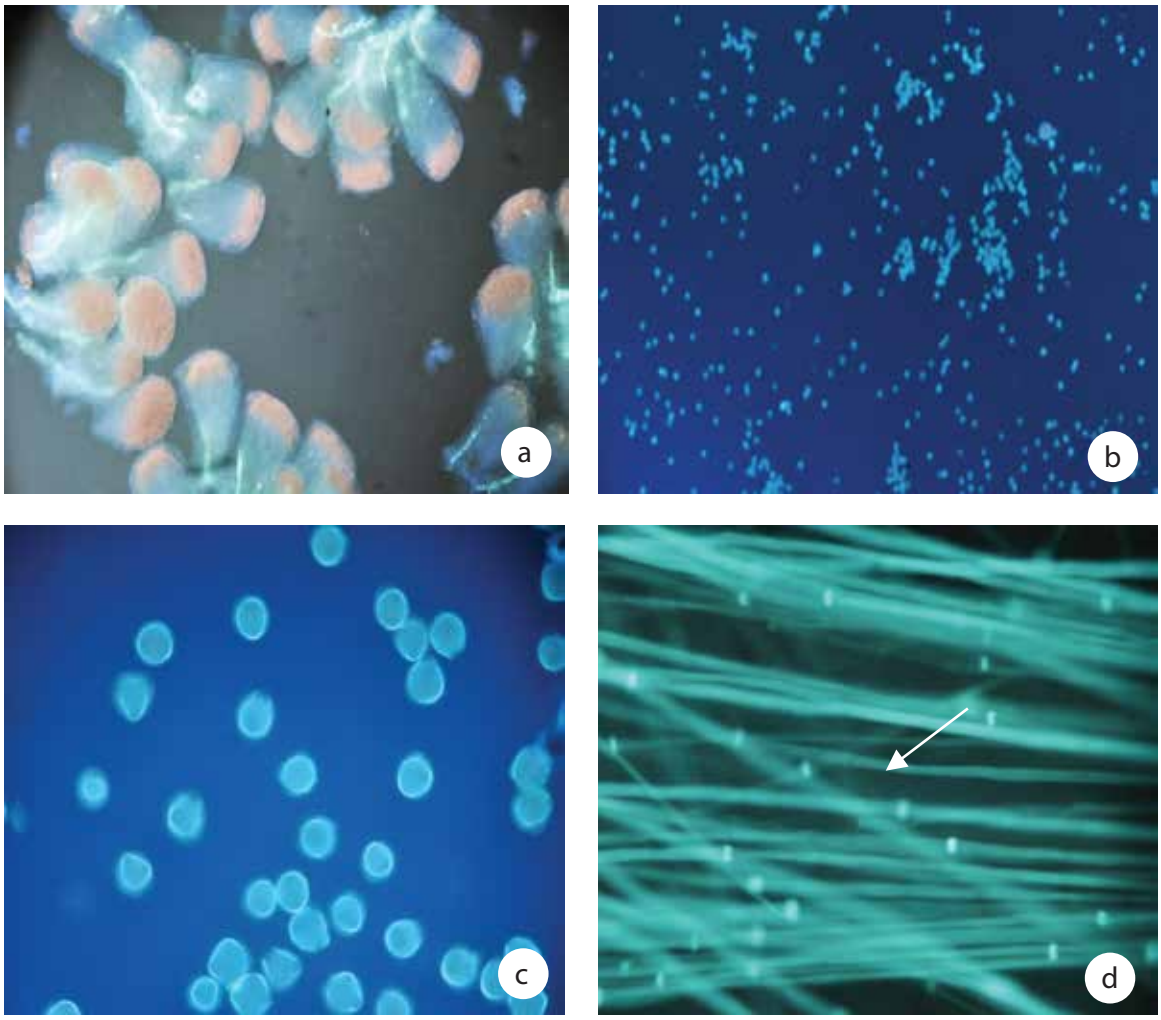


Fig. 37 (a-d): Pollen viability test using FDA stain. (a) Anthers with large number of pollen grains, ×6; (b & c) Fluorescent microscopic views of pollen grains after FDA staining ×10 ; (d) Callose invested the pollen tube (arrow) growing within the style tissue following self-pollination ×16.

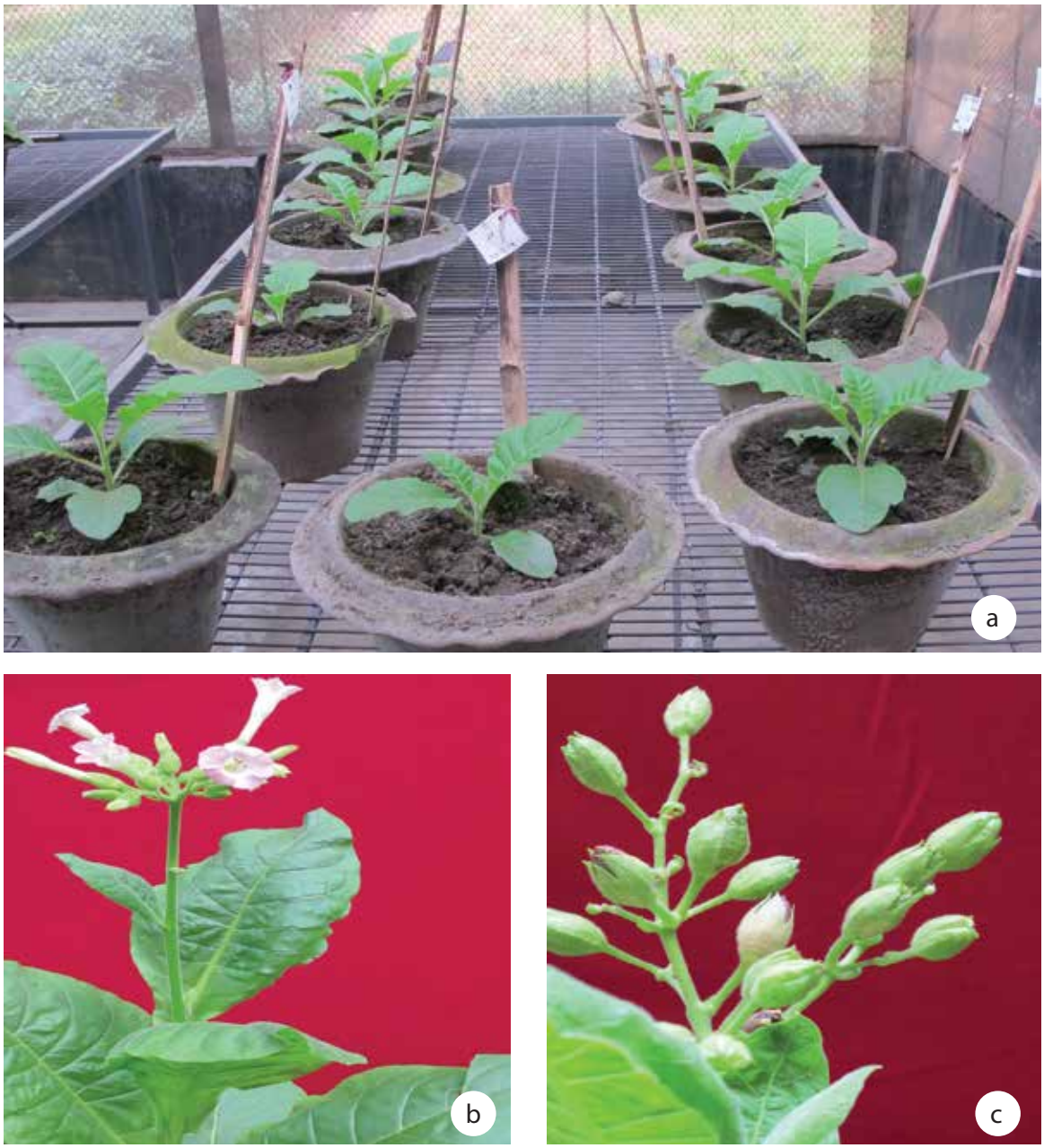


Fig. 38 (a-c): Growing transgenic tobacco plants in earthen pots and formation of viable seeds. (a) T_1 transgenic tobacco plants growing in earthen pot; (b-c) Flower and seed formation of T_1 tobacco plant after 60 days of culture.

4.3 Development of transformation compatible *in vitro* regeneration protocol for mungbean varieties using pBI121GUS-NPTII vector construct (construct I)

In the second phase, several experiments were conducted to obtain an efficient *in vitro* regeneration and *Agrobacterium*-mediated genetic transformation protocols for two locally grown mungbean varieties namely BARI mung-3 and Binamoog-5. Two different types of explants i.e. cotyledon attached decapitated embryo (CADE) and cotyledonary node (CN) were used for this purpose. The *Agrobacterium* strain LBA4404 harboring plasmid pBI121GUS-NPTII (considered as construct I) were used for the establishment of an efficient genetic transformation protocol since it contained two marker genes namely, *GUS* (β-glucoronidase) as screenable marker and *npt* II (neomycin phosphotransferase) as selectable marker gene. Construct I was used to optimize various parameters like, optical density of *Agrobacterium* suspension, suitable incubation and co-cultivation periods for effective transformation. Besides these, the concentration of selectable agent (kanamycin) was also optimized to find out the optimum kanamycin concentration to eliminate control shoots. Through proper selection procedures the putatively transformed shoots were selected and rooted properly. Following adequate development of roots, the plantlets were established in soil following proper hardening. Further molecular characterization of transformed plantlets was carried out through the polymerase chain reaction. The results of the present study obtained from different experiments are presented in the following sections.

4.3.1 *In vitro* regeneration

A transformation compatible *in vitro* regeneration system is a prerequisite for the development of successful genetically engineered plants. In the present study, cotyledon-attached decapitated embryo (CADE) and cotyledonary node (CN) explants were used to develop transformation compatible *in vitro* regeneration system. After successful shoot development, the *in vitro* raised shoots were cultured for *in vitro* root induction and subsequent development of plantlets. Micrografting technique was also applied to scavenge the root induction hurdles faced during *in-vitro* rooting. Rooted or mirografted plantlets were consequently acclimatized in soil following proper hardening and allowed to grow under field conditions.

4.3.1.1 Seed germination and explant preparation

Healthy surface sterilized seeds were germinated in various media such as water agar, MSB_5 and MSB_5 media supplemented with 5.0 µM BAP. Although there weren't any major differences in germination rate of the two varieties in three different media. But it was observed that, the seedlings germinated on hormone supplemented medium (5.0 µM BAP) were healthier and grow vigorously that facilitated the explants preparation easily. Cotyledonary node (CN) explants were derived from 3-days old *in vitro* germinated seedlings and cotyledon attached decapitated embryo (CADE) explants were prepared from surface sterilized overnight soaked seeds of BARI mung-3 and Binamoog-5 varieties (Table 5).

Table 5. Percentage of mungbean seed germination on different culture media

Varieties	Media					
	Water agar		**MSB_5**		**MSB_5 + 5.0 µM BAP**	
	No. of seeds germinated	% of seed germination	No. of seeds germinated	% of seed germination	No. of seeds germinated	% of seed germination
BARI mung-3	47	94	47	94	48	96
Binamoog -5	45	90	46	92	47	94

n = 50 (No. of seed inoculated)

4.3.1.2 *In vitro* regeneration from CADE and CN explants in BARI mung-3 variety

In the present set of experiments, different concentrations of BAP (2.5–10.0 µM) were used with MSB_5 medium to observe their effects on shoot induction from CADE and CN explants of BARI mung-3 variety of mungbean. Data were collected regularly on the following 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 experiments. The MSB_5 medium with reduced concentration of BAP were also used for shoot multiplication and their subsequent development. In this experiment, best response towards multiple shoot regeneration was observed on MSB_5 medium with

5.0 µM of BAP. Regeneration of maximum number of shoots 86% and 75% was observed in 5.0 µM of BAP in case of CADE and CN explants respectively (Table 6). Shoot initiated within 10 -12 days and 12-14 days of inoculation from CADE and CN explants respectively (Fig. 39 a and 40 a). Maximum mean number of shoots obtained per explant was (4.56 ± 0.87) after 30 - 45 days of culture in case of CADE explants. Multiple shoots developed on this medium were found green and healthy (Fig. 39 b, c, d and 40 b, c). The regenerated shoots from CADE (Fig. 39 e, f) and CN explants (Fig. 40 d) developed roots and transferred to soil after proper hardening. The plants produced flower and viable pod after maturation (Fig. 39 g, h and 40 e, f).

Table 6. Effect of different concentrations of BAP in MSB5 medium on regeneration of multiple shoots from two explants of BARI mung-3 variety.

Conc. of BAP (µM)	Explant	No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoots/ explant after 30-40 days of culture ($\bar{x}$±sd)	Average length of shoots after six weeks (cm)
0.0	CADE	50	40	18-20	1.15 ± 0.36	2.35
	CN	50	38	16-18	1.21±0.41	1.60
2.5	CADE	50	66	12-14	1.90±0.77	2.42*
	CN	50	58	14-15	1.58±0.62	2.41
5.0	**CADE**	**100**	**86**	**10-12**	**4.56 ± 0.87**	**3.57**
	CN	100	75	12-14	4.15±0.82	3.38*
7.5	CADE	100	77	11-13	2.59±0.61	3.42*
	CN	100	71	13-15	2.53±0.63	3.35
10.0	CADE	50	60	12-15	2.06±0.74	2.47*
	CN	50	52	14-15	1.76±0.95	2.92

CN = Cotyledonary Node, CADE = Cotyledon Attached Decapitated Embryo.

$\bar{x}$ = mean, sd = standard deviation, *Significant at $p<0.05$

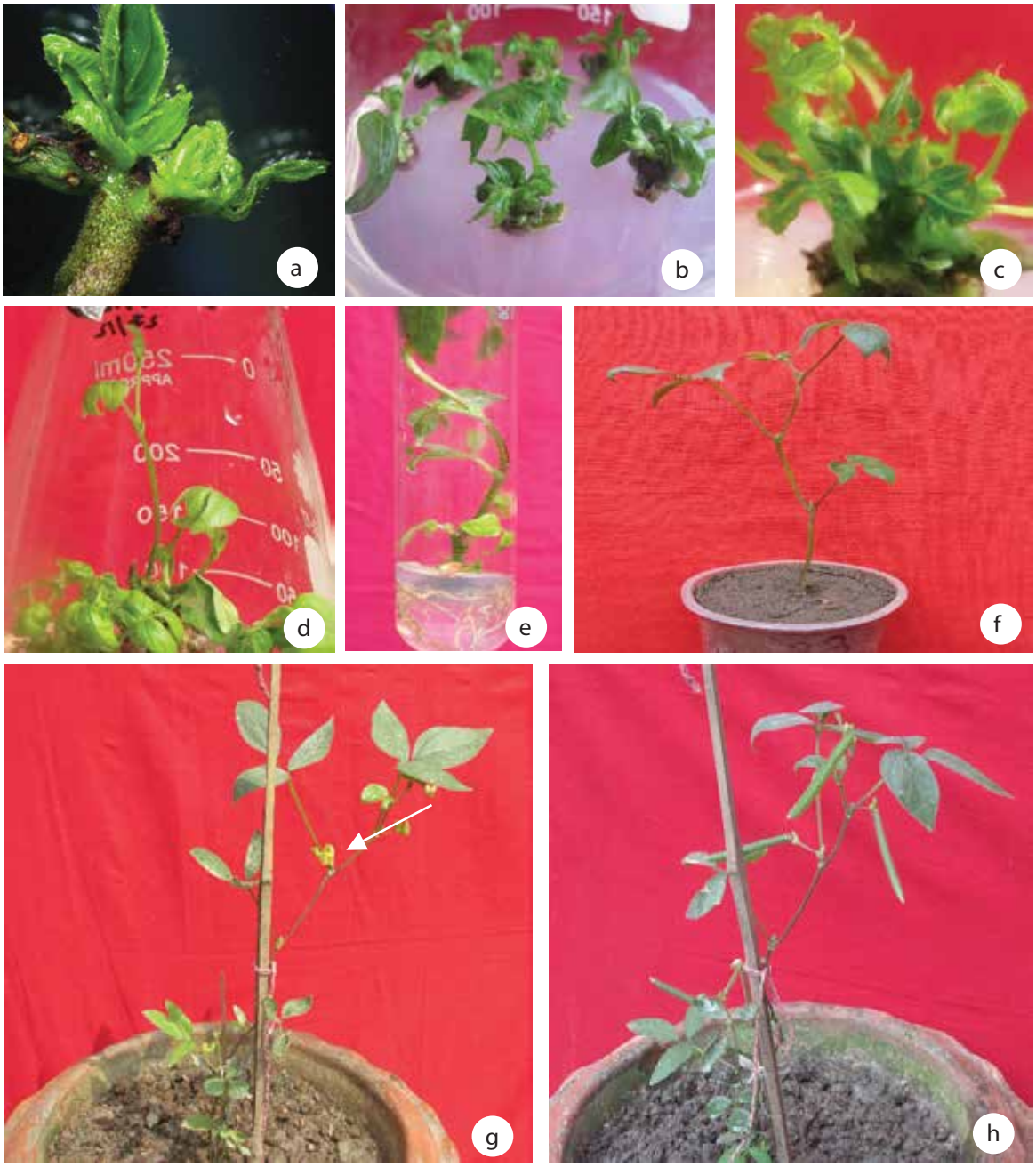


Fig. 39 (a-h): Various stages of development of shoots and pod formation from CADE explants of BARI mung-3 on MSB_5 medium supplemented with BAP. (a) Stereomicroscopic view of initiation of shoots from CADE explants ; (b) shoot initiation in MSB_5 medium supplemented with 5µM BAP; (c-d) Development and elongation of multiple shoots on the same media; (e) Formation of roots from the cut ends of MSB_5 medium containing 2µM IBA; (f) Rooted plantlets transferred to soil in small plastic pot; (g-h) Formation of flower and pods in a large earthen pots.

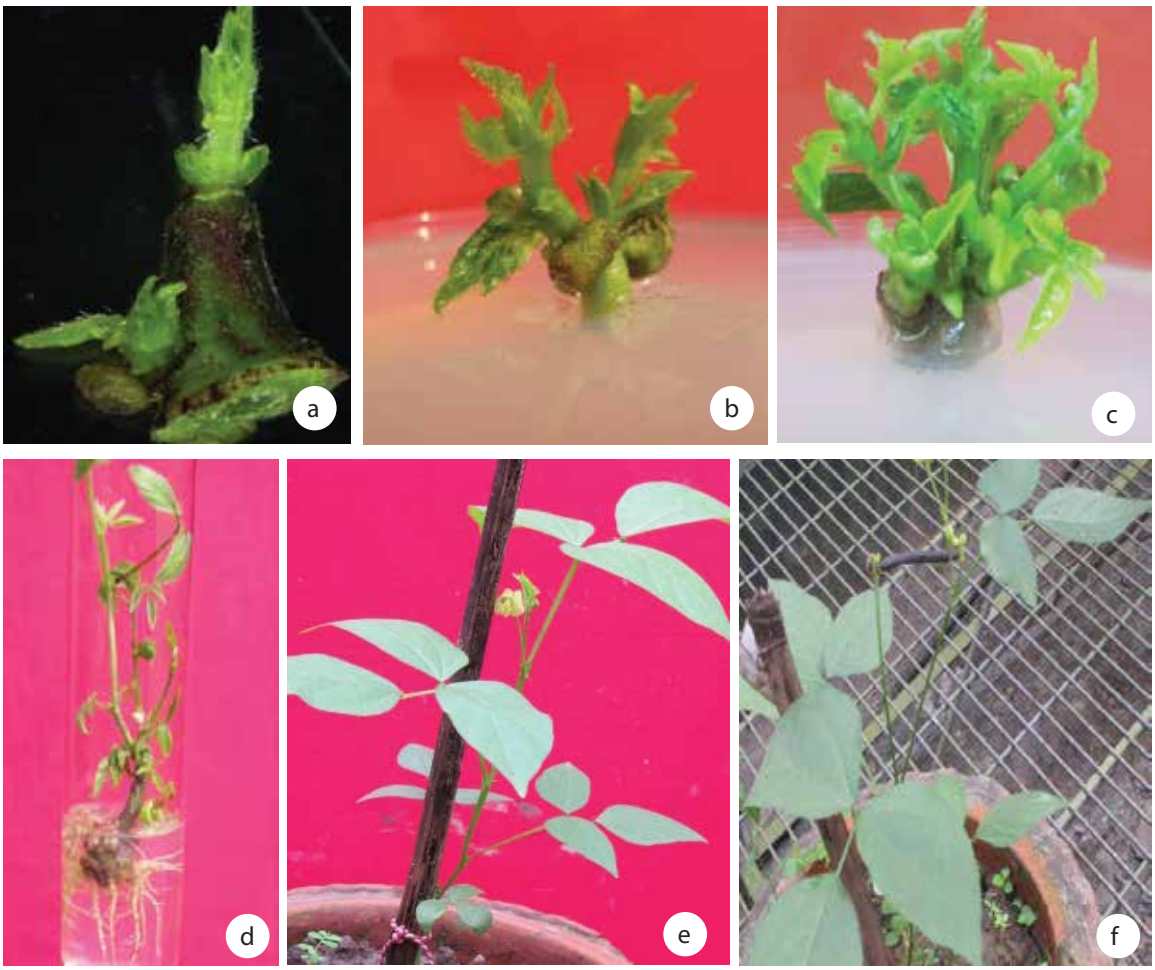


Fig. 40 (a-f): Different stages of *in vitro* shoot regeneration from CN explants of BARI mung-3 on MSB_5 medium supplemented with BAP. (a) Stereomicroscopic view of shoot initiation from CN explant; (b-c) Formation of multiple shoots from cotyledonary node explant on MSB_5 medium with 5 µM BAP; (d)-f) Formation of multiple roots on MSB_5 medium supplemented with 2 µM IBA; (e-f) Formation of flowers as well as pods from the *in vitro* raised plantlets.

4.3.1.3 *In vitro* regeneration from CADE and CN explants in Binamoog-5 variety

In case of Binamoog-5 variety different concentrations of BAP (2.5–10.0 μM) were used with MSB_5 medium to observe their effects on shoot induction from CADE and CN explants of mungbean. Among different concentrations of BAP 5.0 μM showed the best response towards multiple shoot regeneration. The maximum number of shoot regeneration was observed 81% in CADE explants whereas in case of CN explants it was observed 70% (Table 7). Shoot initiated within 8 -10 days and 13-14 days of inoculation of CADE and CN explants respectively.

Table-7. Effect of different concentrations of BAP in MSB_5 medium on regeneration of multiple shoots from two explants of Binamoog-5 variety.

Conc. of BAP (μM)	Explant	No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoots/ explant after 30-40 days of culture ($\overline{x}$±sd)	Average length of shoots after six weeks (cm)
0.0	CADE	50	38	14-16	1.47±0.51	2.47
	CN	50	36	16-18	1.66±0.68	1.5
2.5	CADE	50	58	10-12	1.62±0.49	2.55
	CN	50	54	15-16	2.37±0.49	2.22
5.0	**CADE**	**100**	**81**	**8-10**	**4.41±0.54**	**3.81**
	CN	100	70	13-14	3.33±0.75	3.14*
7.5	CADE	100	72	10-11	3.54±1.16	3.20*
	CN	100	64	14-16	2.78±0.67	2.32
10.0	CADE	50	54	12-13	2.40±0.50	2.33*
	CN	50	50	13-15	2.44±0.50	2.47

$\overline{x}$ = mean, sd = standard deviation, *Significant at $p<0.05$

Maximum mean number of shoots obtained per explant was 4.41±0.54 and 3.33±0.75 after 30-40 days of culture in case of CADE and CN explants respectively. Multiple shoots developed on this medium were found green and healthy (Fig. 41 a, b, c and 42 a, b, c ,d, e). The regenerated shoots developed roots (Fig. 41 d and 42 f) and transfer to soil after proper hardening. The plants produced flower and viable pod after maturation (Fig. 41 e, f and 42 g). The number of regenerated shoots were found to increase by following the subculture in the same medium with reduced concentration of hormone. It was observed that the best average length of shoots after six weeks was 3.81 (cm) from CADE explants. It was also observed that from the base of the regenerated shoots several new shoot buds were developed at later stages of the culture.

Fig. 41 (a-f): Various stages of shoots development and pod formation from CADE explants in Binamoog-5 containing MSB_5 medium with BAP . (a-b) Shoot initiation from CADE explants containing MSB_5 medium supplemented with 5 µM BAP; (c-d) Formation of multiple shoots along with elongated shoots on the same media after 45 days of culture; (e) Formation of roots on MSB_5 medium supplemented with 2 µM IBA; (f) Mature plantlets produced flower and pods after 60 days of culture.

Fig. 42 (a-g): Various stages of development of shoots and pod formation from CN explants of Binamoog -5 . (a-b) Stereomicroscopic view of CN explant and shoot initiation from CN explant after 15 days of culture; (c) shoot initiation from CN explants on MSB_5 medium supplemented with 5 µM BAP; (d-e) Multiple shoot formation along with elongation on the same media; (f) Formation of roots of MSB_5 medium supplemented with 2 µM IBA; (g) The regenerated plantlets transferred to soil in earthen pots and formation of pods.

4.3.1.4 Effect of different concentrations and combinations of BAP and Kn in MSB_5 medium on multiple shoot regeneration from two explants of BARI mung-3 and Binamoog-5 varieties.

MSB_5 medium supplemented with different concentrations and combinations of BAP (5.0 and 7.5 μM) and Kn (0.46, 1.39 and 2.22 μM) were used for induction of multiple shoots directly from two explants of BARI mung-3 and Binamoog-5 varieties. Shoot buds were found to initiate within 8-12 days and 10-12 days of inoculation for BARI mung-3 and Binamoog-5. Among all the concentrations and combinations of BAP and Kn used, highest response towards multiple shoots initiation and development was observed on MSB_5 medium supplemented with 5.00 μM BAP and 2.22 μM Kn from CADE explants. With the mentioned media combination, BARI mung-3 showed 70% response from CN and 80% from CADE explants towards shoot initiation and mean number of shoots obtained per explant was 2.8 in case of CN and 3.8 in case of CADE explants (Table 8). On the other hand, Binamoog-5 showed 74% and 78% response towards shoot initiation from CN and CADE explants respectively and mean number of shoots obtained per explant was 3.8 in case of CN and 3.5 in case of CADE explants (Table 9). It was observed that the nature of multiple shoot regeneration was almost identical when MSB_5 medium was supplemented with either 5.00 μM BAP or 5.00 μM BAP with 2.22 μM Kn (Fig. 43 a, b). Therefore, for further regeneration experiments 5.00 μM BAP was used for multiple shoot regeneration.

Table 8 Effect of different concentrations and combinations of BAP and Kn in MSB_5 medium on regeneration of multiple shoots from different explants of BARI mung-3 variety.

Explant	Conc. of BAP (µM)	Conc. of Kn (µM)	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	5.00	0.46	50	64.00	12	1.50	3.1
CADE			50	68.00	10	2.90	3.0
CN	**5.00**	**1.39**	**50**	**66.00**	**10**	**2.40**	**3.1**
CADE			**50**	**72.00**	**8**	**3.00**	**3.4**
CN	5.00	2.22	50	70.00	12	2.80	3.6
CADE			50	80.00	8	3.80	4.0
CN	7.50	0.46	50	55.00	13-15	2.5	3.1
CADE			50	62.00	12-13	2.5	3.2
CN	7.50	1.39	50	58.00	10-12	3.0	2.6
CADE			50	67.00	10-12	3.5	4.0
CN	7.50	2.22	50	68.00	13-14	2.5	3.2
CADE			50	70.00	13-14	4.0	3.5

CN = Cotyledonary node, CADE = Cotyledon attached decapitated embryo.

Table 9 Effect of different concentrations and combinations of BAP and Kn in MSB_5 medium on regeneration of multiple shoots from different explants of Binamoog-5 variety.

Explant	Conc. of BAP (µM)	Conc. of Kn (µM)	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	5.00	0.46	50	60.00	13	2.50	3.3
CADE			50	68.00	10	3.0	3.0
CN	5.00	1.39	50	66.00	10	3.00	3.1
CADE			50	76.00	12	3.40	3.4
CN	**5.00**	**2.22**	**50**	**74.00**	**12**	**3.80**	**3.8**
CADE			**50**	**78.00**	**10**	**3.50**	**4.2**
CN	7.50	0.46	50	55.00	13-15	2.5	3.1
CADE			50	62.00	12-13	4.5	3.2
CN	7.50	1.39	50	58.00	10-12	3.0	2.6
CADE			50	67.00	10-12	3.5	4.0
CN	7.50	2.22	50	68.00	13-14	2.5	3.2
CADE			50	70.00	13-14	4.0	3.5

CN = Cotyledonary node, CADE = Cotyledon attached decapitated embryo.

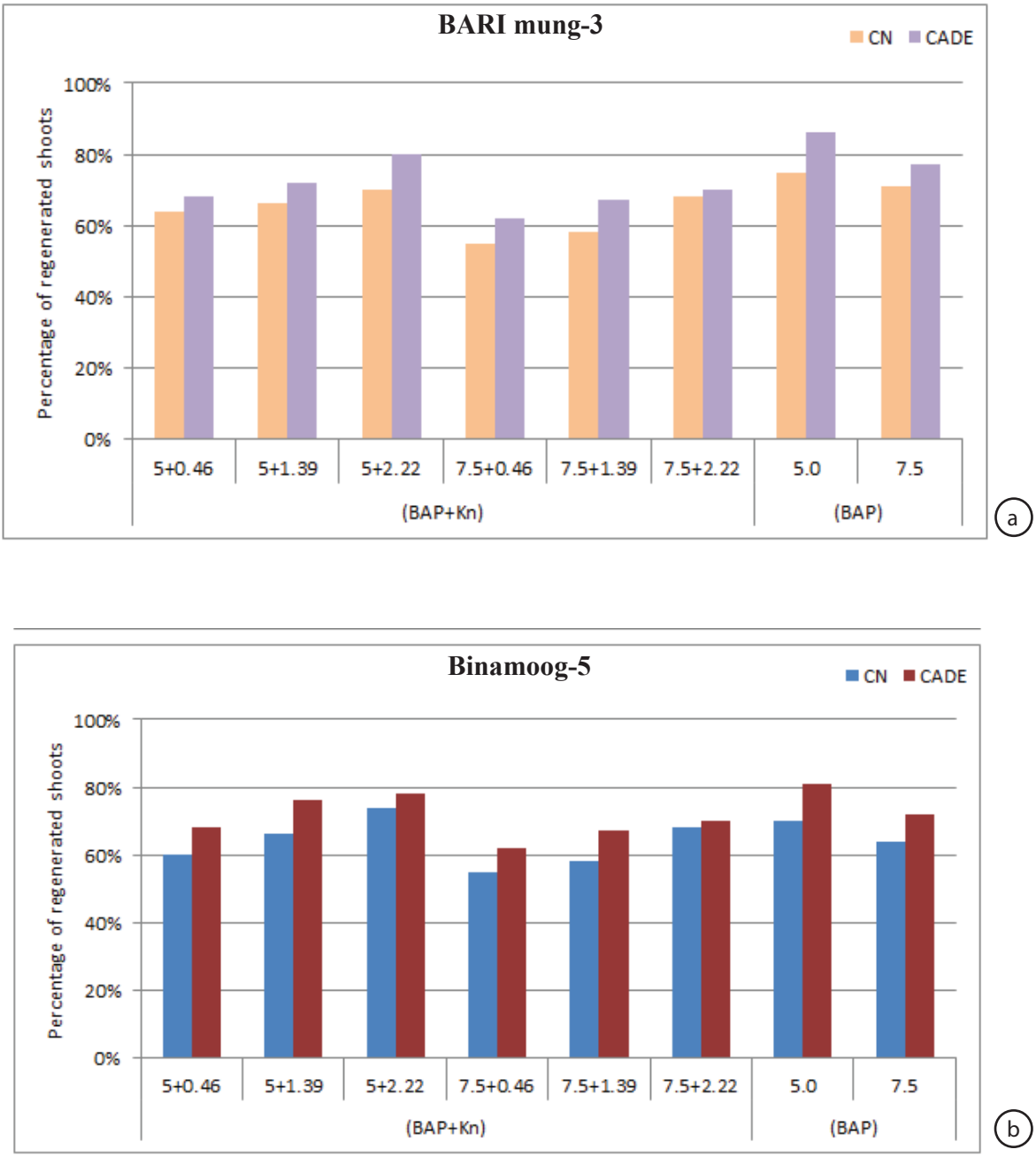


Fig. 43 (a-b): Comparative analysis of CADE and CN explants towards shoot regeneration on MSB_5 medium supplemented with different concentrations of BAP alone and combination of BAP and Kn in two locally grown mungbean. (a) Effect of different concentrations of BAP alone with Kn in MSB_5 medium on regeneration of multiple shoots from two explants of BARI mung-3 variety; (b) Effect of different concentrations of BAP alone with Kn in MSB_5 medium on regeneration of multiple shoots from two explants of Binamoog-5 variety.

4.3.1.5 Rooting of the *in vitro* regenerated shoots

Half strength of MSB_5 medium supplemented with various concentrations of IBA (1.0 -2.5 µM) were used to determine their effect on root induction from the *in vitro* grown shoots of BARI mung-3 variety. For this purpose, 3-5 cm long shoots were excised and cultured on root induction medium. Half strength of MSB_5 medium supplemented with 2.0 µM IBA showed the best response for root formation among the various concentrations of IBA used in this study (Table 10). Maximum number of healthy roots (26.66 %) were obtained with ½ MSB_5 and 2.0 µM of IBA in BARI mung-3 variety (Fig. 44).

Table 10. Effects of different concentrations of IBA with ½ MSB_5 on root formation in BARI mung-3 variety.

Media composition	No. of shoots produced roots	% of shoots forming roots	Days required for root induction	Days required to get well developed roots	Mean number of roots/shoot ($\overline{x}$±sd)
1/2 MSB_5	5	16.66	20-22	35-40	5.2 ± 1.30
1/2 MSB_5 + 1.0 µM IBA	6	20.00	16-18	32-35	5.3 ± 1.21*
1/2 MSB_5 + 1.5 µM IBA	6	20.00	18-20	30-32	6.0 ± 1.26*
1/2 MSB_5 + 2.0 µM IBA	**8**	**26.66**	**13-15**	**30-32**	**6.8 ± 0.71**
1/2 MSB_5 + 2.5 µM IBA	7	23.33	20-22	35-37	5.0 ± 0.58*

N = 30 (number of shoots inoculated for root induction), $\overline{x}$ = mean, sd = standard deviation
*Significant at $p<0.05$

Fig. 44 (a-f): Development of *in vitro* root formation from the regenerated shoots in BARI mung-3 containing ½ MSB_5 medium supplemented with 2.0 µM IBA. (a) In vitro regenerated shoots were transferred to root induction medium; (b-d) Various stages of root formation containing ½ MSB_5 medium supplemented with 2.0 µM IBA; (e-f) *In vitro* regenerated shoots were failed to develop viable roots on the same media.

4.3.1.6 Establishment of plantlets

Formation of root is an indispensable step to produce complete plantlets. Shoots that were regenerated following various experiments during this study did not produce roots spontaneously. Improvement of mungbean crop has been hindered by their extreme recalcitrance to tissue culture. Particularly, the low frequency of rooting is the major limiting factor in the establishment of a successful plantlet regeneration from *in vitro* regenerated shoots of mungbean. The plantlets obtained with insufficient roots from two mungbean varieties were successfully transplanted into small plastic pots containing autoclaved soil, but the survival rate of the transplanted plantlets was found to be quite low. The survived plantlets were transferred to larger clay pots for their further growth and establishment. Under these circumstances, *in vitro* micrografting technique could be an alternative way to enhance *in vitro* root development in mungbean.

4.3.1.7 Micrografting of the *in vitro* regenerated shoots of BARI mung -3 variety

To enhance the survival rate of *in vitro* derived mungbean plantlets micrografting technique was applied. In the present experiments, it was observed that the scions length of 2.5-3 cm (Fig. 45 a) showed better response in micrografting with 55 % of success (Table 11). Whereas 48% success was obtained from the scions of 1.5-2 cm length. For rootstocks, the 14 days-old rootstocks (Fig. 45 b) were found to be more efficient than 7 days-old rootstocks (Table 11). If the age of rootstock is more than 28 days the success rate of grafting is comparatively declined. The average rate of success of grafted shoots was influenced by both scion length and rootstock age. The firm placement of the micro scion onto the rootstock to ensure good contact was essential for the formation of the graft union. Displacement of the micro scion has also caused failure of micrografting. In BARI mung-3 variety the average rate of success graft was 51% (Table 11).

A successful graft union is considered when the rootstock and scion physically join is followed by growth and opening of leaves (Fig. 45 c). After acclimatization the successful grafted plants were transferred in the earthen pots for the development of flowers and pods (Fig. 45 d, e). The propagated plantlets grew very well and did not show any morphological abnormalities. Normal flowering and pod formation occurred in micrografted mungbean plants. When the pods become mature it produces viable seeds (Fig. 45 f).

Table.11 Effect of scion length and the rootstock age on *in vitro* grafting in BARI mung-3 variety

Scion length(cm)	Rootstock age (days)	No. of grafting	Successful grafts	% of successful grafts	Average of successful grafts (%)
1.5-2.0	7	20	7	35	51
2.5-3.0	7	20	8	40	
1.5-2.0	14	20	9	48	
2.5-3.0	**14**	**20**	**11**	**55**	

During these experiments, the growth of callus between scion and rootstock is a key process in the successful graft union development. Cellular division in surfaces of both rootstock and scion was initiated in several points by cells at the most central part of the union, which was extended through 30 days. The re-establishment of vascular continuity through the interface zone is the critical event that determines the compatibility between the rootstock and the scion on the development of graft union formation. The realignment and restoration of the vascular bundles between scion and rootstock was completed 30 days after grafting. Histological analysis of micrografted region under florescent microscope was done and it was observed that a true graft union was formed (Fig. 46 a). The control plant and micrografted plant showed different connection after microscopic observation (Fig. 46 b, c). The successfully developed micrografted plants were transferred to earthen pots and it was observed that the average success rate of transplantation was 81.66% (Table 12) that was higher than the normal root formation.

Table. 12 Success rates of grafting and transplants after 30 days of culture

Lot no.	**No. of grafting**	**No of grafts established**	**Successful transplants**	**% of success (transplants)**	**Average success rate (%)**
Lot-1	23	11	8	72	81.66
Lot-2	24	12	10	83	
Lot-3	20	11	10	90	

Fig. 45 (a-f): Different stages of micrografting from in vitro regenerated shoots of BARI mung-3 and viable pods formation. (a-b) Scions and rootstock were prepared for micrografting , both are cut in V-shape; (c) Arrow indicated successful micrografted region after 35 days of grafting; (d) The successfully micrografted plants were transferred to soil in large earthen pots and produced flower; (e-f) Pod formation from the micrografted plants and development of mature pod after 60 days of culture.

Fig. 46 (a-c): Histological analysis of micrografted plants a) Arrow indicates grafted region which is captured through microscope. b) Anatomical section of micrografted region c) Anatomical section of control plant.

4.3.2 *Agrobacterium*-mediated genetic transformation using pBI121GUS-NPTII gene construct

In the present study genetically engineered *Agrobacterium* strain LBA4404 carrying pBI121GUS-NPTII gene construct were used which contains *GUS* gene as screenable marker and *npt* II gene as selectable marker conferring kanamycin resistance. Marker gene was used to investigate the transformation ability of CADE and CN explants of two mungbean varieties. Results obtained from the optimization of different parameters for *Agrobacterium*-mediated transformation are presented under the following headings.

4.3.2.1 Optimization of different parameters influencing transformation efficiency

Agrobacterium-mediated genetic transformation efficiency has been reported to be influenced by several factors such as optical density (O.D.) of *Agrobacterium* suspensions, incubation and co-cultivation periods for the explants. Optimizations of these conditions were done by monitoring transient expression of the *GUS* reporter gene after co-cultivation of explants with *Agrobacterium* strain LBA4404.

4.3.2.2 Optimization of optical density (O.D.), incubation periods and co-cultivation period of CADE explants in BARI mung-3 and Binamoog-5 varieties through genetic transformation.

One of the most important influencing factors of transformation is optical density of *Agrobacterium* suspension. Overnight grown *Agrobacterium* suspension was used to infect the CADE and CN explants of two mungbean varieties for genetic transformation. The various ranges of optical density (0.4-1.2) were used for this experiment. The optical density 0.6 was found to be the most effective to achieve maximum amount of transformation at the rate of 72 % and 80% in case of CADE explants for Binamoog-5 and BARI mung-3 varieties respectively (Table 13). In case of CN explants, the maximum rate of transformation was 66% and 70% for Binamoog-5 and BARI mung-3 varieties respectively (Table 14). It was also observed that bacterial overgrowth was occurred when CADE and CN explants were co-cultured with an optical density of 1.0 or more than 1. Therefore, bacterial suspension with an optical density of 0.6 was used for all the transformation experiments to avoid bacterial overgrowth in the regeneration medium. Randomly selected explants were subjected to *GUS* analysis and the *GUS* expression in the CADE and CN explants showed blue color whereas control explants showed no blue color (Fig. 47).

Table 13. Influence of optical density (measured at 600 nm), incubation period and co-cultivation period on transformation efficiency of CADE explants of BARI mung-3 and Binamoog-5 variety.

Parameters		Number of explants assayed for GUS activity	BARI mung-3		Binamoog-5	
			Number of explants +ve for GUS assay	% of GUS +ve explants	Number of explants +ve for GUS assay	% of GUS +ve explants
Optical density (600 nm)	0.4	25	10	40	8	32
	0.6	**25**	**20**	**80**	**18**	**72**
	0.8	25	16	64	16	64
	1	25	15	60	14	56
	1.2	25	13	52	11	44
Incubation Period (min)	20	25	10	40	9	36
	30	**25**	**21**	**80**	**19**	**72**
	40	25	18	84	15	80
	60	25	14	88	14	88
Co-cultivation period (Days)	2	25	13	52	12	48
	3	**25**	**20**	**80**	**18**	**72**
	4	25	16	88	14	80

Incubation period in *Agrobacterium* suspension was another important parameter where the two explants were incubated for a definite period to allow the bacteria to infect the plant tissues. In this set of experiment, the effect of different incubation periods was investigated. For this purpose, different incubation periods, such as 20, 30, 40 and 60 minutes were applied using bacterial suspension with a constant optical density of 0.6 in case of two types of explants. Two of the explants showed maximum GUS positive expression when the explants were incubated for a period of 30 minutes in *Agrobacterium* suspension. It was observed that bacterial overgrowth was visualized in culture media when explants were incubated for more than 30 minutes in *Agrobacterium* suspension. Therefore, the optimum incubation period was found at 30 minutes for the two explants (Table 13).

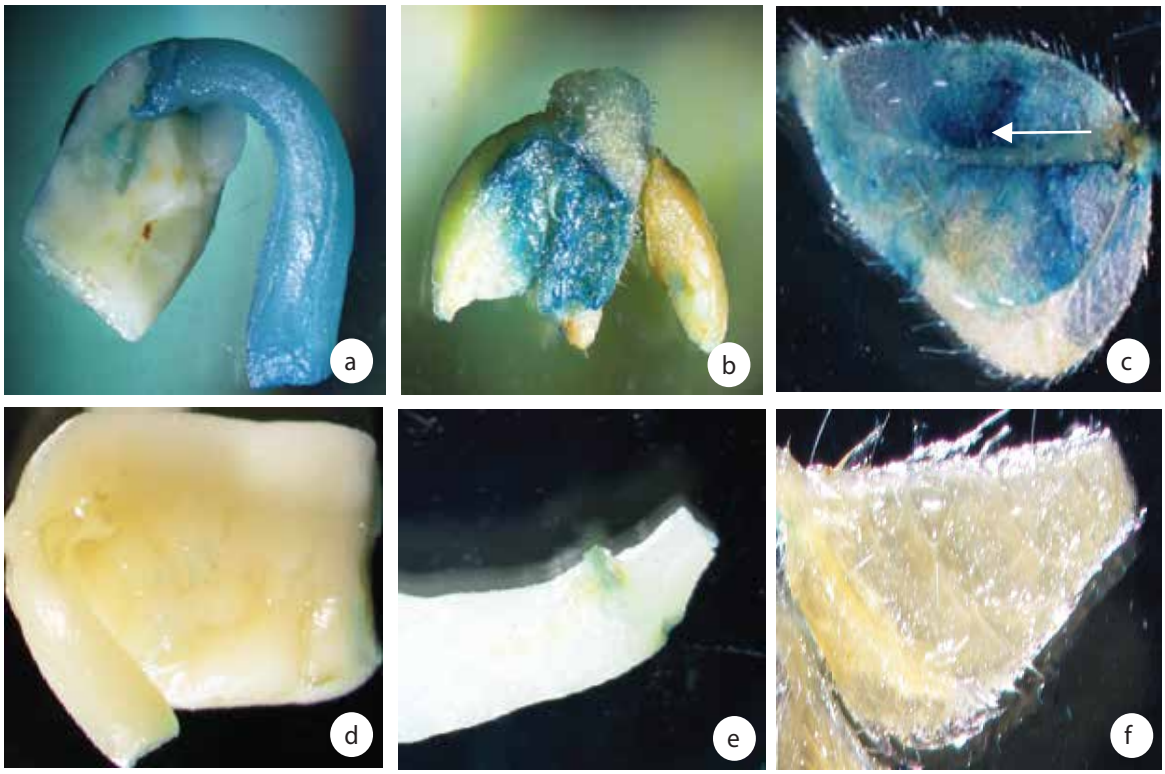


Fig. 47 (a-f): Histochemical GUS expression of infected explants with Agrobacterium strain LBA4404 containing pBI121GUS-NPTII plasmid of BARI mung-3. (a-c) Histochemical localization of GUS activity in CADE , CN and leaf explants showing conspicuous blue color (arrow indicated); (d-f) The absence of GUS activity in control explants showing no blue color.

Co-cultivation period of explants is another important factor which influences transformation efficiency. In this experiment the transformed explants were co-cultivated with 2, 3 and 4 days to obtain specific time period for sufficient transformation of explants. It was observed that the transformation efficiency was found to be variable for different periods of co-cultivation of the explants (Table 13). In these experiments optical density of *Agrobacterium* suspension was maintained at optical density of 0.6. A period of 3 days of co-cultivation period showed the optimum transformation efficiency determined by GUS assay. However, percentage of transformation could be increased with the increase of co-cultivation period but longer co-cultivation period (more than 4 days) produced bacterial over-growth on the co-culture medium. This type of bacterial over-growth was not suitable for survival of co-cultured explants. Therefore, co-cultivation period of 3 days was found to be the most suitable when transformation experiment was performed under optimum condition (Table 13).

4.3.2.5 Responses of the CADE and CN explants of BARI mung-3 and Binamoog-5 varieties towards *Agrobacterium*-mediated genetic transformation

Transformation ability of the two explants, namely, CN and CADE of Binamoog-5 and BARI mung-3 variety was monitored through the expression of the *GUS* gene following incubation for a period of 30 minutes with *Agrobacterium* strain LBA4404 containing vector construct pBI121GUS-NPTII having an O.D. of 0.6 and co-cultivation for 3 days. GUS expression was detected by histochemical assay and such expression was characterized by the formation of indigo blue color within the transformed cells of the infected 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 14. From the Table 14, it can be stated that CADE showed the best response towards transformation that was 72 % and 80 % in case of Binamoog-5 and BARI mung-3 varieties respectively. Whereas the percentage of *GUS* positive explants was 66% and 70% from CN explants in case of Binamoog-5 and BARI mung-3 varieties respectively.

Table 14. Responses of CADE and CN explants of Binamoog-5 and BARI mung-3 varieties of mungbean towards genetic transformation.

Variety	Explant	No. of explants assayed for GUS	No.of GUS+ve explants	% of GUS+ve explants
Binamoog-5	CN	200	133	66.00
	CADE	200	144	72.00
BARI mung-3	CN	150	106	70.00
	CADE	**200**	**155**	**80.00**

4.3.2.6 Influence of acetosyringone concentrations on genetic transformation

Acetosyringone is a compound that induce the *vir* gene expression of *Agrobacterium* and facilitates the transformation process. Acetosyringone is secreted from the wounded portion of dicotyledonous plants that induces *Agrobacterium* to infect the wounded region and gene transferred from the bacterial Ti plasmid to the plant genome. In the current investigation, addition of acetosyringone in the bacterial suspension and co-culture media was found to have direct impact over transformation. Effect of different concentrations of acetosyringone (50 µM, 100 µM, 150 µM and 200 µM) was investigated in the current study. It was observed that the transformation efficiency was increased with the increased acetosyringone concentration in the bacterial suspension and co-culture media up to a certain limit 100 µM concentration (Table15), when a constant optical density of bacterial suspension (OD_{600} 0.6), incubation period (30 min) and co-cultivation periods (3 days) were maintained. Over that limit, depletion in transformation efficiency was observed.

Table 15. Effect of different concentrations of acetosyringone on transformation efficiency in CADE explants of BARI mung-3 variety.

Concentrations of acetosyringone (µM)	Number of explants infected	No. of GUS positive explants	% of GUS positive explants
0.0	40	28	70
50	40	32	80
100	**40**	**36**	**90**
150	40	30	75
200	40	26	65

4.3.2.7 Determination of optimum kanamycin concentration for selection of *in vitro* regenerated control shoots

Agrobacterium strain LBA4404 harboring pBI121GUS-NPTII plasmid contained *nptII* gene conferring resistance to kanamycin. Therefore, kanamycin was used as selective agent during the transformation experiments with that *Agrobacterium* strain. For this purpose, it was necessary to determine the optimum selection level using kanamycin for elimination of the non-transgenic shoots, *in vitro* regenerated control shoots were subjected to selection pressure. The regenerated control shoots were transferred to suitable regeneration medium containing various concentrations of kanamycin. The concentration of kanamycin was increased gradually from 50 mg/l to 200 mg/l in the following manner 50, 100, 150 and 200 mg/l kanamycin. Due to the effects of kanamycin concentration the shoots first became albino and finally died. From this experiment, it was revealed that with the increase of kanamycin concentration, the percentage of survived shoots were found to decrease. The effects of different concentrations of kanamycin on *in vitro* regenerated control shoots have been shown in Fig. 48 a, b, c, d. In this study it was observed that most of the control shoots started to became albino at 50 mg/l kanamycin and finally all control shoots died in presence of 200 mg/l kanamycin within 15 days of inoculation (Fig. 49). Therefore, shoots developed from infected explants surviving in regeneration medium with 200 mg/l kanamycin for more than 15 days were considered to be putatively transformed.

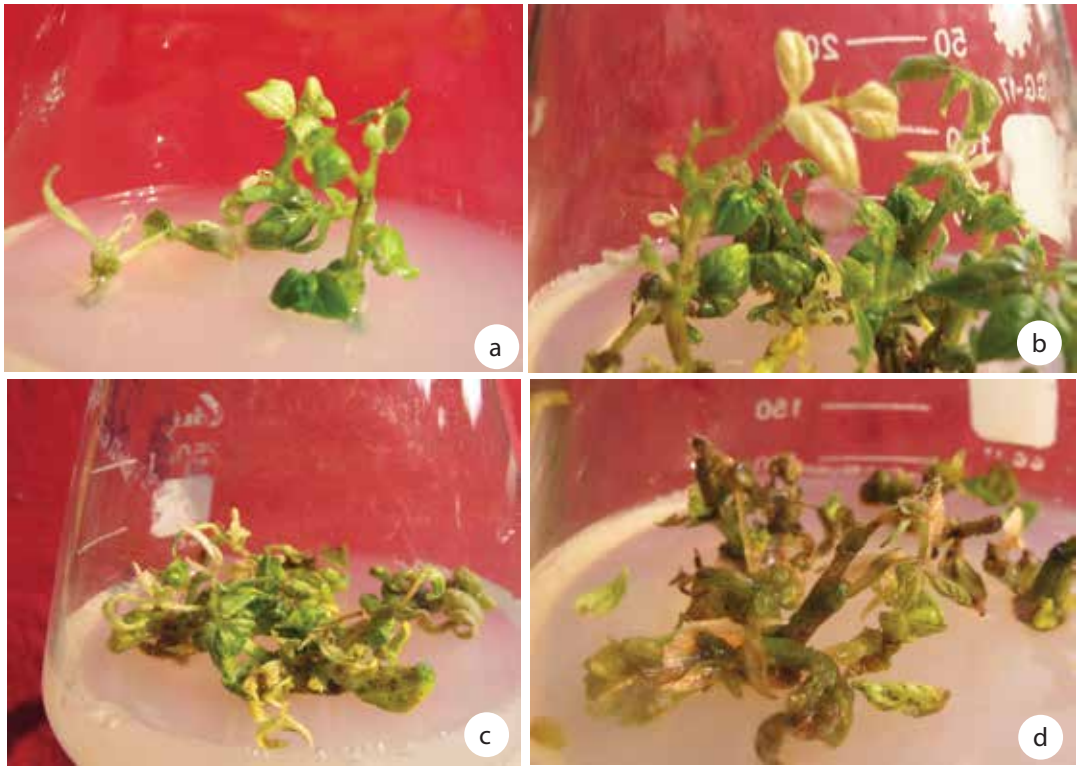


Fig 48 (a-d): Determination of optimum kanamycin concentration for selection of *in vitro* regenerated control shoots in BARI mung-3 . (a-b) *In vitro* regenerated control shoots became albino at 50 and 100 mg/l kanamycin concentration; (c-d) All control shoots were died at 150 & 200 mg/l kanamycin concentration.

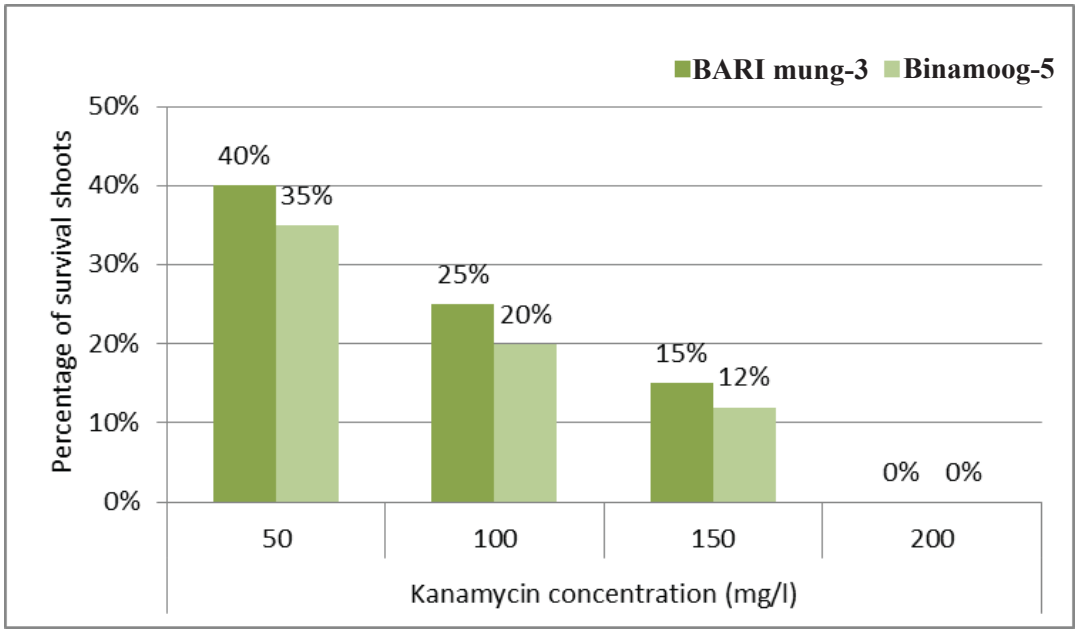


Fig. 49 : Effect of kanamycin concentration on control shoots (non infected) from cotyledon attached decapitated embryo of two mungbean varieties.

4.3.3 *In vitro* shoot regeneration following infection with *Agrobacterium* containing pBI121*GUS*-NPTII gene construct

To obtain *in vitro* regenerated shoots, co-cultivated explants were washed with 300 mg/l ticarcillin for 10 minutes and then transferred to suitable regeneration medium (MSB_5 medium supplemented with 5.0 μM BAP and 100 mg/l ticarcillin). Shoot regeneration medium supplemented with 100 mg/l ticarcillin were used for the elimination of all unwanted bacteria. Selection pressure was not applied immediately after co-cultivation, instead the explants could regenerate (minimum for 15 days) in regeneration medium without the selection pressure. It was observed that if selective agent (kanamycin) was applied immediately after co-cultivation the *Agrobacterium* infected explants did not show further regeneration and in most of the cases the explants failed to survive. When the regenerated shoots were elongated up to 0.2 - 0.5 cm in length the newly developed shoots were transferred to the medium containing kanamycin as selectable agent. In each set of experiment, *in vitro* regenerated control shoots were also maintained to perform various comparative studies between transformed and non-transformed tissues as well as plantlets.

4.3.3.1 Selection of putatively transformed shoots and elimination of non-transformed shoots

For successful transformation a proper and efficient selection procedure is required for obtaining desired transformed shoots. To select the putatively transformed shoots, initially 50 mg/l kanamycin was used as a selection pressure. After 14 days, only green and healthy shoots were sub-cultured on fresh regeneration medium with 100 mg/l kanamycin. Then the kanamycin concentration was raised gradually up to 150 mg/l and then 200 mg/l. During each sub-culture, 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. Finally, those shoots that survived on this selection medium (regeneration medium with 200 mg/l kanamycin) for more than 15 days and remained green and healthy were selected as putative transformed shoots. It was observed that, only few of the CN explants derived shoots were able to survive in presence of higher concentration (200 mg/l) of kanamycin, whereas a few CADE explants derived shoots were recovered after 200 mg/l kanamycin selection. It was observed that all control and non-transformed shoots became albino at 200 mg/l kanamycin concentration and putatively transformed shoots survived at 200 mg/l kanamycin (Fig. 50). A total of 18 kanamycin resistant shoots out of approximately 1000 infected explants in case of Binamoog-5 and 22 out of 1020 in case of

BARI mung-3 were recovered in final selection medium with LBA4404 strain of *Agrobacterium* containing binary plasmid pBI121. Therefore, the frequency of recovery of putative transformed shoots was about 1.8% in case of Binamoog-5, and 2.2% in case of BARI mung-3 varieties (Table 16).

Table 16. Effect of kanamycin on the selection of putatively transformed shoots developed from CN and CADE explants of BARI mung-3 and Binamoog-5 transformed with *Agrobacterium* strain LBA4404 harboring construct I (pBI121GUS-NPTII).

Variety	Explant	No. of explants infected	No. of shoots inoculated	No. of survived shoots in regeneration media with kanamycin different concentrations (mg/l)				% of survived shoots
				50	100	150	200	
Binamoog-5	CN	500	340	210	95	25	6	1.8
	CADE	500	420	232	105	42	12	
BARI mung-3	CN	500	360	218	98	32	7	2.2
	CADE	520	470	245	112	52	15	

CN = Cotyledonary node, CADE = Cotyledon attached decapitated embryo

4.3.3.2 GUS histochemiocal Assay

Parts of leaf were collected from the regenerated putatively transformed mungbean plants and were incubated in GUS staining buffer containing 1mM 5-bromo-4-chloro-3-indolyl β-D-glucuronidase. Following their incubation at 37°C for 2 days visualization of blue colour of GUS expression revealed under stereomicroscope (Fig. 51 a, b). A part of the macerated tissue of explants was also observed under microscope and transformed cells showed blue color and control explants of mungbean showing absence of blue colour (Fig. 51 c, d).

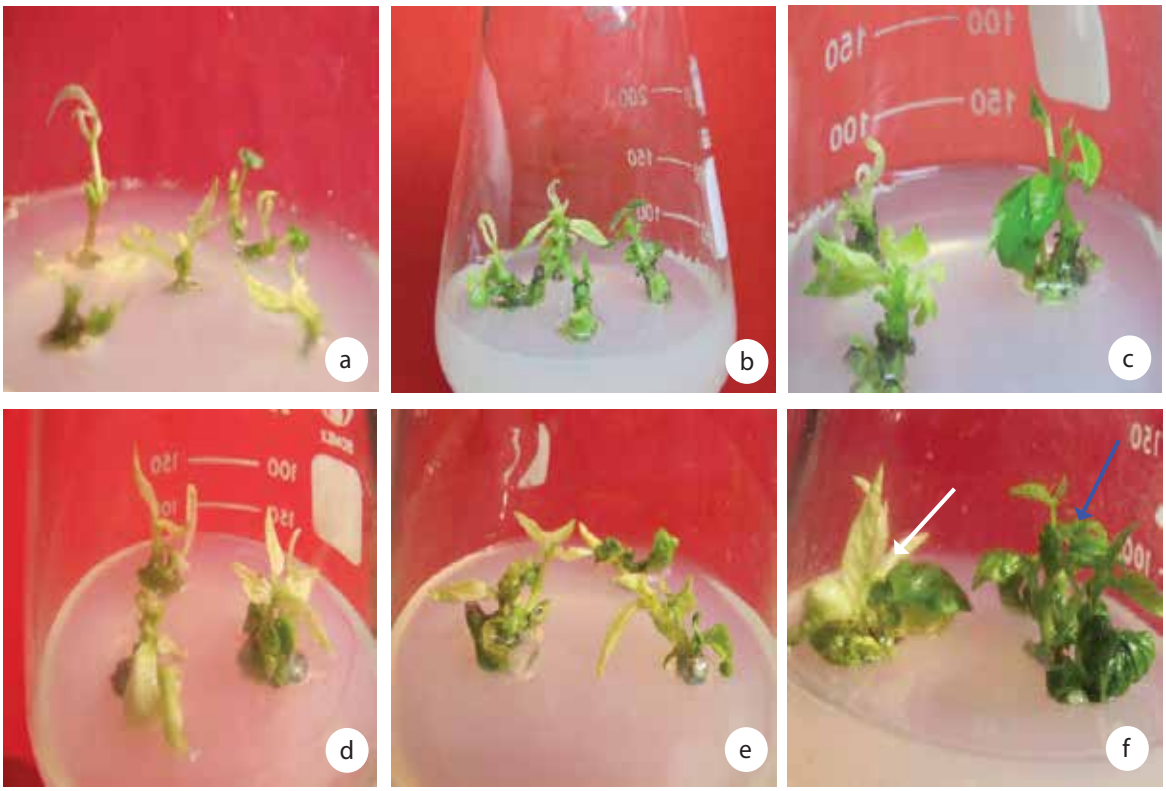


Fig. 50 (a-f): Selection of putatively transformed shoots from CN and CADE explants using LBA4404/pBI121GUS-NPTII construct in BARI mung-3. (a) in case of CN explants, all control shoots became albino after 150 mg/l kanamycin concentration as selection pressure; (b-c) Putatively transformed shoots survived at 150 mg/l kanamycin concentration as selection pressure, where as non-transformed shoots became albino at the same kanamycin selection pressure; (d) In case of CADE explants, all control shoots became albino at 150 mg/l kanamycin concentration; (e-f) Putatively transformed shoots survived at 150 mg/l kanamycin concentration (arrows indicated blue color), where as non infected regenerated shoots became albino at the same medium.

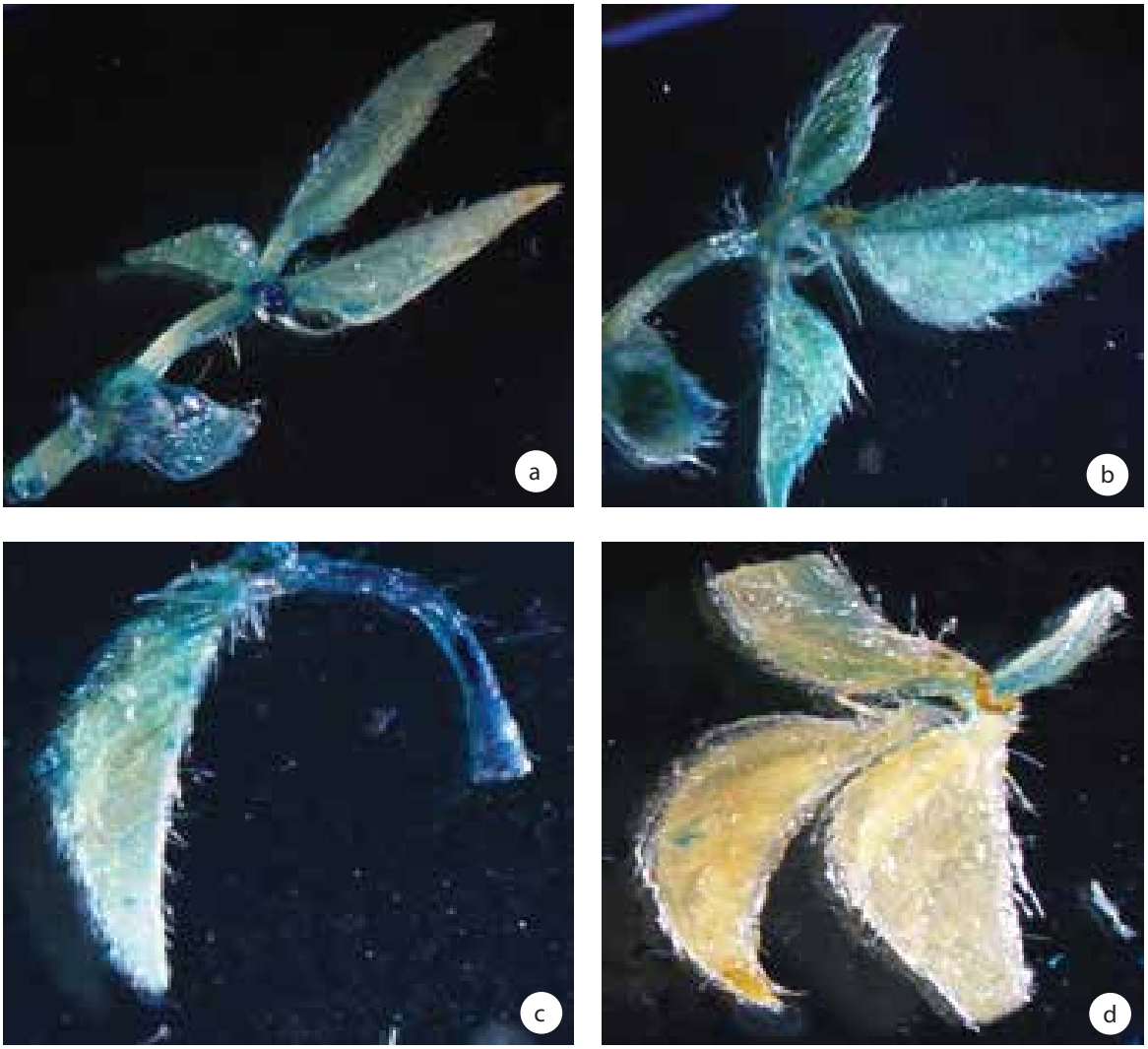


Fig. 51 (a-d): Histochemical localization of GUS expression in different parts of putative transformed shoots from CADE explants in BARI mung-3. (a-c) Expression of GUS gene in developing shoots and leaves showing conspicous expression of blue color all over the shoots and leaves; (d) Note that, the control shoot without the expression of blue color.

4.3.3.3 Analysis of transgene integration

Transgene integration was confirmed through PCR analysis by amplification of *GUS* and *npt*II genes present within the genomic DNA of the transformed plantlets of mungbean. For this purpose, total genomic DNA was isolated from the leaf of putatively transformed shoots as well as control shoots (Fig. 52 a). Plasmid DNA from genetically engineered *Agrobacterium* construct pBI121GUS-NPTII was isolated to use as positive control during PCR analysis. Specific primers were used for the amplification of *GUS* and *npt*II genes. The amplified DNA was analyzed through agarose gel electrophoresis. This observation indicated the presence of transgene within the DNA of transformed shoots. Among 24 shoots, only 6 shoot showed identical bands to positive control in case of *npt II* gene and only one shoot showed band identical to positive *GUS* gene used as positive control (Fig. 52 b, c).

4.3.4 *Agrobacterium*-mediated in planta genetic transformation

Mungbean exhibited recalcitrant nature towards *in vitro* regeneration as well as *Agrobacterium*-mediated genetic transformation. *Agrobacterium*-mediated genetic transformation has been reported to be mostly hampered due to the absence of an effective and reproducible *in vitro* regeneration system. Under these circumstances, in planta genetic transformation was carried out using *Agrobacterium* strain LBA4404 containing pBI121GUS-NPTII gene construct. These experiments were conducted to develop a protocol for in planta genetic transformation in mungbean. BARI mung-3 was found to be more susceptible to mungbean yellow mosaic virus disease therefore, BARI mung-3 was chosen as the plant material. Overnight water soaked sprouted seeds were used as material during in planta genetic transformation. Optimization of incubation period and vacuum infiltration were carried out for in planta genetic transformation.

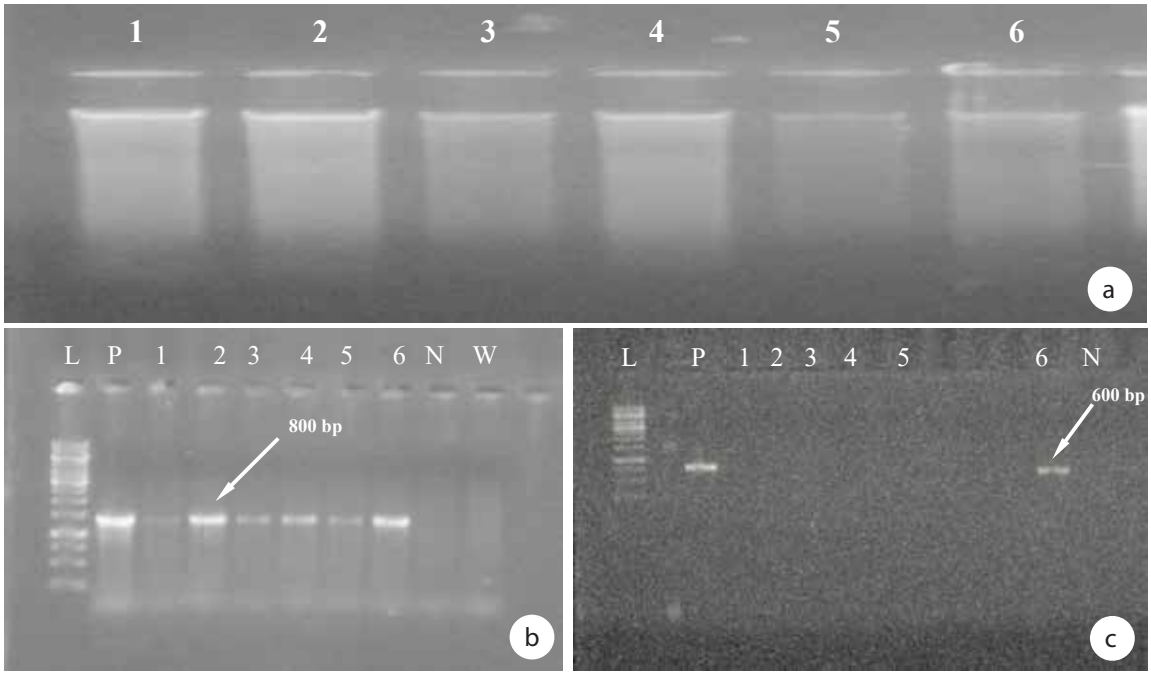


Fig. 52 (a-c): Genomic DNA isolation and PCR confirmation of *nptII* and *GUS* gene in putatively transformed plants of BARI mung-3 using gene specific primers. (a) Electrophoretic seperation of genomic DNA isolated from six putatively transformed shoots; (b) PCR amplification of *nptII* gene; lane L refers to 1 Kb DNA ladder, lane 1-6 showing amplified positive bands of transformed shoots, which is identical to the positive control (lane P); lane N showing non transformed shoots and lane W water control (c) PCR amplification of *GUS* gene in lane 6 showing amplified band which is identical to the positive control (lane P), lane N-non showing transformed shoot.

4.3.4.1 Optimization of incubation period for *Agrobacterium*-mediated in planta genetic transformation

The whole embryo (Fig. 53 a) was injured at the embryonal axis point with a sterile 0.5 ml syringe and incubated in bacterial suspension 0.6 O.D. in MSB_5 medium for different incubation periods (15, 30, 45, 60 and 120 mins). It was observed that, the transformation efficiency increases with the increasing duration of incubation period (Table 17). The duration of incubation period was found to have a direct impact on the transformation efficiency. But long-time incubation of the embryo to the bacterial suspension enhanced bacterial overgrowth and softening of the cells that reduce the capacity of further growth of the plants. Considering these facts, an incubation period of 60 min was found to be more suitable which was validated through GUS assay. (Table 17).

Table 17. Optimization of incubation period for in planta genetic transformation

Incubation Period (min)	No. of explants assayed for GUS assay	No. of explants showed GUS positive response	% of GUS positive response
15	75	7	9.33
30	75	15	20.0
45	75	18	24
60	**75**	**30**	**40**
120	75	33	44

4.3.4.2 Optimization of vacuum infiltration period during the in planta genetic transformation

The in planta genetic transformation was mostly affected by the duration of vacuum infiltration and it was observed that it had a direct impact on the in planta genetic transformation. The infected embryos were then subjected to vacuum infiltration for several periods of durations (0.5, 1, 2, 3, 4 and 5 mins) at Optical Density 0.6. It was observed that 2 mins of vacuum infiltration increased the transformation efficiency up to 15 %. Vacuum infiltration for more than 2 mins was found to cause depletion on transformation efficiency (Table 18).

Table 18. Effect of vacuum infiltration on in planta genetic transformation

Vacuum Infiltration Duration (mins)	No. of explants assayed for GUS assay	No. of explants showed GUS positive response	% of GUS positive response
0.0	75	21	34
0.5	75	27	42
1	75	36	48
2	**75**	**39**	**55**
3	75	30	40
4	75	18	24
5	75	12	16

4.3.4.3 In planta genetic transformation in BARI mung-3 using pBI121GUS-NPTII vector construct

The young embryo was inoculated with *Agrobacterium* suspension (O.D. 0.6) along with 100 μM acetosyringone. The needle was dipped in the *Agrobacterium* suspension and pinched on the top of young embryo. Injured whole embryo were subjected to vacuum infiltration for 2 mins and then these vacuum infiltrated embryos were incubated for 60 minutes in *Agrobacterium* culture. After incubation, the embryos were washed 3-4 times with autoclaved sterile distilled water and dried on a filter paper. Then the embryos were cultured on ½ MSB_5 medium devoid of any kind of hormone (Fig. 53 b). After 15 days, the well rooted plants were transferred to small plastic pot for proper hardening (Fig. 53 c, d). The survived plants were transferred to earthen pots until the formation of flowers and pods (Fig. 53 e, f).The pod produced mature seeds after 55 days of *Agrobacterium* infection (Fig.53 g).

4.3.4.4 Analysis of GUS expression of the putatively transformed plants

Leaves and roots of the in planta transformed plants were collected and analyzed for GUS histochemical assay to check whether the gene had successfully integrated into nuclear genome of the plant or not. The experimented parts of the in planta transformed plants showed GUS positive blue color where the control plant parts had no GUS positive response (Fig. 54 a, b). On the other hand, chimeric expression of the GUS gene was detected on leaf and root tissues of some plants through stereomicroscopic observation (Fig. 54 c, d, e, f). The T_0 seeds were germinated and developed T_1 plants and randomly collected 100 T_1 plants were subjected to PCR but no PCR positive bands were found within the T_1 plants were detected using the marker gene. Therefore, no further in planta genetic transformation experiments were carried out with the antiviral construct (pBI121CP-AC2).

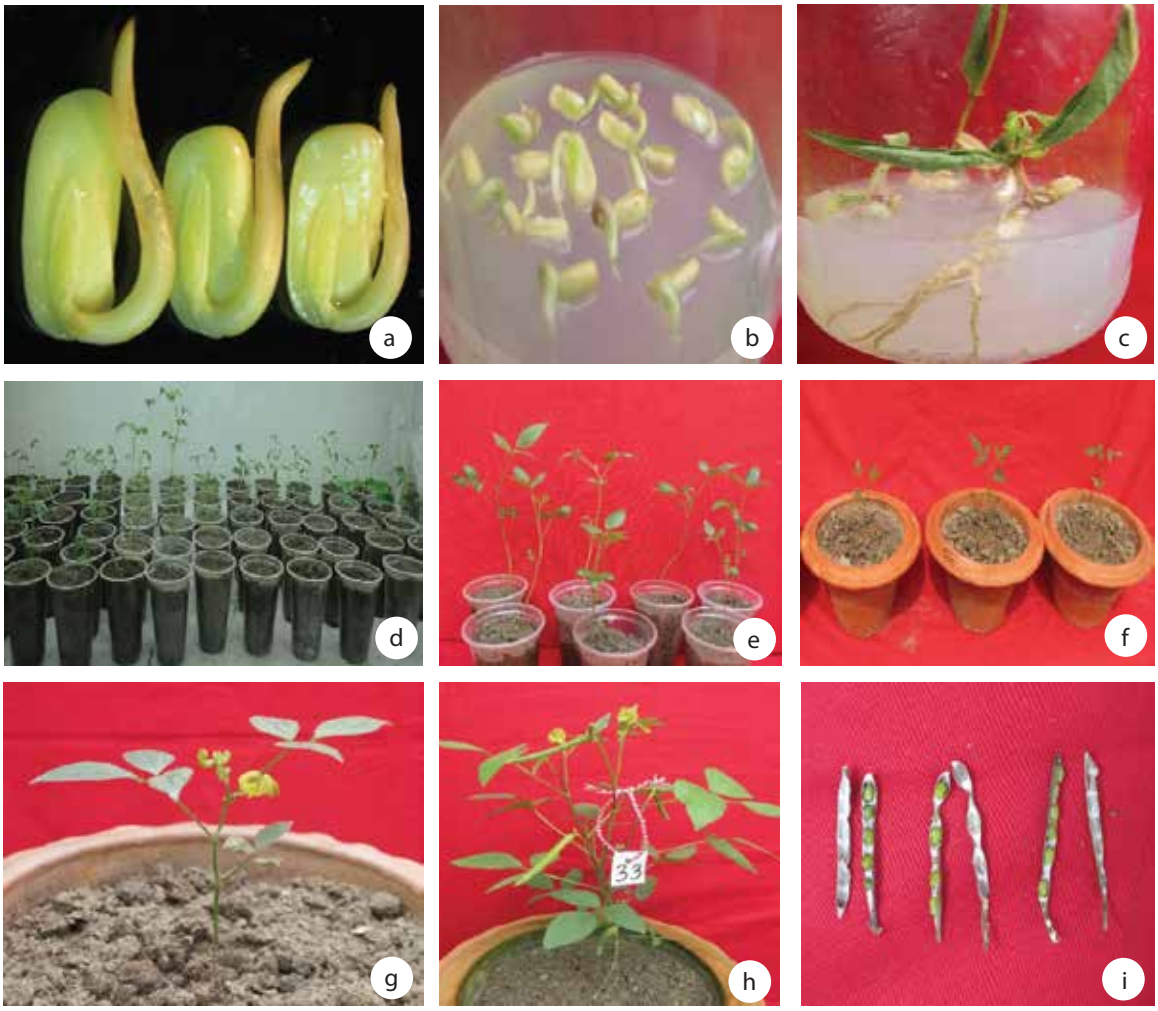


Fig. 53 (a-i): Development of in Planta genetic transformation technique from overnight germinated whole embryo in BARI mung-3 transformation with *Agrobacterium* strain LBA4404/pBI121GUS-NPTII. (a) Fluorescent microscopic view of whole embryo from overnight germinated seeds; (b) The infected whole embryo were cultured on ½ MSB_5 media without any growth regulators; (c) Formation of well developed roots from in vitro germinated plants on 1\2 MSB_5 media without any rooting hormone; (d-e) Infected plants were transferred to soil containing small plastic pots for hardening; (f) hardened plants were transferred to larger earthen pots containg soil and maintained at double net house for biosafety regulation; (g-i) In vivo flower and pod formation from the putative transformants and fully developed mature pod.

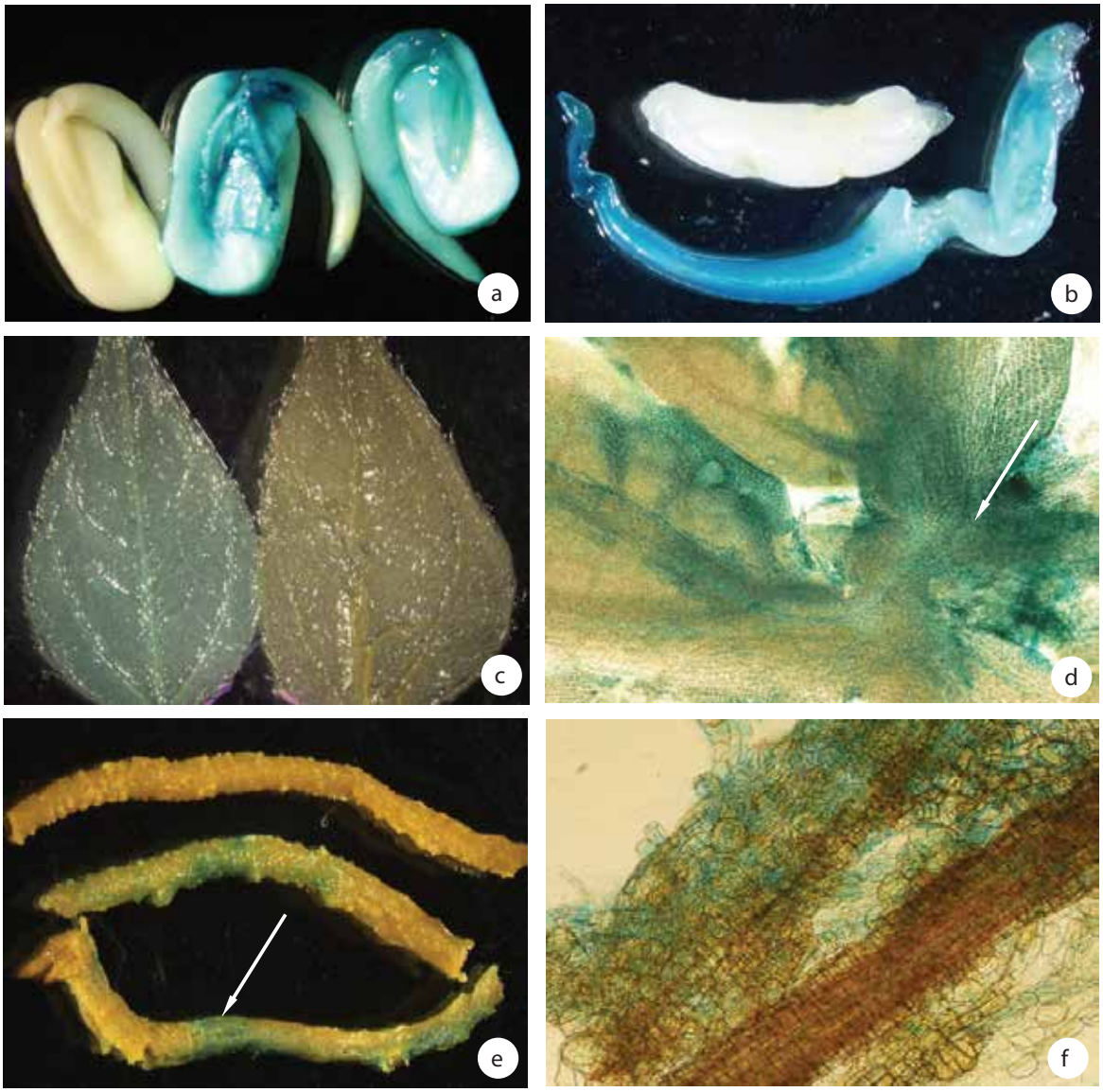


Fig. 54 (a-f): Expression of gus gene in whole embryo, leaf and root tissues of the successfully putative transformed plants. (a-b) Successfully putatively transformed whole embryo showing positive blue colour along with control explant showing negative response in GUS histochemical assay; (c) stereomicroscopic view of the transformed leaf tissue along with control leaf ; (d) light microscopic view of the transformed tissues showing GUS positive blue colour (arrow indicated); (e) Chimeric GUS expression observed in the putatively transformed roots where as control root showing negative GUS expression under stereomicroscope; (f) macerated cells of root tissue from successfully putatively transformed plant showing GUS positive response.

4.4 *Agrobacterium*- mediated genetic transformation into BARI mung-3 variety using pBI121CP-AC2 antiviral construct

The ultimate objectives of this study is to develop yellow mosaic virus resistance mungbean line. Therefore, *Agrobacterium*-mediated genetic transformation was carried out using newly developed antiviral construct pBI121CP-AC2 (section 3.5.4) to integrate coat protein (*CP*) gene and silencing suppressor gene (*AC2*) into the genome of BARI mung-3 variety. In BARI mung-3 variety CADE and CN explants were used for development of transgenic mungbean line. For this purpose, genetic transformation experiments were carried out using the protocol developed by pBI121GUS-NPTII construct.

4.4.1 *In vitro* regeneration and selection of putatively transformed shoots of BARI mung-3 variety using kanamycin selection pressure

After infection with pBI121CP-AC2 antiviral construct the CADE and CN explants were maintained under co-cultivation for three days in co-cultivation medium supplemented with 100 µM acetosyringone. The co-cultivated explants were washed with sterile distilled water containing 300 mg/l ticarcillin for 20 minutes, for the elimination of *Agrobacterium* attached on the surface of the explants. Following this step, the explants were transferred to MSB_5 medium containing 5 µM BAP for the regeneration of shoots (Fig. 55 a). In case of mungbean, selection pressure was not applied immediately after co-cultivation, instead the explants were allowed to initiate regeneration for 15 days in regeneration medium without the selection pressure containing only 100 mg/l ticarcillin. When the multiple shoots elongated up to 0.2 - 0.5 cm in length the explants with the newly developed shoots were transferred to the medium with kanamycin selective agents (Fig. 55 b, c, d). During the study the regeneration of shoots from infected explants were carried out following the regeneration protocol established for both the explants such as CADE and CN. Shoots regenerated from non-infected explants became albino and could not survive in the selection medium since they did not possess the kanamycin resistant gene. The 100 mg/l kanamycin concentration was applied as the first selection pressure then the concentration of kanamycin was increased gradually like 150 mg/l and 200 mg/l. Shoots those remained green, healthy and survived on the medium containing final selection pressure (200 mg/l) for 30 days were maintained as putatively transformed shoots. Elimination of non-transformed shoots and the selection of putatively transformed shoots from CN and CADE explants respectively in the present study have been shown in (Fig. 56 a- f) and (Fig. 57 a - f).

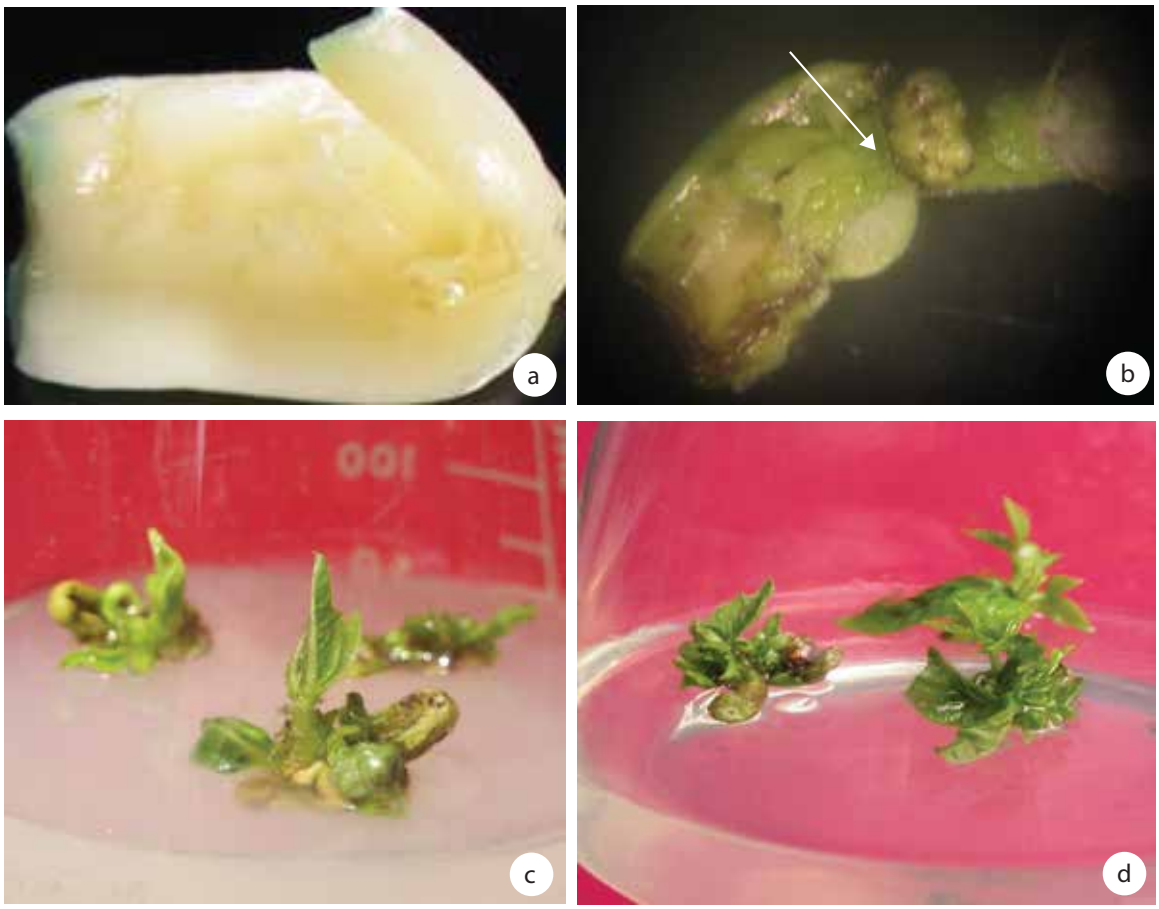


Fig. 55 (a-d): In vitro shoot regeneration and selection of putatively transformed shoots from CADE explants using antiviral construct LBA4404/pBI121CP-AC2. (a) Infected CADE explant on selection free regeneration medium; (b-c) Shoot initiation (arrow indicated) and elongation of shoots from infected explants ; (d) Elongated shoots were transferred to regeneration medium supplemented with 100 mg/l kanamycin as a selection pressure.

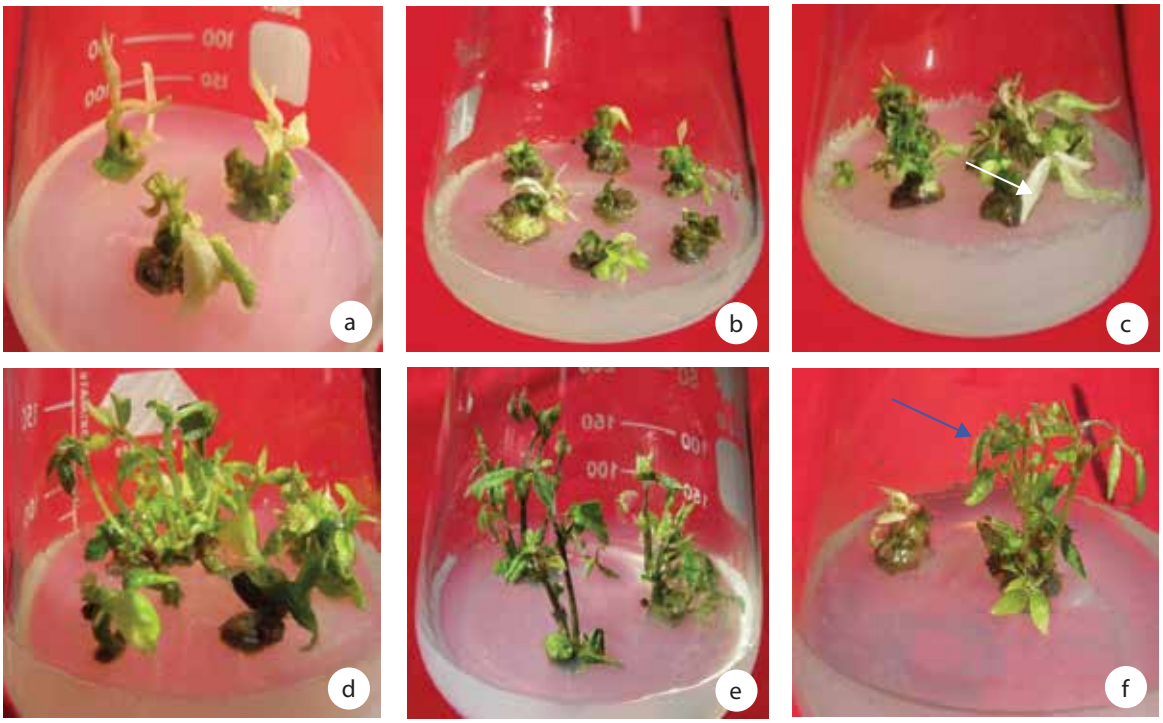


Fig. 56 (a-f): Selection of putative transformants and elimination of non transformed shoots in case of CN explants. (a) Control shoots of BARI mung-3 regenerated from CN explants showing albino (leading to death) following selection pressure 150 mg/l kanamycin concentration; (b-c) Shoots regenerates from infected CN explants cultured on regeneration medium with 100 mg/l kanamycin concentration, the non transformed shoots failed to survive in presence of 100 mg/l Kanamycin selection pressure (arrow indicated); (d-f) Putatively transformed shoots survived on selection medium containing 200 mg/l kanamycin (arrow indicated).

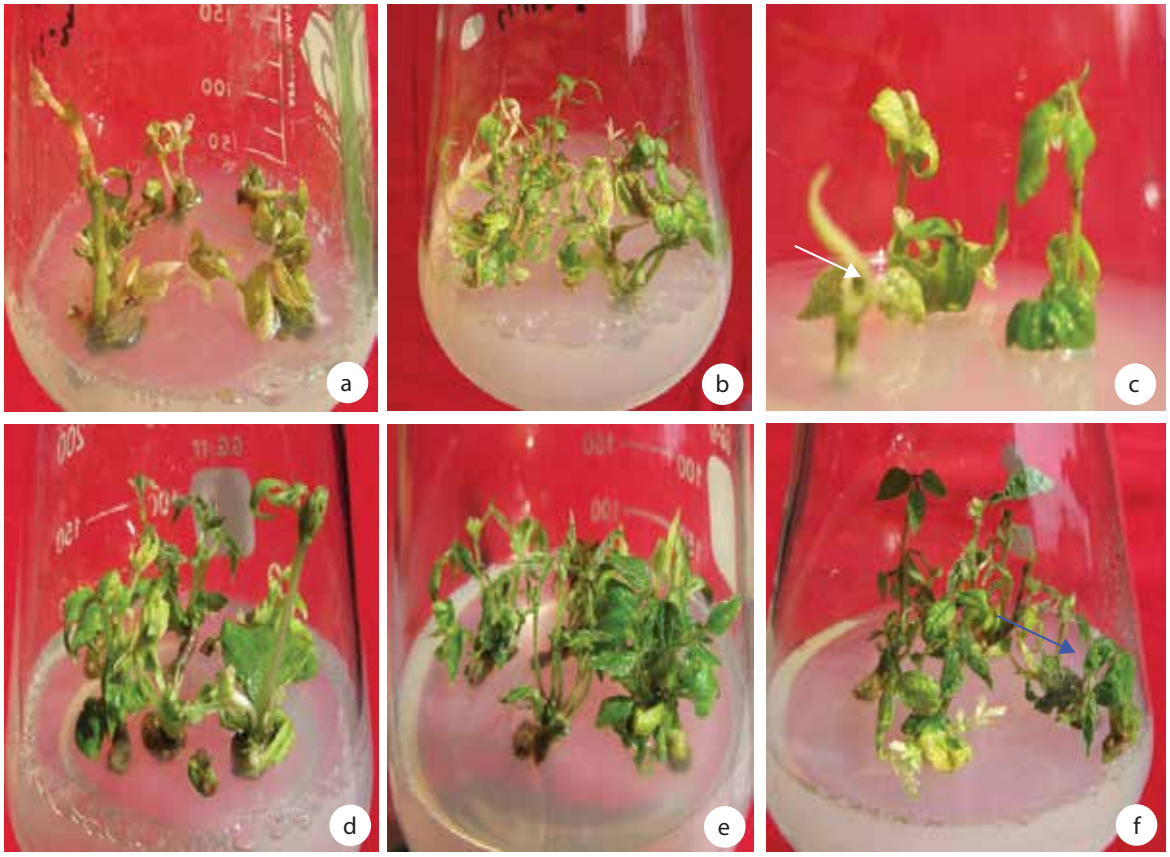


Fig. 57 (a-f): Selection of putative transformants and elimination of non transformed shoots in case of CADE explants. (a) Control shoots of BARI mung-3 regenerated from CADE explants showing albino (leading to death) following selection pressure 150 mg/l kanamycin concentration; (b-c) Shoots regenerates from infected CADE explants cultured on regeneration medium with 100 mg/l kanamycin concentration, the non transformed shoots failed to survive in presence of 100 mg/l Kanamycin selection pressure (arrow indicated); (d-f) Putatively transformed shoots survived on selection medium containing 200 mg/l kanamycin (arrow indicated).

The results of transformation experiment are shown in Table 19. In case of CADE explants, out of approximately 1120 infected explants, 35 shoots were recovered on final selection medium. Therefore, the frequency of recovery of putatively transformed shoots from CADE explants was about 3.1%. In CN explants the frequency of recovery of putatively transformed shoots was about 1.96% (Table 19).

Table 19. Effect of kanamycin on the selection of putatively transformed shoots developed from CN and CADE explants of BARI mung-3 transformed with *Agrobacterium* strain LBA4404 harboring construct II (pBI121CP-AC2).

Explants	No. of explants infected	No. of survived shoots in culture (SEM) with Kanamycin (mg/l)			% of survived shoots
		100	150	200	
CADE	1120	155	75	35	3.1
CN	562	92	45	11	1.96

CADE = cotyledon attached decapitated embryo, CN = Cotyledonary node

4.4.2 Root formation and transplantation of putatively transformed plants to soil

Shoots that survived under the kanamycin selection pressure were separated and sub-cultured in root induction medium containing half strength of MSB_5 with 2.0 µM IBA as well as 50 mg/l ticarcilin. It was observed that the shoots which survived in higher concentration of kanamycin (200 mg/l) showed lower response towards root induction. Altogether 46 shoots survived through selection were subjected for root induction. It was observed that out of 66 shoots 6 of them showed positive responses towards induction of roots. In case of CADE explants 35 shoots survived under selection were subjected for roots induction. Out of 35 shoots 4 of them showed response towards root induction (Fig. 58 a). But no rooted plantlets of CN explants were survived following their acclimatization in soil (Table 20). Among these 6 rooted plantlets 2 rooted plantlets were survived following their transplantation and hardening in soil (Fig. 58 b, c).

Table 20. Root formation and survival rate of plantlets

Explants	No. of shoots for root induction	Days required for root initiation	No of shoots produce root	% of root formation	No. of plants survived after transplantation	% of survival rate
CADE	35	20 - 45	4	11.42	2 (4)	50
CN	11	22-30	2	18.18	Plants failed to survive	0.0

4.4.3 PCR analysis for the amplification of *CP-AC2* genes and efficiency of transformation by CADE explants

Molecular analysis through PCR amplification was performed to confirm the presence of *CP-AC2* genes in putative transformants. The genomic DNA from the putatively transformed shoots along with their control shoots was isolated for the amplification of *CP-AC2* gene through Polymerase Chain Reaction (PCR). Plasmid DNA of pBI121CP-AC2 was used as positive control during PCR analysis. In case of CADE explants out of 35 shoots, only 6 shoots showed positive bands, identical to the band of positive control (Fig. 59). In case CN explants out of 11 shoots no PCR positive shoots were found. Among the 4 rooted plantlets, 2 plantlets showed the PCR positive amplification towards *CP-AC2* gene. The results of PCR analysis and transformation efficiency for the integration of *CP-AC2* gene has been presented in Table 21.

21. Transformation efficiency of BARI mung-3 variety using CADE explants.

No. of infected explants	No. of shoots survived at 200 mg/l kanamycin	No. of PCR + ve plants	% of transformation efficiency
1120	35	6	0.53

4.4.4 PCR for the amplification of *pic* A gene

This experiment was conducted to verify the probable presence of *Agrobacterium* cells in T_0 plants. PCR amplification was performed using *picA* gene specific primers. For the amplification of PCR, 5'-ATG CGC ATG AGG CTC GTC TTC GAG-3' was used as forward primer and 5'-GAC GCA ACG CAT CCT CGA TCA GCT-3' used as reverse primer. Among the six transformed plants no bands were found to amplify after PCR amplification, identical to the band of positive control (Fig. 60). Only band was found to amplify in lane of positive control. Thus, it was concluded that the transformed plantlets did not have any *Agrobacterium* contamination.

4.4.5 Development of T_0 and T_1 transgenic plants

The transformed plantlets of BARI mung-3 were successfully maintained following biosafety protocols in double layered insect proof net house for further analysis. The survived plants were transferred to earthen pots. After 50 days from the date of shoot initiation the plants were started flowering and finally pod formation occurred (Fig 58 d, e, f). After maturity only two seeds were produced in a single pod. (Fig. 58 g). The T_0 seeds were germinated in ½ MSB_5 medium and produce T_1 transgenic plants. The plants were transferred to earthen pots and grow until maturity (Fig 61 a, b, c). The mature plants produced flower, pod and viable seeds (Fig 61 d, e, f). In case of T_1 transgenic plants *CP-AC2* gene amplification were found showing band, identical to the band of positive control (Fig. 62).

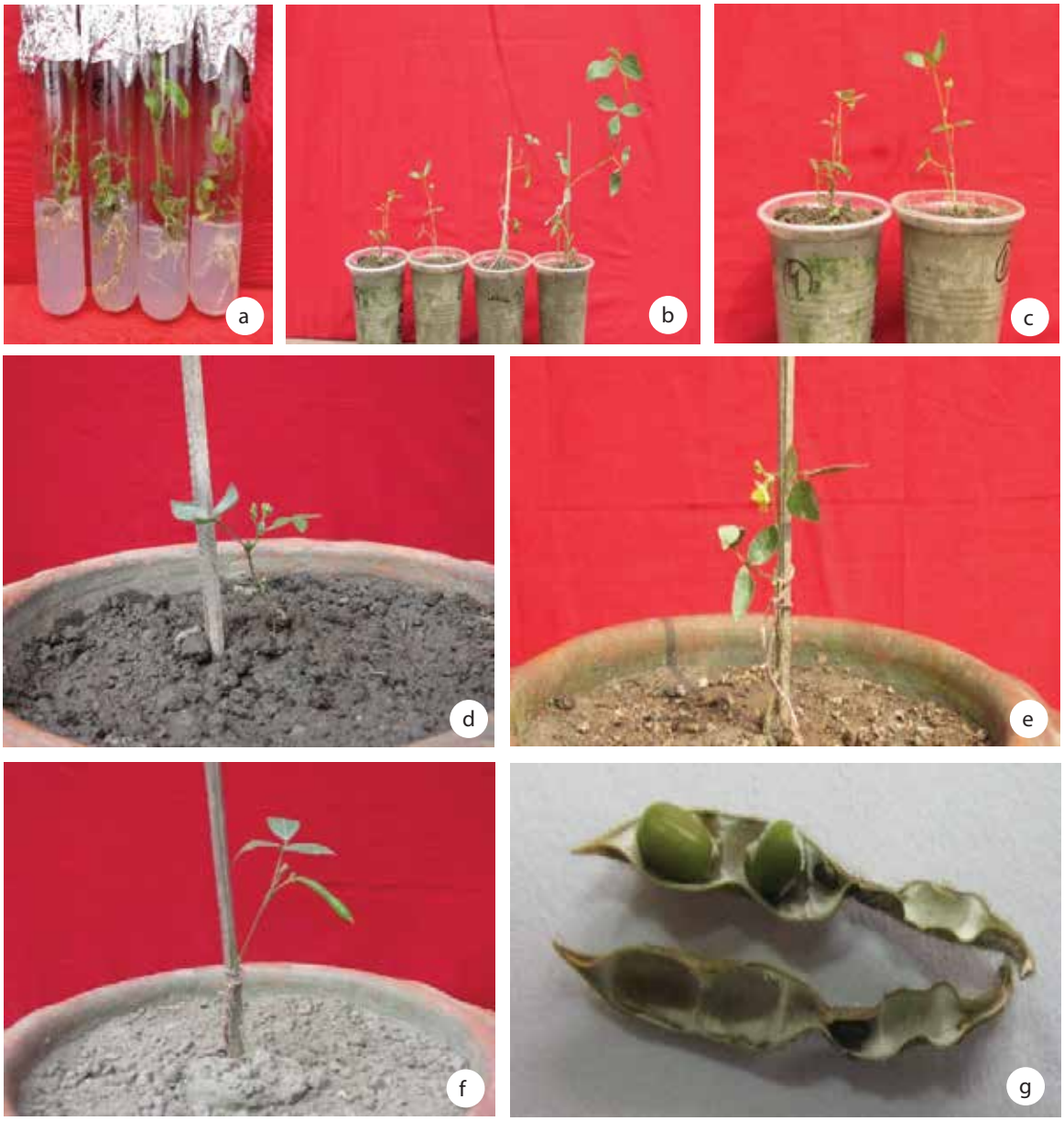


Fig. 58 (a-g): Development of transformed mungbean T_0 progenies. (a) Formation of multiple roots on ½ MSB_5 medium supplemented with 2 µM IBA of BARI mung-3; (b-c) The regenerated plantlets transferred to soil in small plastic pots for hardening; (d-e) Formation of flower in large earthern pots; (f) Mature plants formation of pods after 55 days of transformation; (g) Mature seed formation .

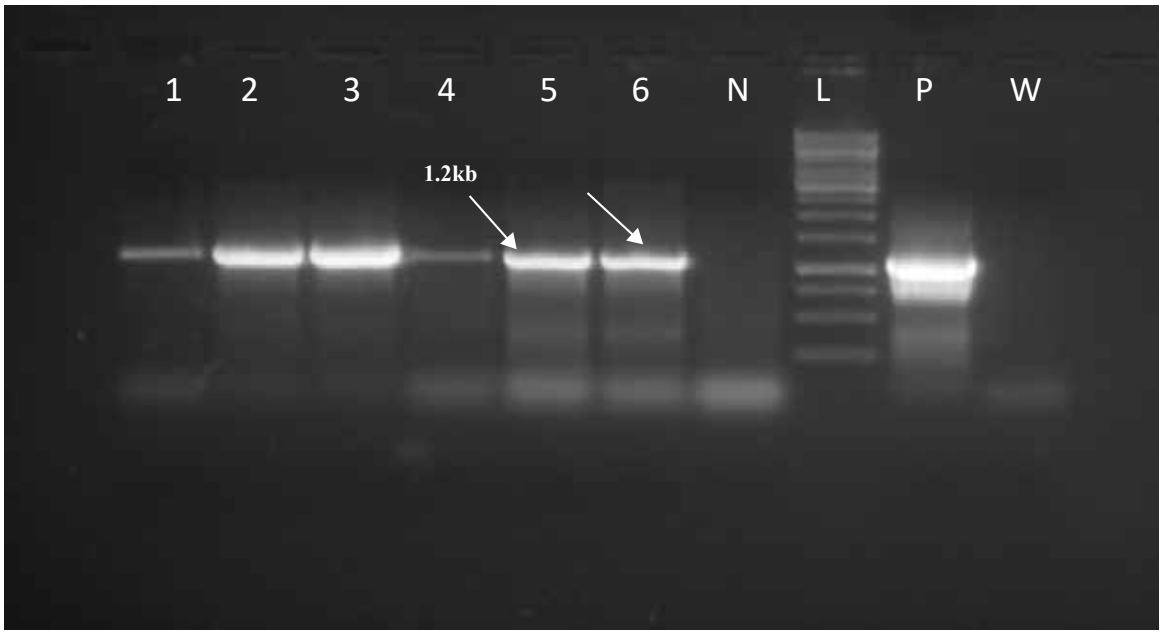


Fig 59: PCR confirmation of the *CP-AC2* gene from six T_0 plantlets of BARI mung-3 ; lane L refers to 1 kb DNA ladder; lane 1-6 produced corresponding bands, identical to the band obtained from positive control lane P; no amplification from negative control and water control (lane N and lane W).

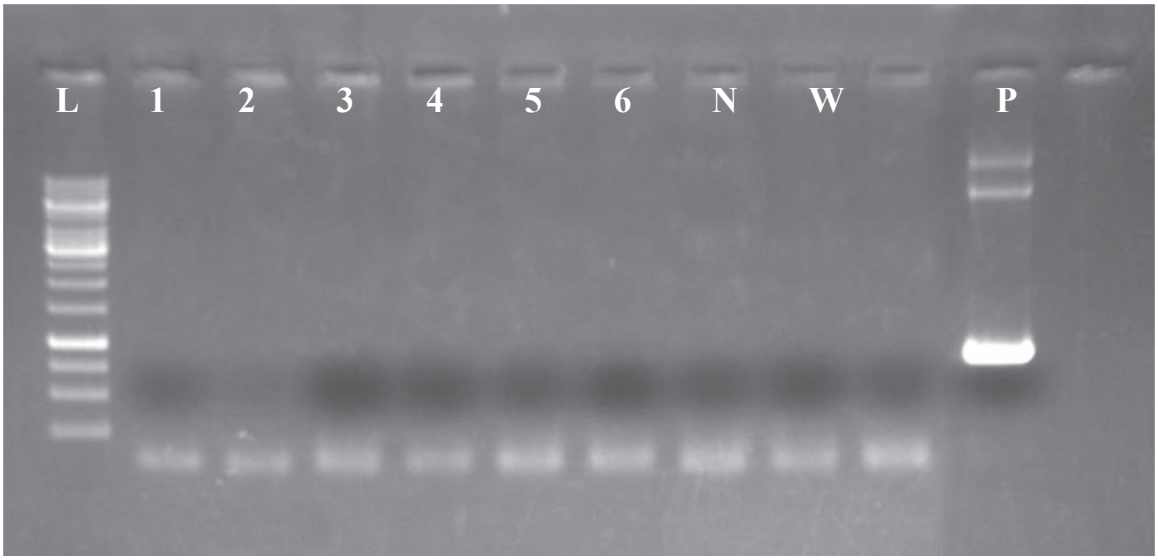


Fig. 60: PCR amplification of *Pic A* gene with pic A primer from six T_0 transformants; lane L refers to 1 Kb DNA ladder, lane 1-6 genomic DNA of transformed shoots, note that no bands were amplified in genomic DNA lane 1-6 after PCR amplification, only one band was found to amplify in lane p (positive control), lane N showing non transformed shoot and lane W showing water control).

Fig. 61 (a-f): Development of T_1 transgenic mungbean plants from the T_0 seed in BARI mung- 3 cultured on MSB_5 medium. (a) T_0 mungbean seed were germinated in MSB_5 medium without any growth regulators; (b) The developed T_1 plantlets were transferred to soil containing larger earthern pot; (c-d) T_1 plant devloped flower and pod after maturity; (e-f) The mature pod produced only one viable seeds after 60 days of culture.

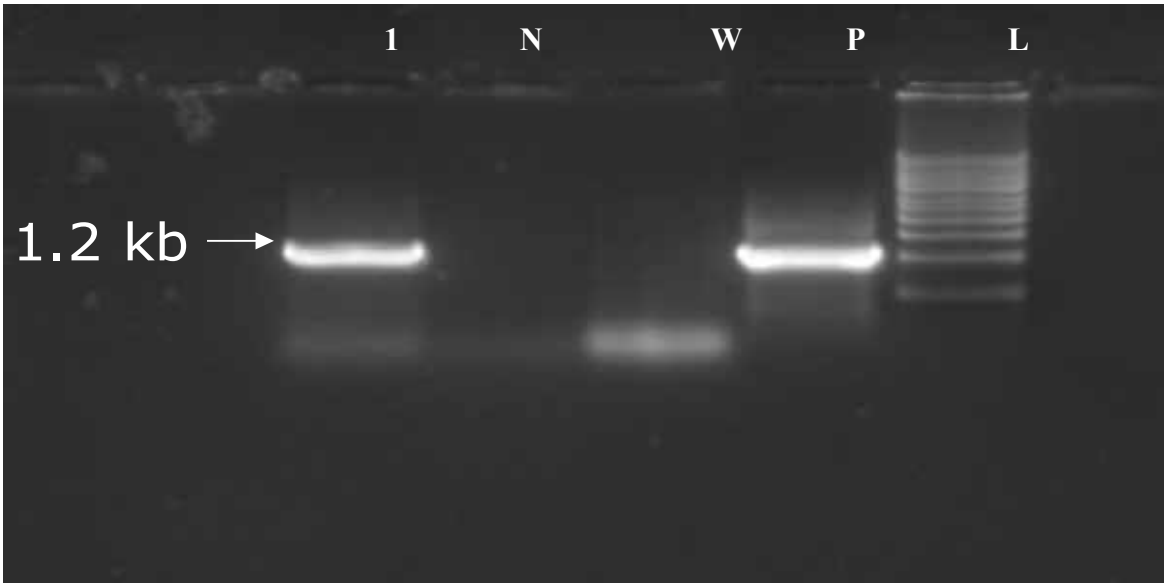


Fig. 62: PCR confirmation of *CP-AC2* gene in T_1 transgenic plants of BARI mung-3 using CP forward and AC2 reverse primer; lane L refers to 1 Kb DNA ladder, lane1 showing amplified band which is identical to the positive control (lane P). lane N and W showing non transformed plant and water control respectively.

5. DISCUSSION

The overall objective of the present investigation was to develop yellow mosaic virus resistant mungbean [*Vigna radiata* (L.) Wilczek] line through genetic transformation. To fulfill the objectives of this investigation several experiments, namely, cloning of the MYMV genome, development of antiviral gene construct (pBI121CP-AC2) and evaluation of the gene expression capacity of pBI121CP-AC2 construct in the model tobacco plant was carried out. Moreover, protocols for *in vitro* regeneration and genetic transformation using a marker gene were developed for two mungbean varieties cultivated in Bangladesh. Finally, insertion of the newly developed antiviral gene construct into desired mungbean plants was carried out and consequently confirmation of its integration in the regenerated mungbean plants was performed through molecular approaches. The results so far obtained through these investigations has been discussed in this chapter.

Currently, there are some yellow mosaic disease tolerant mungbean varieties are available, but a complete yellow mosaic virus resistant mungbean variety has not been produced so far (Nair et al. 2017). Antiviral strategies, e.g. RNAi, has been reported to be an effective methodology to control plant viruses (Gupta and Mukherjee 2019). There are reports of using antiviral strategy in developing transgenic soybean and cowpea plants against MYMIV (Veer et al. 2013, Kumar et al. 2017). Nevertheless, till date no antiviral strategy has been reported to develop transgenic MYMV resistant mungbean variety. Therefore, in the current study a new antiviral gene construct (pBI121CP-AC2) was developed to achieve RNAi mediated resistance against yellow mosaic viruses. This gene construct contains a viral coat protein gene (*CP*) and a silencing suppressor gene (*AC2*) in an antisense orientation under cauliflower mosaic virus 35S (CaMV35S) promoter in a pBI121 binary vector which confer the resistance of mungbean yellow mosaic virus. For the development of a new antiviral construct yellow mosaic virus infected leaf samples were collected based on its characteristic symptoms and to confirm the presence of MYMV infected plants.

Yellow mosaic symptomatic samples were identified through PCR using conserved region primers designed after alignment of the available CP sequences in NCBI database. More than 90% nucleotide sequence identity has been suggested as a guideline for

predicting viral strain and less than 90% for distinct begomovirus strain (Rybicki 1998). Rolling circle amplification technique (RCA) were conducted for the amplification and characterization of full length MYMV genome. The presence of ~2.7 kb product observed in the gel electrophoresis in case of DNA samples isolated from infected mungben leaves implied the presence of MYMV genome. The ~2.7 kb fragments obtained from Bam HI and Pst I restriction digestion were attempted to clone into suitably digested pGreen0029 vector into the Bam HI and Pst I restriction recognition sites. From the isolated six samples in which four were able to be cloned successfully. All the clones were subsequently sequenced using vector specific forward and reverse primers. The sequence obtained from the automated sequencing of the clone was analyzed through NCBI-BLAST search and found to have more than 90% sequence similarity with already reported MYMV strains existed in the database, Similar identification process was demonstrated by Pandey et al. (2010), Pratap et al. (2011), Idris et al. (2007) and Kamal et al. (2015).

Full genome sequences of the four clones obtained through automated sequencing were analyzed using the software bioedit version 7.0. Database searches with other geminivirus sequences were carried out by NCBI-BLAST program (http://blast.ncbi.nlm.nih.gov). BLAST search results revealed that the nucleotide sequences of the three clones had similarities and identities with DNA-B genomes of the mungbean yellow mosaic virus (MYMV) and these three clones were derived through restriction digestion using Bam HI. The other one that had been digested with Pst I, had sequence similarities and identities with DNA-A genomic component of mungbean yellow mosaic virus. To develop the gene construct pBI121CP-AC2, the CP and AC2 sequences were amplified from the DNA-A clone.

The nucleotide sequence analysis of DNA-A of the MYMV with ten selected DNA-A sequences of geminiviruses in NCBI database through BLAST search indicated that the isolated DNA-A has highest sequence identity (96.48%) with mungbean yellow mosaic India virus [Bangladesh] DNA-A, complete sequence [Accession number AF314145.1] and lowest sequence identity (95.83%) with mungbean yellow mosaic India virus segment DNA A, complete sequence [Accession number KU950430.1]. At the same time DNA-A clone was also analyzed and showing the presence of two ORFs (AV1 and AV2) on the virion sense strand and four ORFs (AC1, AC2, AC3 and AC4) on the complementary-sense strand. The AV1 ORF which encodes CP was mapped to

nucleotide positions 302 to 1075 (257 amino acids). The AV2 ORF was mapped to nucleotide positions 142 to 492 (116 amino acids). The largest ORF in DNA A, which encodes AC1 or Rep was mapped to nucleotide positions 2612 to 1524 (362 amino acids). The AC2 ORF which encodes TrAP was mapped to nucleotide positions 1624 to 1217 (135 amino acids). The AC3 ORF which encodes the REn protein overlaps with the AC2 ORF and was mapped to nucleotide positions 1476 to 1072 (134 amino acids). The smallest ORF in DNA A which encodes AC4 was mapped to nucleotide positions 2461 to 2162 (99 amino acids).

The full-length sequence of MYMV DNA-B identified in this study were further compared with published DNA-B sequences of other geminiviruses reported worldwide. The nucleotide sequence analysis of DNA-B of the MYMV with ten selected DNA-B sequences of geminiviruses in NCBI database through BLAST search indicated that isolated DNA-B had highest sequence identity (99%) with mungbean yellow mosaic India virus segment DNA B, complete sequence [Accession number KU950431.1] and lowest sequence identity (96%) with mungbean yellow mosaic virus isolate LBG623 segment DNA-B, complete sequence [Accession number KF928962.1]. This full-length DNA-B sequences identified in this study had similarity with BV1 and BC1 gene encoding nuclear shuttle protein or movement protein. One ORF was on the virion-sense strand (BV1) and other was on the complementary-sense strand (BC1). The BV1 ORF which encodes the nuclear shuttle protein (NSP) was mapped to nucleotide positions of 419 to 1189 (256 amino acids). The BC1 ORF which encodes the movement protein (MP) mapped to the nucleotide positions of 2117 to 1221 (298 amino acids). The isolated full length DNA B sequence has been published in NCBI database (accession number KY303697.1, Bhajan et al. 2017)

The developed DNA-A clone was subjected to PCR using MYMV coat protein (CP) specific primers (MYMV cpFwd and MYMV cpRev) which were designed during this study (Section 3.1.1). Sequence analysis of the amplified coat protein gene (*CP*) showed maximum (98%) sequence similarities with the coat protein gene of mungbean yellow mosaic India virus (MYMIV) Bangladesh strain and was designated as MYMIV- BD accession number (AF31414.1). Islam et al. (2012) found 97 % sequence identity with mungbean yellow mosaic virus coat protein gene in Bangladesh strain. Pandey et al. (2010) has reported similar findings in identifying ToLCV, a genus of begomovirus causing tomato leaf curl disease. In Begomovirus, *AC2* gene is a viral

transcription factor that trans activates the late viral genes AV1, encoding coat protein (CP) and BV1, encoding nuclear shuttle protein (NSP) on DNA-A and DNA-B, respectively (Haley et al. 1992, Guerrero et al. 2020). Similarly, amplification of *AC2* gene from the DNA-A clone was done following PCR using *AC2* gene specific primers. The obtained PCR product was sequenced and subjected to NCBI-BLAST and maximum (98 %) sequence similarity was observed with the *AC2* gene of mungbean yellow mosaic India virus (MYMIV) and accession number is AF31414.1. After confirmation through sequence analysis of the MYMV coat protein (*CP*) gene and *AC2* gene, these two genes were cloned in pBI121 backbone vector in an antisense orientation in developing a new antiviral construct (pBI121CP-AC2). This construct was developed based on post-transcriptional gene silencing mechanism (Bhattacharjee et al. 2019) where the *AC2* and *CP* genes supposed to produce antisense mRNA. Following the infection of MYMV the viral mRNA product was delivered in the sense orientation produced dimer with the antisense mRNA.

Since tobacco (*Nicotiana tabacum*) is a model plant for *Agrobacterium*-mediated genetic transformation, the efficiency of the newly developed antiviral construct (pBI121CP-AC2) was checked by using tobacco plant. Tobacco has been consistently proved to be used as one of the model plants in tissue culture (Garner1951, Skoog and Miller 1957). In the present study, highest number (90%) of healthy multiple shoots was obtained on MS medium supplemented with 4.44 µM BAP and 0.54 µM NAA in *Nicotiana tabacum* cv. Petit Havana. This was the best medium in terms of percentage of callus formation, shoot regeneration and the number of shoots per explant (Encina et al. 2001, Gowher et al. 2007). Rahman et al. 2010, reported leaf segment explants from 10 - 15 days old seedling was suitable for *in vitro* regeneration of *Nicotiana* spp. Formation of roots at the base of *in vitro* regenerated shoots is an important step to produce plantlets. For root induction and proliferation, MS medium and half strength of MS medium without hormonal supplement were used for *Nicotiana tabacum* cv. Petit Havana. MS medium without hormonal supplements was found to be effective in producing roots from 90% of regenerated shoots of tobacco.

Since the antiviral construct (pBI121CP-AC2) contained kanamycin resistance gene transformation experiments were mediated through kanamycin selection pressure. It was observed that albino shoots were developed along with green shoots after transferring

them into MS medium containing 100 mg/l kanamycin. During optimization of regulatory factors, it was found that maximum transformation was obtained when explants were incubated for 20 - 25 minutes in bacterial suspension with OD 0.3 to 0.5 and followed by a co-cultivation period of 2 - 3 days for the *Agrobacterium* LBA4404/pBI121CP-AC2. It was observed that, all control shoots died in the selection medium in presence of 100 mg/l kanamycin. To recover the transformed shoots, gradual elimination of non-transformed shoots and shoot buds was done through separating green shoots from albino shoots and allowing their further growth on fresh regeneration medium containing 100 mg/l kanamycin. The shoots that survived on the medium containing 100 mg/l kanamycin were considered as putative transformants. Konstantinova et al. (2003) reported that for selection of transgenic tobacco, leaf explants were cultured on regeneration medium containing 100 mg/l kanamycin producing the optimum response.

The transgenic nature of the transformed tobacco plantlets was confirmed through the application of specific molecular techniques like polymerase chain reaction (PCR) analysis. This was conducted to confirm the integration of viral diseases resistant gene (*AC2* and *CP*) in transformed tobacco plant. The DNA isolated from both of transformed and non-transformed shoots was subjected to PCR for the amplification of *AC2* and *CP* gene. Amplified DNA was analyzed through agarose gel electrophoresis. From the gel it was observed that the single band (1200 bp) formed in the transformed plantlets of *N. tabacum* were identical to the amplified positive bands. This result indicated that the *CP-AC2* gene was inserted in the genomic DNA of transformed tobacco plantlets**.**

In the present study pollen viability was examined observed for the developed transgenic tobacco plant and through this study 90% of the pollen grains were found viable. The germination rate of the transgenic seeds was also evaluated. For this purpose, seeds of the transgenic tobacco plants were germinated in the MS media containing 10% sucrose and 0.01% boric acid and in this case 60% germination was recorded for these seeds. These findings were almost identical to that with non-transgenic tobacco plants. Reale et al. (2006), reported the percentage of pollen viability and germination ability as 80 and 55% respectively in olive cultivars.

After successful integration of the newly developed antiviral construct in tobacco plants, in the next phase of experiments, attempts were made to integrate it into mungbean plants. For this purpose a suitable *in vitro* regeneration protocol for mungbean was developed before taking any attempts towards genetic transformation. Most of the earlier studies revealed that the grain legumes are recalcitrant in nature towards *in vitro* regeneration and genetic transformation (Bajaj and Ghosal 1981, Mroginski and Kartha 1984, Fatokun et al. 2002, Tuteja et al. 2013). However, recent information indicated that a number of studies have been conducted towards the transformation and *in vitro* regeneration of different cultivars of mungbean across the globe. Due to its recalcitrant nature success rate of the *in vitro* regeneration and genetic transformation of mungbean in developing transgenic plant is limited (Nisbet and Webb 1990, Sainger et al. 2015). However, several attempts have been made in regenerating plantlets from a number leguminous plants including *Pisum sativum* L., *Cicer arientum* L., *Phaseolous vulgaris* L., *Vigna unguiculata* L., *V. radiata* L. Wilczek, *Arachis hypogaea* L. and *Lens culinaris* Medik. (Sarker et al. 2003, Pniewski and Kapusta 2005, Mohamed et al. 2006, Hoque et al. 2007, Ghasemi et al. 2008, Verma et al. 2009, Aasim et al. 2011, Vadawale 2011, Rozan and Hassan 2016). Although considerable success has been achieved in regeneration of plantlets in these plants, very few efficient regeneration protocols are presently available to use them in transformation experiment. This seriously hindered the progress toward the implementation of gene transfer technology to improve leguminous crops.

Shoot regeneration via callus formation has been tried by some previous workers using various concentrations and combinations of hormonal supplements in nutrient media. However, their attempts to regenerate shoots from the induced callus were reported to be 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. Under these circumstances the plants have been regenerated through tissue culture *via* organogenesis using a wide range of explants of mungbean like hypocotyl and primary leaves (Jaiwal et al. 2001, Mahalakshmi et al. 2006) double cotyledonary node (Yadav et al. 2010) cotyledonary node (Baloda et al. 2017), immature cotyledons (Patra et al. 2018) and cotyledonary node and cotyledon attached decapitated embryo (Bhajan et al. 2019).

During the present study *in vitro* plant regeneration was carried out through organogenesis using two different types of explants, namely, cotyledonary node (CN) and cotyledon attached decapitated embryo (CADE) from two mungbean varieties cultivated in Bangladesh. Seed germination rate and time required for germination were almost identical for both varieties used in these experiments. The seed germination rate was found to be higher and healthy seedlings were developed in MSB_5 medium supplemented with BAP containing 3% sucrose with 0.8% agar after 4-5 days of culture. The performance of *in vitro* regeneration using the explants from these seedling were found to be effective. This result agrees with that of Yadav et al. (2010), but did not support the findings of Afrin (2009).

The various factors on which *in vitro* shoot regeneration depends include the composition of culture medium, proper concentration of growth regulators and the responses of explants as well as the genotype of the plant material used. Most of the researchers used BAP in shoot induction medium (Yadav et al. 2010, Hoque et al. 2007, Khatun et al. 2008). 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 2002). Therefore, in the present investigation MSB_5 medium supplemented with different concentrations of BAP (0.00–10.0 μM) were tried for shoot regeneration from CADE and CN explants. It was observed that maximum mean number of shoots per CADE explant was 4.56 ± 0.87 in case of BARI mung-3 and that was 4.41 ± 0.54 in case of Binamoog-5. Shoot initiation started from 10 - 15 days of culture of the explants but maximum number of shoots was observed after 60 -70 days of inoculation. Multiple shoots developed on this medium were found green and healthy. When explants were cultured on 5 μM BAP and the percentage of responsive explants was 86% and 81% respectively. The best average length of shoots in BARI mung-3 was 3.57 whereas in Binamoog-5 the average length of shoot was 3.81 after six weeks of culture. Increasing concentration of BAP did not produce enhanced number of shoots, rather decreased the regeneration efficiency (Kadri et al. 2014). However, the number of shoots per explants was low in CN explants in both varieties. To study the effect of shoot regeneration from CADE and CN explants, different concentrations and combinations of BAP (5 - 7.5μM) and Kn (0.46 - 2.22μM) were employed in MSB_5 medium. Multiple shoot induction from CADE explant was obtained on MSB_5 medium supplemented with 5 μM BAP and 2.22 μM Kn in case of

BARI mung-3 that are almost similar in case of only 5 μM BAP used. The results presented here clearly demonstrated that only BAP supplemented with MSB_5 medium was effective in regenerating multiple shoots directly from CADE and CN explants. During this study, following each subculture the length of regenerated shoots was found to increase and increased the number of multiple shoots with the reduced concentration of BAP. Zahan (2007) reported almost similar findings in shoot regeneration from cotyledon explants on mungbean. Patra et al. 2018 reported that, the maximum percentage of mungbean shoot regeneration (72.4%) was achieved on MS medium supplemented with 2.0mg/l BAP and 1.5mg/l NAA after six weeks of culture of cotyledonary tissue.

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 MSB_5 medium supplemented with different concentrations of IBA. Most of the previous workers reported that successful root induction can be achieved in ½ MS medium supplemented with different concentrations of IBA (Sagare and Mohanty 2014, Hoque et al. 2007, Mahalakshmi et al. 2006, Vats et al. 2014). The regenerated shoots exhibited very low rooting efficiency (26.66%) in half strength of MSB_5 containing 2.0 μM IBA. Amutha et al. (2003) used IBA for rooting in *Vigna radiata*. There are number of reports depicting problems in rooting of *in vitro* regenerated shoots in chickpea (Banko and Stefani 1989, Shankar and Mohan Ram 1990, Malik and Saxena 1992) and in lentil (Polanco and Ruiz 1997, Sarker et al. 2003). The loss of regeneration potential was found to associate with the inability in producing effective root system during the *Agrobacterium*-mediated genetic transformation resulted very low efficiency of plant transformation (Sanjaya et al. 2006). The major limiting factor in the establishment of a successful plant regeneration system from *in vitro* regenerated shoots for mungbean is the low frequency of rooting. In general, putative transformants are difficult to produce root (Pickardt et al. 1995) as they are subjected to high concentrations of antibiotics for prolonged periods during selection. During the present experiments with mungbean transformants which maintained for prolonged periods under antibiotic stress similarly had very slow response to rooting. Thus hampered the development of complete transformed plantlets of mungbean. Under these circumstances, micrografting could be an alternative to solve the problem of effective root development in mungbean.

Micrografting has been successfully applied to various crops i.e. cotton, lentil, chickpea, fruit trees, woody species and a closely related member in the Leguminosae family (Gulati et al. 2001, Yadav and Singh 2012). Successful micrografting of shoots has been reported previously in other grain legumes i.e. lentil (Gulati et al. 2001), chickpea **(**Yadav and Singh 2012), *Vicia narbonensis* (Pickardt et al. 1995) and *Cicer arietinum* (Krishnamurthy et al. 2000). In mungbean, the rate of successfully grafted shoots was influenced by both scion length and rootstock age. In these experiments, it was observed that the scions of 2.5 – 3.0 cm in length showed better response in micrografting with 55 % of success rate, whereas 48% success was obtained from the scions of 1.5 – 2.0 cm in length. Sanjaya et al. (2006) reported about graft success (60%) for sandalwood using 1-2 cm long scions derived from nodal shoot segments.

The firm placement of the micro scion onto the rootstock to ensure good contact was essential for the formation of the graft union. Displacement of the micro scion has also caused failure of micrografting. In mungbean plants the average rate of successful graft was 51%. The growth of callus is a key process in the development of the graft union because the scion physically joins to the rootstock. According to the observation, cellular division in surfaces of both rootstock and scion was initiated in several points by cells at the most central part of the union, which was extended through 30 days. The re-establishment of vascular continuity through the interface zone was the critical event that determined the compatibility between the rootstock and the scion on the development of graft union formation. The true union is achieved only after the xylem and phloem have made perfect contact. The realignment and restoration of the vascular bundles between scion and rootstock was completed 30 days after grafting. Microscopic observations revealed the development of graft union between scion and root stock.

The present investigation dealt with the integration of desired gene through genetic transformation for mungbean varieties growing 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 soybean (Zhang et al. 2016, Li et al. 2017), chickpea (Kar et al. 1996, Indurker et al. 2010, Tripathi et al. 2013), peanut (Tiwari and Tuli 2012, Mehta et al. 2013, Chen et al. 2015) and lentil (Das et al. 2012, Bermejo et al. 2012). Reports are also available for using several other methods of genetic transformation of plants including microprojectile bombardment, electroporation, sonication, chemical method of transformation etc.

(Magbanua et al. 2000, Livingstone et al. 2005, Niu et al. 2009). All these methods have not been found to be equally effective for the specific plant of interest. Generally, *Agrobacterium*-mediated genetic transformation has been considered as the most convenient and cost effective than other techniques. It is the most popular method for genetic transformation because of high co-expression of introduced genes, potentially low copy number and preferential integration into active transcription regions (Dodo et al. 2008, Tiwari et al. 2011). Therefore, the *Agrobacterium*-mediated genetic transformation technique was followed to integrate desired genes (*CP-AC2*) in mungbean.

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). Reports are also available on transient GUS expression using various infected explants of local varieties of mungbean such as BARI mung-3 and Binamoog-5 (Sarker and Siddiqua 2004, Zahan 2007). Most of these studies were carried out mainly for the development of a protocol for *Agrobacterium*-mediated genetic transformation of different varieties of mungbean. The transformation experiments of the present study have been designed following the method reported by Bhajan et al. (2019). In this part of investigation transformation experiments were performed using cotyledonary node (CN) and cotyledon attached decapitated embryo (CADE) from BARI mung-3 and Binamoog-5 varieties of mungbean. These explants showed greater potential in regenerating enough healthy shoots. Yadav et al. (2012) also demonstrated efficient regeneration in mungbean using CN explant. Bhajan et al. (2019) also noticed that CADE explant was efficient for transformation in mungbean. For successful transformation, choice of *in vitro* regeneration system is of prime importance. Single cell involved plant regeneration has been considered as a successful event in achieving transformation and through this system of regeneration there are least chances of chimera formation in developing transgenic plants.

In the present transformation study two different binary vector plasmid pBI121GUS-NPTII and pBI121CP-AC2 were used. The pBI121GUS-NPTII containing *GUS* (β–Glucoronidase) and *nptII* (Neomycin phosphotransferase) genes (considered as construct I) and pBI121CP-AC2 containing *nptII* (Neomycin phosphotransferase), *CP* (coat protein) and *AC2* gene (considered as construct II). Construct I was mainly used for the optimization of a suitable transformation protocol which includes various parameters

required for *Agrobacterium*-mediated genetic transformation, such as the optical density of *Agrobacterium* suspension, suitable incubation and co-cultivation periods required for effective transformation. Construct II was used for the integration of *CP-AC2* gene into mungbean genome in antisense orientation with an aim to develop yellow mosaic virus disease resistant mungbean lines. Attempts were also taken for selection and regeneration of putatively transformed plantlets.

Agrobacterium-mediated genetic transformation procedure is believed to be influenced by several factors (Mansur et al. 1993). Factors that influence successful transformation, such as type of *Agrobacterium* strain, genotype (host) compatibility and responsiveness of explants toward *Agrobacterium* infection, optical density (O.D.) of *Agrobacterium* suspensions, incubation and co-cultivation period were optimized in conducting transformation experiments. Moreover, the efficiency of transformation and transgenic plant production depends on the establishment of suitable protocols including the co-cultivation of host tissue, regeneration and selection of transgenic plantlets. In the present study, CN and CADE explants of both the varieties were used for the evaluation of various factors influencing transformation efficiency of mungbean.

During optimization of the above-mentioned factors it was found that the maximum transformation efficiency was observed with bacterial suspension having an optical density of 0.6 at 600 nm in both explants. Based on the results of histochemical GUS assays it can be reported here that 30 minutes of incubation period followed by 3 days of co-cultivation were found to be the most effective towards transformation using CN and CADE explants in both the varieties of mungbean studied. In both varieties CADE explants exhibited the better transformation efficiency than that of CN explant. In *Vigna radiata* Jaiwal et al. (1998, 2001), and Mirza and Tazeen (2004) reported that optical density of 1.0 and 3 days of co-cultivation period were considered as optimal condition for genetic transformation. Islam and Islam (2010) found that 45 minutes incubation and O.D of 1.3 at 600nm and 72 hours co-cultivation period was the best in achieving desired transformation. 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. Influence of regulatory factors on the

transformation efficiency of various explants was also observed by Yadav et al. (2012) in mungbean, Li et al. (2017) in soyabean. In this study, it was found that, such longer period of incubation (more than 45 minutes in case of construct I & II) and co-cultivation (more than 3 days) reduced survivability of explants and lead bacterial overgrowth in culture medium thus hampering the proper growth of infected explants.

The composition of co-culture media particularly the hormonal supplements is considered as an important factor in obtaining regeneration from infected explants. Schroeder et al. (1993) reported that in case of *Pisum sativa* presence of growth regulators in the co-cultivation media enhanced recovery of putative transgenic plants. In chickpea, Kar et al. (1996) reported that absence of growth regulators in co-cultivation media greatly reduced transformation efficiency and recovery of transgenic plants. Similarly, in the present study presence of growth regulator in co-culture media also found to enhance the induction of adventitious shoots and found to improve the health of regenerated shoots. Yadev et al. (2010) reported similar effective results 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. For this reason, 5.0 µM BAP was used in co-culture media.

Both the constructs (pBI121GUS-NPTII and pBI121CP-AC2) used in this investigation contained *npt* II gene within its T-DNA region and this gene confers kanamycin resistance to the transformed cells. Therefore, for the selection of transformed tissue kanamycin was applied to the regeneration medium for the selection of transformants. To determine the level of selection agents (kanamycin) in medium, different concentrations of kanamycin were tested. In several investigation, kanamycin was applied immediately after co-cultivation for the selection of transgenic shoots/cells (Venkatachalam et al. 2000, Vasudevan et al. 2007 and Chopra et al. 2011). However, in the present study it was found that addition of kanamycin immediately after co-cultivation greatly hampered the growth of the explants. It was observed that even in the presence of lower concentration of kanamycin, co-cultivated explants failed to regenerate. Following the reports of Iqbal et al. (2012), Anuradha et al. (2008) selection agent was added when the shoot buds started showing their first signs of emergence. Therefore, a pre-culture period and a delayed selection with kanamycin were followed in obtaining regeneration from explants with high rate of transformation efficiency.

However, presence of kanamycin greatly hampers growth of the explants and as a result many putative transformants was 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 of culture 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, Cardi et al. 1992).

In the present investigation different concentrations of kanamycin were applied to screen the transformed shoots effectively. Non-transformed shoots were started to become albino in presence of 100 mg/l kanamycin. To establish the initial selection pressure 100 mg/l kanamycin was applied and this concentration of kanamycin increased gradually up to 200 mg/l to optimize the proper selection pressure. For this purpose, kanamycin concentration was gradually increased from 50 to 200 mg/1. During this investigation, it was found that all the control shoots failed to survive at 200 mg/1 kanamycin within 15-20 days of culture. Shoots that survived on the selection pressure of 200 mg/l for 20 - 25 days were considered as putative transformed shoots. Sharma and Anjaiah (2000) reported that 200 mg/ml kanamycin as optimum for the selection of transformed shoots raised from cotyledon explants. Sarker et al. (2003) also increased kanamycin concentration gradually from 50 - 200 mg/l in BM-2 and BM-4 varieties of lentil. Therefore, the shoots survived in the medium containing 200 mg/1 kanamycin were considered as putatively transformed. It may be mentioned here that till now the transformation efficiency in legumes is very low 0.03 - 5.1% (Yan et al. 2000). Using kanamycin as a selectable marker Chopra et al. (2011) got the transformation efficiency of 0.9%, Celikkol et al. (2009) got the transformation efficiency of 2.3% in case of lentil, Pniewski and Kapusta (2005) got the transformation efficiency of 4.1% in case of pea. Shoots that survived in the 200 mg/l kanamycin selection pressure were subjected to root induction medium. 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 colored 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 few transformed shoots. A few 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 indicates 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 (Ottavani et al. 1993). After sufficient development of roots from the selected shoots, the plantlets were successfully transplanted 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.

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 *GU*S and *npt II* gene. Amplified DNA was analyzed through agarose gel electrophoresis. From the gel it was observed that in case of *npt* II a single band of 800 bp was observed in six transformed plantlets identical to the amplified DNA of *Agrobacterium* strain LBA4404/pBI121 (positive control). On the other hand, in case of GUS a single band of 750 bp was observed in one transformed plantlet identical to the amplified DNA of the bacterial strain. This result indicated that the *GUS* and *nptII* genes were inserted in the genome of

only one transformed plantlet. The insertion of single gene was also in reported previous studies (Hassan 2006). Here, it may be pointed out that the selection procedure developed during this study has been found to be effective in recovering transformed plantlets. The protocol of *Agrobacterium*-mediated genetic transformation developed through the present investigation can be used for the production of transgenic mungbean plants for virus resistant mungbean line.

Legumes are reported as highly recalcitrant plants towards *in vitro* regeneration and genetic transformation and highly genotype specific (Bajaj and Gosal 1981; Mroginski and Kartha 1984). Reports are also available regarding the problems towards *in vitro* rooting of legumes (Kar et al. 1996, Roy et al. 2001, Jayanand et al. 2003, Chakraborti et al. 2006 and Anwar et al. 2008) which is the main problem towards the genetic improvement of legumes through tissue culture-based techniques. Scientist have developed a technique to transfer transgenes in plants through avoiding the *in vitro* regeneration step called 'in planta genetic transformation'. This method was first developed in Arabidopsis by Feldman and Marks (1987). Recently genetic improvement of various plants was achieved which are non-responsive or less responsive towards *in vitro* regeneration following in planta genetic transformation method (Rohini and Rao 2000, Park et al. 2005, Keshamma et al. 2008, Kapildev et al. 2016). Since, legumes are recalcitrant towards *in vitro* regeneration and genetic transformation, several scientist have integrated foreign genes in different legume crops, e.g. *Glycine max* (Hu and Wang 1999), *Arachis hypogaea* (Rohini and Rao 2000), *Raphanus sativus* cv. Kosena (Park et al. 2005), pigeon pea (Rao et al. 2008) and blackgram (Kapildev et al. 2016). However, no such report is present for mungbean. Considering such hassles towards the *in vitro* regeneration of mungbean, in this study the efficacy of *Agrobacterium*-mediated in plant genetic transformation method was also evaluated for the plant of interest using a marker gene.

At this step of investigation, BARI mung-3 was chosen as the plant material because BARI mung-3 was found to be more susceptible towards several diseases, like,mungbean yellow mosaic virus, *Cercospora* leaf spot etc (Farooq et al. 2018). The *GUS* gene expression was enhanced upto certain level (OD_{600} 0.7) of bacterial suspension. If the optical densities was enhanced more than OD_{600} 0.7 then the depletion of *GUS* gene expression was observed. Therefore, OD_{600} 0.7 was recommended as the most effective

for in planta genetic transformation in this study. Three days co-cultivation period was found to show the highest GUS positive response. Most of the authors reported that 3 days of co-cultivation were more appropriate for genetic transformation in *Vigna radiata* (Mirza and Tazeen 2004, Islam and Islam 2010, Yadav et al. 2012). During plant transformation the T-DNA transfer machinery of *Agrobacterium* was induced by phenolic compounds such as acetosyringone. Acetosyringone is a signal molecule produced by tobacco cells (Vernade et al. 1988). Addition of acetosyringone not only to the induction medium, but also to the *Agrobacterium* co-culture medium, improved transformation frequencies (Mullins et al. 2001, Rho et al. 2001). In this experiment, addition of acetosyringone to the induction medium and co-cultivation media was found to increase the transformation efficiency. Different concentrations of acetosyringone (25, 50, 75, 100, 125 and 150 µM) were used in both induction and co-culture media. The effect of the addition of acetosyringone was clearly observed. A concentration of 100 µM acetosyringone in both the media showed highest response in GUS histochemical assay, however, the *GUS* gene expression was observed to deplete in more than 100 µM acetosyringone in both the media. So, in this study, 100 µM of acetosyringone in induction media and co-culture media was reported to be the most suitable to increase the transformation efficiency. Apart from incubation period, acetosyringone concentration, vacuum infiltration is also reported to enhance the transformation efficiency (Kapildev et al. 2016). Therefore, in this phase of investigation duration of vacuum infiltration was also optimized along with incubation period. For the induction of *vir* gene acitivity of *Agrobacterium*, tobacco leaf extract was added 5h before the infection (Rao et al. 2008). Apart from that, in this phase of experiment, Winan's AB medium was used which is also reported to enhance the activity of *vir* gene (Rao et al. 2008).

To observe the impact of vacuum infiltration towards genetic transformation efficiency, the explants were subjected for vacuum infiltration in a vacuum chamber attached with a vacuum pump before inoculating in the induction medium for several times (0s, 30s, 1 min, 2 min, 3 min, 4 min and 5 min). The effect of vacuum infiltration towards genetic transformation was also checked by GUS histochemical assay. It was seen that, the transformation efficiency was increased upto 56% percent with a vacuum infiltration duration of 2 min, more prolonged duration resulted depletion in *GUS* gene expression. The explants were hydrolyzed and macerated to observe the *GUS* gene expression under the microscope and chimeric expression of the gus gene was observed in both the root

and leaf tissue. All these observations and positive response in GUS histochemical assay indicated towards the successful integration of the gene of interest. However, after PCR analysis no PCR positive bands were found. Detection of integrated gene in the targeted host plant is an integral part of developing transgenic plants. Most trusted way to detect the integration of transgene is through polymerase chain reaction (PCR) analysis. Hence the in planta genetic transformation method had not been followed in case of antiviral gene construct (pBI121CP-AC2) in this study.

The goal of the experiment was to develop yellow mosaic virus resistant transgenic mungbean line through the integration of coat protein (*CP*) and silencing suppressor (*AC2*) sequences in antisense orientation mungbean genome. In this regards, *Agrobacterium*-mediated genetic transformation was carried out using newly developed antiviral gene construct pBI121CP-AC2 in BARI mung-3 variety for development of transgenic mungbean line following the protocol developed by Bhajan et al. (2019). BARI mung-3 is the most popular mungbean variety in Bangladesh, but they are highly susceptible to yellow mosaic disease (Faruq and Islam 2010). So, the integration of coat protein (*CP*) gene and silencing suppressor gene (*AC2*) into the genome of BARI mung-3 variety via genetic transformation techniques to combat mungbean yellow mosaic diseases has been shown to be an effective strategy for rapid development of resistant plant to pathogens. The percentage of survived shoots on 200 mg/l kanamycin selection pressure was 3.1% in CADE explants and 1.96 % CN explants in mungbean. Based on the results of survived shoots on selection medium of two explants, it was observed that the transformation efficiency was higher for kanamycin selection in CADE explants than CN explants. High survival rate of putatively transformed shoots using *Agrobacterium* strain LBA4404 has been reported in pigeon pea (Rao et al. 2008), in pea (Orczyk and Orczyk 2000), in papaya (Azad et al. 2013) and in mungbean (Yadav et al. 2012). Shoots that survived in higher concentration of selection medium were transferred to root induction medium. It was observed that no root induction was initiated when putatively transformed shoots were cultured on root induction medium containing kanamycin. For this reason, only ticarcillin was used in root induction medium to control the overgrowth of *Agrobacterium*. These findings agreed with the results of Anuradha et al. (2006). Sharma and Anjaiah (2000) also cultured the elongated shoots on root induction medium without any antibiotic.

In the present investigation the transgenic nature of the transformed plantlets of T_0 and, T_1 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 *CP-AC2* gene present in antiviral gene construct (pBI121CP-AC2). PCR amplified DNA was analyzed through agarose gel electrophoresis. From the gel it was observed that the single band formed (with product size 1200 bp) in each of the transformed plantlets were identical to the respective amplified DNA of bacterial strain (positive control). This result indicated that the *CP-AC2* gene was integrated in the genomic DNA of transformed plantlets. It is well known that T-DNA transfer to plant cells occurs in a defined direction, starting from the right border to the left border (Becker et al. 1992, Zambyski 1992). In the present study, experiments were also conducted to nullify the probable presence of *Agrobacterium* cells in T_0 plants. Pic A is an *Agrobacterium tumefaciens* chromosomal locus, identified by Mu d11681 mutagenesis (Rong et al. 1990). It was observed that among the transformed plants of mungbean varieties, no bands were found to amplify after PCR amplification using Pic A primer, but positive bands were observed in positive control. Thus it may be concluded that the transformed plantlets did not have any *Agrobacterium* contamination. Tiwari and Tuli (2012) reported the use of bacterial vir G gene specific primers to exclude any false positives due to persistent *Agrobacterium* cells in T_0 plants.

Through the present research, it has been possible to raise 6 plantlets that survived in pot and all of them were screened positive for of *CP-AC2* gene. The T_0 mungbean plants were grown in double layered insect proof net house prepared following proper bio-safety regulation. The seeds of T_0 mungbean plants were successfully germinated to raise T_1 plants. The promising transgenic events identified in this research need further testing for developing advanced transgenic lines. From the foregoing discussion, it may be concluded that during the present investigation it has been possible to optimize the *in vitro* regeneration system for two varieties of mungbean growing in Bangladesh. One notable findings of the present investigation was to develop a new antiviral vector construct and could establish micrografting technique from the *in vitro* regenerated shoots of mungbean. Through this investigation it has been possible to bypass the *in vitro* rooting system in mungbean which was very difficult to induce. Finally it has been possible to develop transgenic mungbean plantlets using both marker and antiviral gene constructs.

In regards of *Agrobacterium*-mediated genetic transformation in mungbean against yellow mosaic disease, this is the first report on the successful development of transgenic mungbean using the antiviral construct (pBI121CP-AC2) in Bangladesh. However, the frequency of transformation efficiency using both marker and antiviral genes was rather low which needs to be addressed in the future work of mungbean genetic transformation. Using this transformation protocol further investigation will be carried out by other scientist in future for the development of transgenic mungbean plants, so that the proper bioassay for the virus resistance can be carried out.

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Website Consultation

- http://blast.ncbi.nlm.nih.gov
- www.dhcrop.bsmrau.net
- avrdc.org/intl-mungbean-network

7. APPENDIX – A

ABBREVIATIONS

The following abbreviations have been used throughout the text:

%	:	Percentage
+ve	:	Positive
µ	:	Micron
µg	:	Microgram
µl	:	Micro liter
µM	:	Micromole
1 N	:	1 Normal
A.	:	*Agrobacterium*
AC2	:	Silencing suppressor
B_5	:	B_5 basal medium
BAP	:	6-benzylaminopurine
BARI	:	Bangladesh Agriculture Research Institute
BBS	:	Bangladesh Bureau of Statistics
BINA	:	Bangladesh Institute of Nuclear Agriculture
bp	:	base pair
C	:	Centigrade / Celsius
$CaCl_2$	:	Calcium chloride
CaMV	:	Cauliflower Mosaic Virus
cDNA	:	Complementary DNA
CH	:	Casein hydrolysate
cm	:	Centimeter (s)
CTAB	:	Cyle tetramethyl ammonium bromide
CP	:	Coat Protein
CADE	:	Cotyledon attached decapitated embryo
CN	:	Cotyledonary node
dNTP	:	Deoxy nucleoside tri-phosphate
DCN	:	Double cotyledonary node
e. g.	:	Example gratia, for example
et. al.	:	et alil and others
etc	:	et cetra, and the rest
FAO	:	Food and Agriculture Organization

FAOSTAT	:	FAO Corporate Statistical Database
Fig/s	:	Figure / Figures
FW	:	Fresh weight
FDA	:	Fluorescin diacetate
g	:	gram (s)
GM	:	Genetically modified
GUS	:	ß-glucoronidase
Ha (s)	:	Hectare
$HCCL_3$	:	Chloroform
HCL	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
Hr (s)	:	Hour (s)
i. e.	:	id est = which to say in other words
IAA	:	Indole- 3 – acetic acid
IBA	:	Indole- 3 – butyric acid
Kan	:	Kanamycin
Kb	:	Kilo base pair
Kcal	:	Kilocalorie
Kn	:	Kinetin (6- furfurylaminopurine)
KNO_3	:	Potassium nitrate
l	:	Litre
LB	:	Liquid Broth
lb / sq. inch	:	Pound per square inch
m	:	Meter (s)
M	:	Molar
mg / l	:	Milligram per liter
mg	:	Milligram
min (s)	:	Minute (s)
ml (s)	:	Milliliter (s)
mm	:	Milimeter
mM	:	Millimolar
MS	:	Murashige and Skoog Medium 1962
MT	:	Metric tonne
MW	:	Molecular weight
MOA	:	Ministry of Agriculture
MYMV	:	Mungbean yellow mosaic virus

MP	:	Movement protein
Na_2 – EDTA	:	Sodium salt or ferric ethylene diamine tetra acetate
NAA	:	α- naphthalene acetic acid
NaOH	:	Sodium hydroxide
NH_4NO_3	:	Ammonium nitrate
nm	:	Nanometer
No.	:	Number
NOS	:	Nopaline synthase
*npt*II	:	Neomycine phosphotransferasae II
NSP	:	Nuclear shuttle protein
OD	:	Optical density
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of Hydrogen
rpm	:	Rotation per minute.
PDR	:	Pathogen derived resistance
RISC	:	RNA induced silencing complex
RCA	:	Rolling circle amplification
sec.	:	Second
Sp. / Spp.	:	Species
t	:	Ton
T- DNA	:	Transfer DNA
T_0, T_1	:	Transgenic lines (First generation inbreed progeny)
US	:	United States
US$	:	United States dollar
USDA	:	United States Department of Agriculture.
UV	:	Ultraviolet Wavelength
v / v	:	Volume by volume
Var. (s)	:	Variety (s)
Vir	:	Virulence region
Viz	:	Namely
w / v	:	Weight by volume
Wt.	:	Weight
X – gluc	:	5-bromo-4-chloro-3-indolyl glucoronide
YEP	:	Yeast Extract Peptone
YMB	:	yeast extract Mannitol Broth
YMD	:	Yellow mosaic disease

APPENDIX - B

Murashige and Skoog (MS) Medium 1962

Components	Concentration
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
$MgSO_4.4H_2O$	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
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30,000.00

pH adjusted to 5.8 before autoclaving.

APPENDIX- C

MSB_5 medium

Macro and Micronutrients of MS medium and B_5Vitamins

(Gamborg et al.1968)

Components	Concentration
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
$MgSO_4.4H_2O$	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	
Nicotinic acid	1.00
Pyrodoxine-HCl	1.00
Thiamine-HCl	10.00
Inositol	100.00
Sucrose	30,000.00

PH adjusted to 5.8 before autoclave