ANALYSIS OF FACTORS INFLUENCING SUCCESSFUL *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION IN TWO DIFFERENT EXPLANTS OF PEANUT (*Arachis hypogaea* L.) VARIETY BINA CHINABADAM-2

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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Dedicated to my Parents

DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled **"Analysis of factors influencing successful *Agrobacterium*-mediated genetic transformation in two different explants of peanut (*Arachis hypogaea* L.) variety BINA Chinabadam-2"** submitted by the undersigned has been carried out under the supervision of Dr. Aparna Islam, Associate Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree of diploma.

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List of Abbreviations

BAP	6-Benzylaminopurine
BINA	Bangladesh Institute of Nuclear Agriculture
EDTA	Ethylenediaminetetraacetic acid
GUS	β-glucuronidase
MS	Murashige and Skoog (1962) medium
NOS	Nopaline synthase
OD	Optical density
X-Gluc	5-bromo-4-chloro-3-indolyl D-glucuronic acid
YEP	Yeast Peptone Media

Abstract

Peanut (*Arachis hypogaea* L.) is a nutritious oil-seed crop. It has low productivity due to its susceptibility towards many biotic and abiotic factors. It has incompatibility towards conventional breeding techniques, thus imposing a barrier towards its germplasm improvement. Under the situation genetic engineering in peanut offers an alternative improvement technique in order to achieve improvement in its germplasm, thereby, enhancing its productivity. This study was carried out to analyze various transformation factors to determine their optimal condition so that a proper protocol can be established to carry out transformation in two explants, namely, decapitated half embryo and whole embryo of BINA Chinabadam-2. The explants were prepared and infected in *Agrobacterium* culture media. The explants were then allowed to co-cultivate in MS media containing 2mg/l BAP and 1mg/l kinetin. 25% of the explants were analyzed for transient expression by GUS histochemical assay and the rest were transferred to regeneration media containing 200mg/l cefotaxime. High transformation efficiency was received with minimum of 1 hour of infection duration, 24 hours of co-cultivation and optical density (600nm) of close to 1.0 in case of decapitated half embryo explants. One day old explants gave good response. For whole embryo with cotyledon attached, pre-culture of explants had negligible effect while an optical density of 1.0 gave best result which is in accordance to the results received for decapitated half embryo.

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Chapter I: Introduction

1. Introduction

1.1 Background

Groundnut (*Arachis hypogaea* L.), a self-pollinated grain legume, is an essential oil seed crop. It is cultivated in 24 million ha all over the world for the purpose of extracting edible oil and also to be used as a source of healthy food. Its kernels are rich in oil (48–50%) and protein (25−28%), and are source of several vitamins, minerals, antioxidants, biologically active polyphenols, flavonoids, and isoflavones (Janila *et al.*, 2013).

Grain legumes do not possess a wide genetic base since they are mainly self-pollinated (Chandra and Pental, 2003). It is hypothesized that, the species which is recognized as peanut in the present day, is the result of a single hybridization event between two diploid species, namely, *Arachis duranensis* and *A. ipaensis* (Higgins and Dietzgen, 2000). The varieties of peanuts which occupy the Asian lands have a narrow genetic base and minimal genetic diversity which resulted into their susceptibility to many pests and diseases (Anuradha *et al.*, 2006; Iqbal *et al.*, 2012).

The main attackers of the peanut are fungi. Some common fungi-related diseases in peanuts in Bangladesh are Late leaf spot (LLS) caused by *Phaeoisariopsis personata* , Early leaf spot (ELS) caused by *Cercospora arachidicola*, Hori and rust caused by *Puccinia arachidis* Spegazzini (Janila *et al.*, 2013). Iqbal *et al.* (2012) have stated wilt caused by *Fusarium oxysporum* to be highly destructive. *Sclerotium rolfsii* causes stem and pod rot which is a major risk to peanut production in tropical regions, particularly, where expansion of irrigated peanut cultivation is taking place (Janila *et al.*, 2013). Peanut bud necrosis disease (PBND) and peanut stem necrosis disease (PSND) are viral diseases that are economically threatening the cultivation in India. On the other hand, peanut stripe potyvirus (PStV) is mainly common in East and South East Asia.

Another important concern for peanut production all over the world is the environmental stresses. It is predicted that the future climactic change will bring about more extreme weather; especially drought condition will be more prevalent in the

tropical and sub-tropical regions of the world which will make peanut production extremely difficult (Sun *et al.*, 2013). Therefore, it is necessary to genetically manipulate peanuts to utilize their full potential. Moreover, using genetic engineering technique it would be possible to introduce novel characteristics into peanut. It will help in the development of improved peanuts by letting the incorporation of genes from sources apart from *Arachis* germplasm straight into the commercial cultivars without varying other traits (Higgins and Dietzgen, 2000).

1.2. Genetic manipulation in peanut: a brief review

It has been reported that grain legumes have restrained proneness towards genetic manipulation *in vitro* and are, in general, recalcitrant to transformation (Hassan *et al.*, 2007). In most grain legumes, ability to employ regular transformation protocols has been restricted. Poor regeneration ability (especially via callus) and shortage of compatible gene delivery method have been pointed out to be the reasons behind the low rate of success. However, some success has been accomplished in soybean (Chandra and Pental, 2003).

The two commonly applied methods of delivering genes into plant cells are:
1) via *Agrobacterium tumefaciens*, and
2) via particle bombardment method using a gene gun.

It has been reported that higher success rate of transformation has been achieved in zygotic embryo of peanuts using gene gun (4.5%) as opposed to only 1.8% with *Agrobacterium* transformation method (Higgins and Dietzgen, 2000). However, still *Agrobacterium*-mediated transformation method has been used in this study because of wider availability and cost effectiveness. Gene guns are expensive and unavailable in the facility where the study was carried out.

The *Agrobacterium*-mediated transformation technique is extensively affected by various factors at different stages of the experiment (Fig. 1). For this reason, one of the crucial necessities for success in transformation is to find a consistent transformation protocol (Sarker, *et al.*, 2003).

According to Sharma and Anaijah (2000), there has not been significantly noteworthy success with genetic transformation of *Arachis* species. Their opinion is based on the fact that an efficient protocol to regenerate whole plant via *in vitro* regeneration of adventitious shoot buds from transformed tissue is almost non-existent. This indicates the importance of compatibility between explant regeneration and transformation.

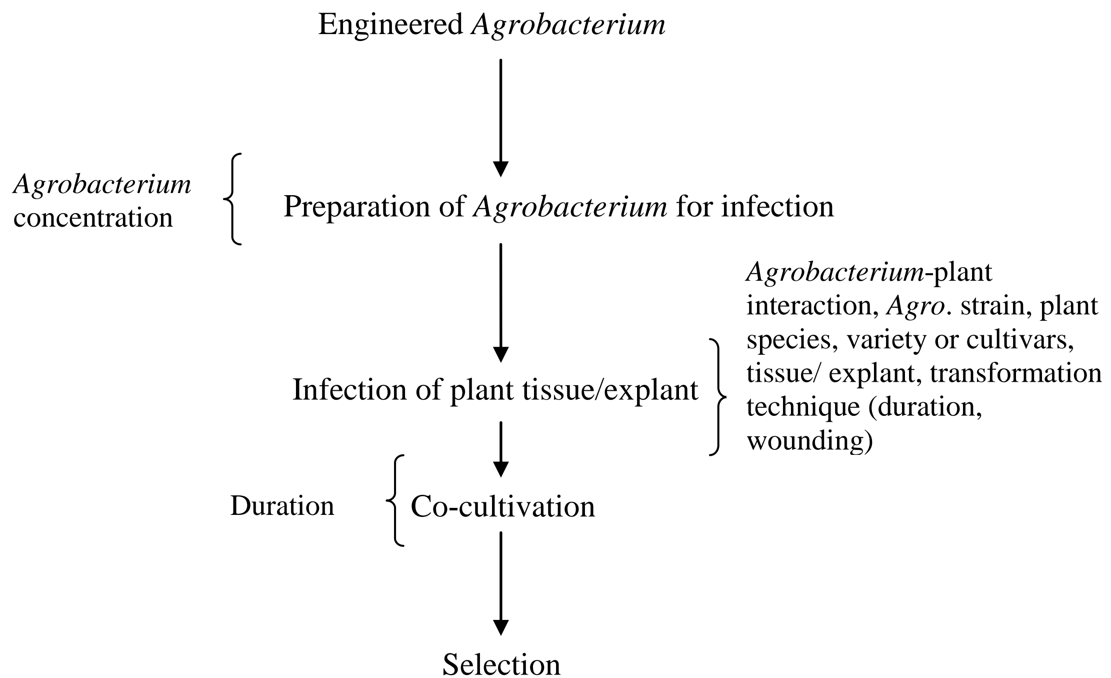


Figure 1: Steps of *Agrobacterium*-mediated transformation

Even though there have been reports of successful transformation of *Arachis* species, there have not been many reports of works being done with the local peanut varieties of Bangladesh. Most of the works reported with peanut involved Chinese and Indian varieties (Sharma and Anjaiah, 2000; Anuradha *et al.*, 2006; Tiwari and Tuli, 2011) using cotyledonary nodes (Anuradha *et al.*, 2006; Iqbal *et al.*, 2012) and cotyledons as explants (Tiwari and Tuli, 2011). In Bangladesh, there are reports of transformation like in peanut variety DM-1 (Sarkar and Islam, 1999, 2000). In this case the *Agrobacterium*-mediated transformation in epicotyl, hypocotyl and leaflet explants was reported, but they could establish *in vitro* regeneration protocol only from leaflet explant. Later on, another work was reported using leaflet as explants in the variety Tridana by the same research group (Sarker *et al.*, 2000). However, the use of decapitated half-embryo as an explant for transformation in peanuts has not been

reported yet. But, decapitated embryo is one of the best explants for transformation of lentils, another recalcitrant leguminous crop (Hassan *et al.*, 2007) which may be a good option for peanut regeneration.

After identification of a compatible explant, it is essential to evaluate factors involved in gene transfer prior to taking any transformation project. There have been numerous reports of transformation factor analysis in various species. Factors, such as, effect of explant maturity, effect of plant growth regulators present in the co-cultivation media, and length of co-cultivation period were optimized to establish a transformation protocol of common bean (*Phaseolus vulgaris*) (Zhang *et al.*, 1997). Factors affecting transformation in chickpea were optimized by Akbulut and his colleagues (2008). Islam and her coworkers reported the effects of optical density at 600nm, incubation period for infection and co-cultivation period in three Bangladeshi tomato varieties along with one Indian tomato variety (Islam, et al., 2010). Earlier, similar study was reported by Sharma *et al.* (2009), where they optimized factors, like, bacterial density and co-cultivation time in three Indian varieties of tomato. They determined bacterial density by direct enumeration of bacteria instead of indirect method, such as, measuring optical density using a spectrophotometer. Pérez-Piñeiro *et al.* (2012) has also cited several references stating the importance of optical density at 600nm in order to determine the bacterial density during infection of wheat explants. For wheat, the crucial optical density to result in efficient transformation has been found to be approximately 0.6 to even as high as 1.3. However, they also reported that too high an optical density resulted in overgrowth of bacteria during infection and co-cultivation and thus, cause death of the explant (Pérez-Piñeiro *et al.*, 2012).

Co-cultivation duration is an important factor in plant transformation. Zhang and coworkers (1997) carried out transformation in common bean seedlings and mentioned that optimum co-cultivation period was genotype dependent. Depending on the genotype, it can vary from 2 days to 5 days (Zhang *et al.*, 1997). According to Sharma *et al.* (2009), 72 hours was the optimum co-cultivation duration for transforming cotyledon explants of tomato. In case of peanuts, with cotyledonary nodes as explants, the reported optimum co-cultivation period was 72 hours (Anuradha *et al.*, 2006; Iqbal *et al.*, 2012). For leaflet as explants, 72 hours of co-cultivation is optimum as well (Sarker *et al.*, 2000). Sharma and Anjaiah (2000) used

cotyledons of peanut as explant and mentioned a co-cultivation duration of two days for optimum transformation.

Akbulut *et al.* (2008) reported to have varied infection time or as they stated to be the co-cultivation in liquid induction medium while carrying out *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) seedlings . They applied a variety of infection duration (2h, 8h, 16h and 24h) and concluded 16h and 24h as optimum. They also pointed out that the longer the infection period, higher is the rate of transformation but this also resulted in bacterial overgrowth which ultimately diminished viability of seedlings. Optimization of infection duration has been reported in the herb *Artemisia absinthium* where root, leaf and hypocotyls were used as explants (Mannan *et al.*, 2009). Mannan and his colleagues (2009) determined the optimum duration of infection to be five minutes. Paramesh *et al.* (2010) reported varying infection time in tomato where they used cotyledonary leaf and hypocotyl explants for transformation. According to Sarker and Islam (1999) and Sarker *et al.* (2000), 60 minutes of infection time is optimum for transformation in leaflet explants of peanuts. In case of decapitated half-embryo of peanuts, variation of infection duration has not been reported and hence it is a necessary objective. Rohini and Rao (2000) have already optimized the necessary factors for regeneration of transgenic plants from whole-embryo of peanut. They worked with the variety TMV-2 and discovered that following a period of pre-culture on semisolid MS basal medium for two days, the optimum time of infection is between 16 and 24 hours with sufficient pricking using a sterile needle.

1.3 Objectives

A popular peanut variety, BINA Chinabadam-2, has previously not been reported for any plant transgenic, thus biotechnological research. Therefore, aim of the present study is to analyze all the variables that affect transgene expression to establish a proper working protocol that can be followed to introduce foreign DNA in this peanuts variety.

This study investigated the effects of various factors, such as, optical density at 600nm wavelength, age of explants, duration of infection and co-cultivation period on the transformation efficiency of peanuts. Two different explants, decapitated half-embryo and decapitated whole-embryo of the variety BINA Chinabadam-2 were used to conduct this study and the transformation frequency was observed with GUS histochemical assay to monitor transient expression.

Chapter II: Materials and Methods

2. Materials and Methods

2. 1 Materials

2.1.1 Plant material: BINA Chinabadam-2

The seeds of the variety BINA Chinabadam-2 were collected from Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh for this study. BINA Chinabadam-2 was developed by carrying out random mutagenesis in Dhaka Chinabadam-1 through two consecutive gamma irradiations. The former has much higher productivity than the Dhaka Chinabadam-1 variety. The seeds of BINA Chinabadam-2 are 28% larger than Dhaka Chinabadam-1. They are tolerant to collar rot disease caused by *Aspergillus* sp. and frogeye leaf spot disease caused by *Cercospora* sp.

2.1.2 Agrobacterium strain: LBA4404 (pBI121)

Agrobacterium-based plasmid vectors allow the transformation of a wide range of plant species by capitalizing on a natural bacterial system to introduce DNA into the nuclear genome of plants. *Agrobacterium tumefaciens* is a soil bacterium. It is pathogenic to a range of dicotyledonous plant species, causing the formation of crown galls or tumors at or close to infection sites. The proliferated tissue in the tumor provides the bacterium with unusual amino acids (opines), which are an important carbon and nitrogen source, at the expense of the host plant. Genes required to establish a tumor and to bring about opine biosynthesis are transferred from *Agrobacterium.* As part of this sophisticated parasitism, *Agrobacterium* transfers a discrete portion of its DNA (T-DNA) into the nuclear genome of the host plant. Most of the machinery necessary for this T-DNA transfer resides on a tumor-inducing (Ti) plasmid. This Ti plasmid includes the T-DNA itself, delimited by 25 bp imperfect repeats and ~35 virulence (*vir*) genes, clustered together into a *vir* region. The combined action of the *vir* genes achieves the delivery of the T-DNA to the nucleus of the host plant cell.

For analyzing the factors, genetically engineered *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pBI121 of 14kDa was used. The binary vector contains the following genes within the right border and the left border (Fig. 2):

1. The *udiA* gene encoding GUS (β-glucuronidase) driven by CaMV promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation through the GUS histochemical assay.
2. The *nptII* gene encoding neomycin phosphotransferase II conferring kanamycin resistance, driven by NOS promoter and NOS terminator.

The bacterium also contains plasmid pAL4404 which is a disarmed Ti plasmid (132kDa) containing the virulence genes.

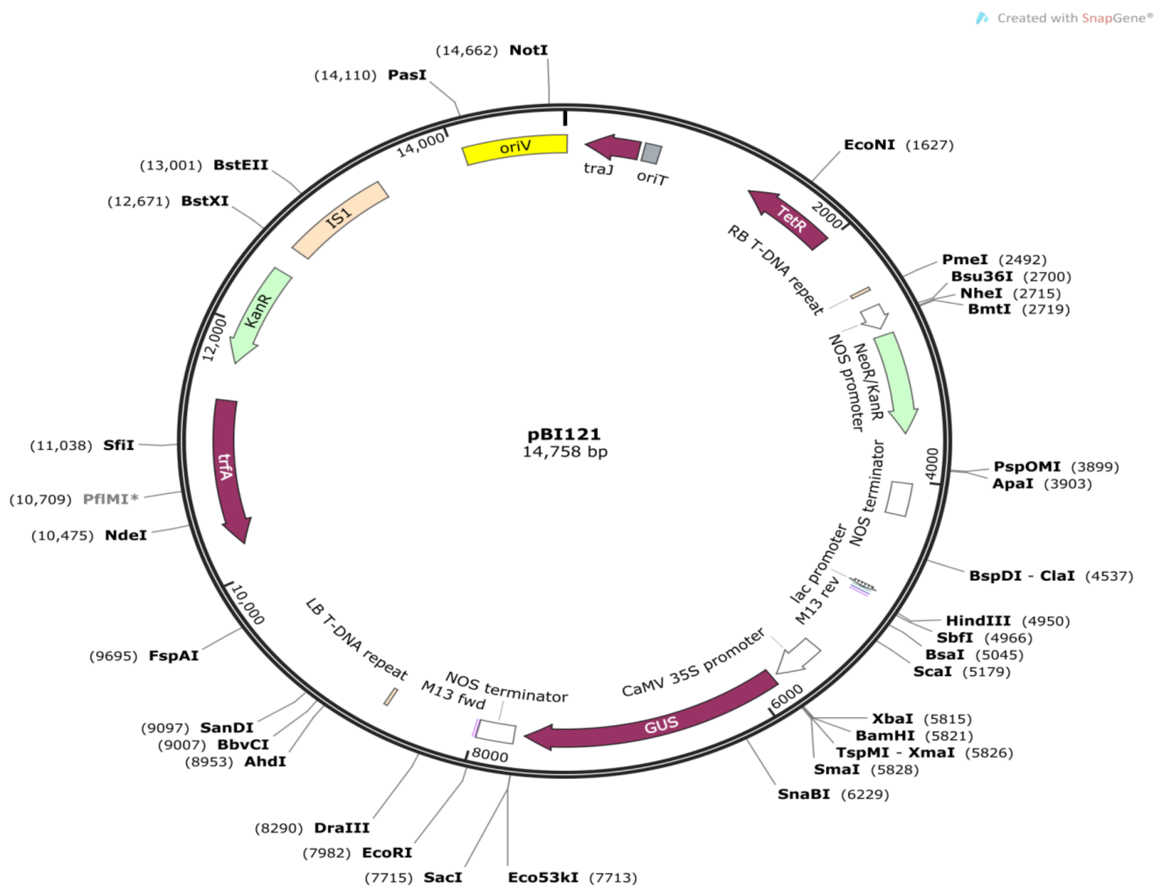


Figure 2: Genetic map of binary vector plasmid pBI121

(Source: http://www.snapgene.com/resources/plasmid_files/plant_vectors/pBI121/)

2.1.3 Co-cultivation media

After infection, the explants were allowed to co-culture along with the *Agrobacterium* in Murashige and Scoog (1962) media (MS media) with 2 mg/l BAP and 1 mg/l Kinetin. Different components are required to prepare the stock solution in MS media. The components and amounts used are given below:

Component	**Amount**
Macro nutrients (10x)	mg/l
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.2H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170
Minor salts (100x)	mg/l
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron EDTA solution (100x)	mg/l
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organics (100x)	mg/l
Nicotinic acid	0.5
Pyridoxin HCl	0.5
Thaimin HCl	0.1
Glycine	2.0

After preparation of the stock solutions, MS media is prepared following the following composition:

Component (Stock concentration)	Amount (for 100 ml)
Macro nutrients (10x)	10 ml
Minor salts (100x)	1 ml
Iron EDTA solution (100x)	1 ml
Organics (100x)	1 ml
Myo-inositol	0.01 g
Sucrose	3 g

All the components mentioned above were added to a conical flask and volume was brought up to 100 ml with ddH_2O. The pH was adjusted to 5.8 with 1N NaOH or HCl as needed. For solid medium, agar was added in 0.6% (w/v) ratio.

2.1.4 Bacterial medium

YEP (Yeast Peptone Media) was used to culture bacteria. It was prepared according to the composition mentioned below:

Component	Amount (for 1 liter)
Yeast Extract	10 g
Peptone	10 g
Sodium Chloride	5 g
Agar	13 g (for solid media)

2.2 Methods

2.2.1 Explant preparation

The explants used in the present study were decapitated half-embryo and decapitated whole-embryo attached to cotyledon. The seeds were taken in conical flasks and sterilized by washing in double-distilled, autoclaved water for 2 minutes, followed by 1 minute of 70% ethanol wash. They were again washed in double-distilled,

autoclaved water for three times with 1 minute duration of each wash and then immersed in 0.1% aqueous mercuric chloride for 18 minutes and placed in a shaker at 180 r.p.m. After pouring off the mercuric chloride, they were again washed in double-distilled, autoclaved water 3 to 4 times until all the bubbles disappeared.

The seeds were kept immersed in double-distilled autoclaved water for 24 hours, 48 hours and 72 hours to obtain explants of age 1 day, 2 days and 3 days, respectively. For culture of decapitated half-embryos, sprouted seeds were split open and both root and shoot parts of the mature zygotic embryos were cut and each of the half embryos were excised off the cotyledons to obtain decapitated embryo explants. For whole embryo explants, the testa was removed and the cotyledons were split such that the embryo was attached to one of the cotyledons and the embryo along with the cotyledon was used as the explants.

2.2.2 Agrobacterium culture preparation

Colony of *Agrobacterium tumefaciens* strain LBA4404 was inoculated into YEP media containing the antibiotic kanamycin (200 mg/l) for selection and kept overnight to achieve an optimum bacterial density. The bacterial density was determined via an optical enumeration method by measuring the solution's optical density at 600 nm using a spectrophotometer from PG Instruments.

2.2.3 Transformation technique

Freshly cut decapitated half-embryo explants were infected by dipping into the bacterial suspension into a conical flask. The conical flask was sealed with paraffin film and covered with aluminum foil. Finally, they were placed in a shaker at 28°C with 180 r.p.m. The flask was retrieved every 30 minutes and the explants were transferred to the co-cultivation media in order to ensure durations of infection for 30 minutes, 1 hour, 1 hour 30 minutes and 2 hours.

The infected explants were allowed to cultivate along with the *Agrobacterium tumefaciens* for 24 hours, 48 hours and 72 hours under 25°C with 16/8 hours photoperiod before placing them on regeneration media containing 200 mg/l cefotaxime to kill the bacteria. Approximately 25% of the explants were retained to

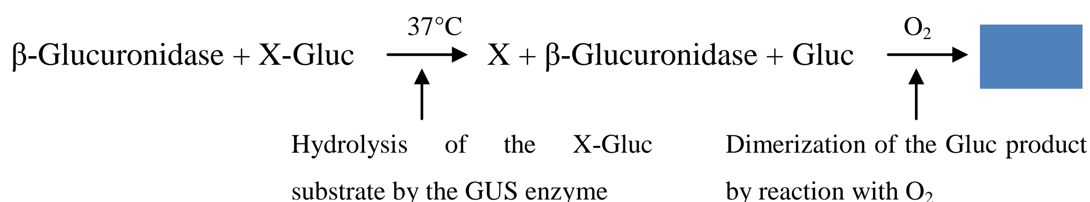
perform GUS histochemical assay before transferring them into antibiotic containing media.

The decapitated whole-embryos with attached cotyledon were allowed to pre-culture before infection. The embryos with one cotyledon attached were incubated on semisolid MS basal medium for two days before infection. The obtained explants were then infected by dipping them into a solution of *Agrobacterium* containing media in a Petri dish and wounded by excision of one cotyledon and pricking by using a sterile needle. The explants along with the *Agrobacterium* solution were transferred into a conical flask and kept in a shaker for infection duration of 20 hours. Afterwards 3 out of the 18 explants were analyzed using GUS histochemical assay. The rest of the explants were transferred to MS media containing 200 mg/l cefotaxime. The same procedure was carried out with explants that were not subjected to pre-culture. A range of optical density was used and both pre-cultured and non pre-cultured explants were subjected to the same procedure.

2.2.4 GUS assay

GUS assay was carried out by incubating the explants from the supposed transformants in a buffer encompassing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-D-glucuronic acid), 100 mM sodium phosphate (pH 7.2), 0.1% Triton X-100, 0.5 mM potassium ferricyanide and 10% methanol overnight at 37°C.

The primary reaction product, 5-bromo-4-Chloro-3-indolyl is colorless and soluble. This product was then oxidized and dimerized in the visualization reaction to form an insoluble, intensely blue final reaction product (indigo). The presence of ferri- and ferrocyanide in the incubation medium is a critical point for the visualization reaction in this procedure. It accelerates the formation of the final reaction product. It also protects the formed indigo from further oxidation, which would convert it to colorless or yellowish products.



Chapter III: Results

3. Results

In the present study, GUS histochemical assay was the principle analytical test used to determine the effects of various transformation factors. Transient expression within the decapitated half- or whole-embryo was studied to determine the effect of various factors.

3.1 Explant: Decapitated half-embryo

In the present investigation decapitated embryo of BINA Chinabadam-2 were infected with LBA4404 *Agrobacterium* strain harboring pBI121 binary vector. Following *Agrobacterium* infection, explants were subjected to GUS histochemical assay. Following color development transient transformation was measured (Fig. 3)

3.1.1 Effect of age of explants on transformation efficiency

Decapitated half-embryo obtained from 1 day old seeds showed 75% positive results with GUS histochemical assay while explants retrieved from 2 and 3 days old seeds showed 50% and 57.3% transformation efficiency, respectively (Table 1).

Table 1: Effect of age of the decapitated half-embryo on transformation efficiency in BINA Chinabadam-2 variety.

Age of embryo	Optical density	No. of explants used in GUS assay	Percentage of GUS positive explants
1 day	1.6	8	75
2 days	1.6	8	50
3 days	1.6	14	57.33

3.1.2 Effect of bacterial cultural density on transformation efficiency

Bacterial cultures of varying densities were used to conduct this study in order to determine the value of the optimum density required to ensure maximum

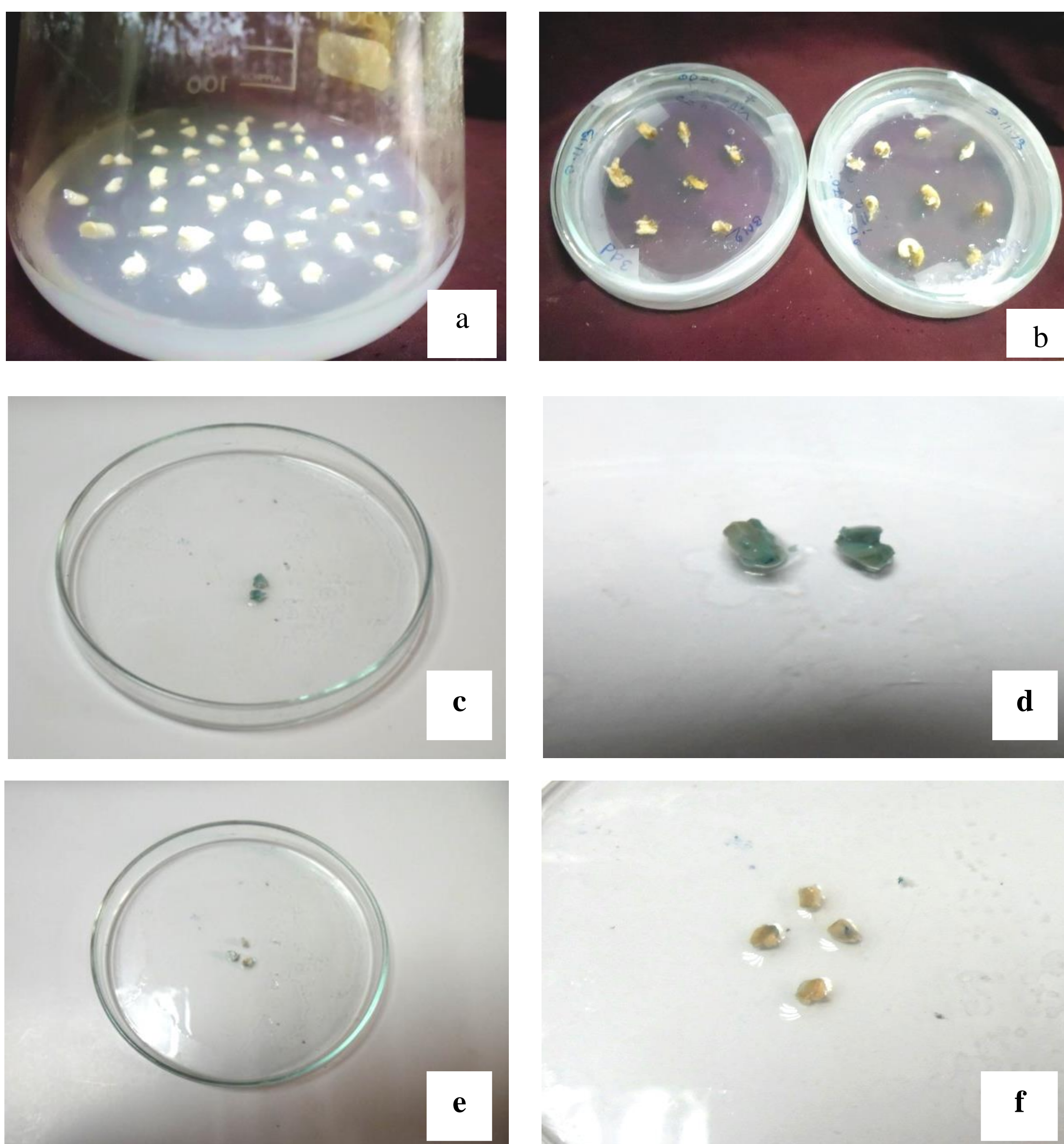


Figure 3: Factors affecting transformation of decapitated half-embryo of BINA Chinabadam-2 variety. **a.** Co-cultivation of decapitated half embryo in MS media containing 2 mg/l BAP and 1 mg/l kinetin. **b.** Transfer of explants to regeneration media containing 200 mg/l cefotaxime after co-cultivation. **c-d.** Explants following infection with pBI121 plasmid containing LBA4404 strain for 120 minutes having OD_{600} of 1.37, 24 hours of co-cultivation period showed blue color after GUS histochemical assay. **e-f.** Control explants showing expected negative result after GUS histochemical assay.

transformation efficiency. The range of OD_{600} used was between 0.5 and 1.7 and the optimal value of OD_{600} at which maximum transformation was observed, was at 0.7.

At this value, 100% explants were found to be GUS positive for the infection duration of 60, 90 and 120 minutes (Table 2).

3.1.3 Effect of infection duration on transformation efficiency

Infection period was found to influence transformation event immensely. Cent percent transformation was achieved in many cases (Table 2). The pattern that was observed is that, high percentage of GUS positive explants can be obtained by allowing a short infection duration with high bacterial density or a long duration in case of low bacterial density. 100% GUS positive explants were obtained at OD_{600} of 1.67 after infection duration of 30 minutes only, while at lower optical densities, minimum of 60 minutes of infection time was required in order to receive the same transformation efficiency (Fig. 4). High optical density results in overgrowth of bacteria and therefore, transformation efficiency decreased. While the optical density was low, a short infection of 30 minutes was not enough to give positive result in GUS histochemical assay.

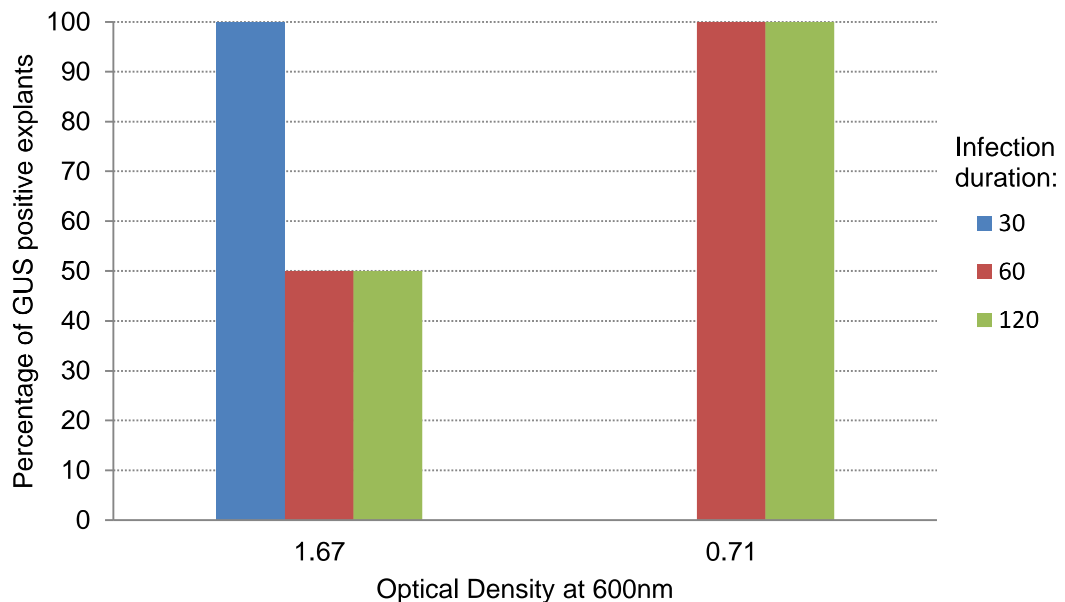


Figure 4: Effect of various infection durations on transformation efficiency with respect to bacterial densities.

3.1.4 Effect of co-cultivation period on transformation efficiency

The decapitated half-embryo explants were allowed to co-cultivate for 24, 48 and 72 hours. According to the data obtained (Table 2) the mean percentage of GUS-positive explants for 24 hours of co-cultivation were substantially higher than 48 or 72 hours of co-cultivation period (Fig. 5). Hence, it can be concluded that 24 hours of co-cultivation period is optimum for transformation of decapitated half-embryo of BINA Chinabadam-2 variety.

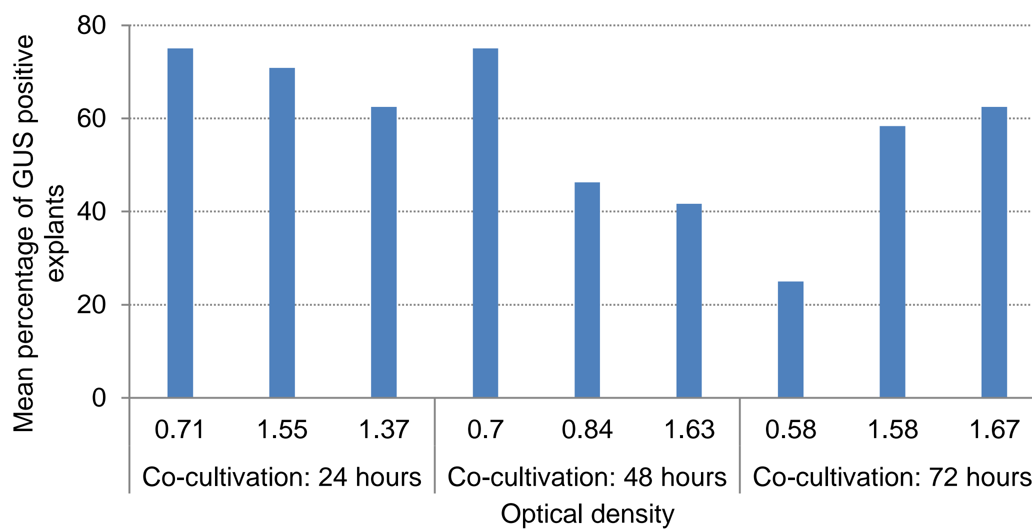


Figure 5: Effect of duration of co-cultivation on transformation efficiency of decapitated half-embryo of BINA Chinabadam-2 variety at different optical densities

Table 2: Effects of bacterial density, co-cultivation duration and duration of infection on decapitated half-embryo of BINA Chinabadam-2 variety.

Co-cultivation duration (hours)	Optical density (OD_{600})	Infection time (mins)	Percentage of GUS-positive explants	Mean percentage of GUS positive explants
24	1.37	30	50	62.5
		60	50	
		90	50	
		120	100	
	1.554	30	50	70.83
		60	100	
		90	33.33	
		120	100	

Co-cultivation duration (hours)	Optical density (OD_{600})	Infection time (mins)	Percentage of GUS-positive explants	Mean percentage of GUS positive explants
	0.707	30	0	75
		60	100	
		90	100	
		120	100	
48	0.7	30	0	75
		60	100	
		90	100	
		120	100	
	0.84	30	50	46.25
		60	50	
		90	60	
		120	25	
	1.63	30	50	41.67
		60	50	
		90	0	
		120	66.67	
72	1.67	30	100	62.5
		60	50	
		90	50	
		120	50	
	1.58	30	33.33	58.33
		60	50	
		90	100	
		120	50	
	0.58	30	50	25
		60	0	
		90	0	
		120	50	

3.2 Explant: Decapitated whole-embryo with one attached cotyledon

3.2.1 Effect of pre-culture on transformation efficiency

Pre-cultured decapitated whole embryo with attached cotyledon explants showed 100% GUS-positive response with an optical density of 1.0 (Fig. 6). When optical density was varied to 0.58 and 1.4, in both cases, percentage of GUS-positive explants decreased to 50%. However, non pre-cultured explants showed 100% GUS activity, with an optical density at and higher than 1.0 (Table 3).

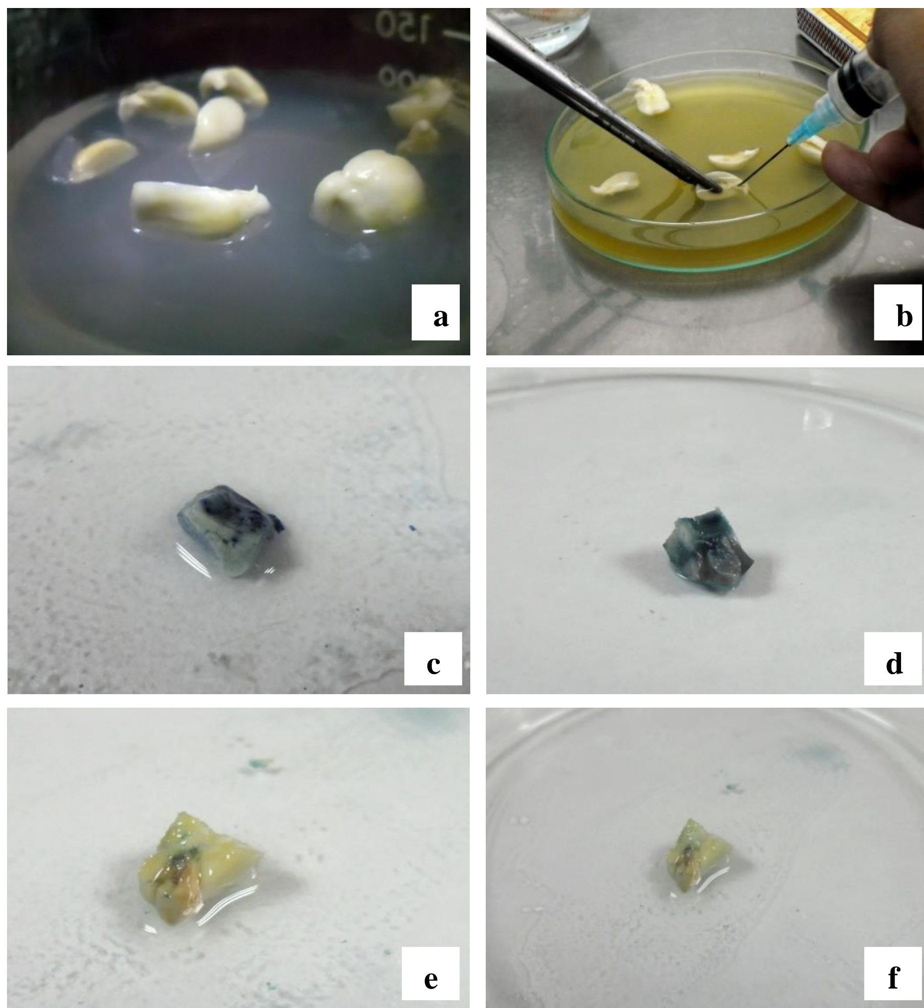


Figure 6: Factors affecting transformation efficiency of decapitated whole-embryo of BINA Chinabadam-2. **a.** Pre-culture of explants on semi-solid MS medium **b.** Infection of explants via pricking with needle **c.** Explant following pre-culture of 2 days with OD_{600} of 1.0 showed positive result after GUS histochemical assay. **d.** Explant without pre-culture having OD_{600} of 1.4 showed positive result after GUS histochemical assay. **e-f**. Control explants showing negative result after GUS histochemical assay.

3.2.2 Effect of bacterial density on transformation efficiency of decapitated whole-embryo with attached cotyledon

Maximum transformation efficiency was received with an optical density of 1.0 (Table 3). Using a lower optical density, that is 0.58, reduced transformation efficiency to 50%. Increasing the optical density to 1.4 also reduced the transformation efficiency to 50%. However, transformation efficiency remained 100% with an optical density of 1.4 as well when the explants were not pre-cultured.

Table 3: Effects of pre-culture and optical density on transformation efficiency of decapitated whole-embryo with attached cotyledon of BINA Chinabadam-2 variety.

	Optical density (OD_{600})	**No. of explants taken for GUS assay in transient transformation**	**Percentage of GUS-positive explants in transient transformation**
With pre-culture	0.58	2	50
	1.0	3	100
	1.4	2	50
Without pre-culture	0.58	2	50
	1.0	2	100
	1.4	2	100

Chapter IV: Discussion

4. Discussion

Transformation is immensely influenced by several factors. In the present study, several factors were studied for two explants, namely, decapitated half-embryo and decapitated whole-embryo attached with one cotyledon.

Usually a range of bacterial density is used to conduct studies of its effect on transformation efficiency. The findings resemble the negatively skewed data received by Sharma and his colleagues (2009) while carrying out transformation in tomato. The different bacterial densities they used were 0.5×10^8, 1.0×10^8, 2.0×10^8, 5.0×10^8 and 10.0×10^8 and the maximum transformation efficiency was found at 1.0×10^8 which is close to the minimum value in the range. Data received in the present study also resemble similar result with maximum transformation efficiency being received at OD_{600} of 0.7 within the range of 0.5 to 1.7. This is also in line with the findings by Sharma and Anjaiah (2000) who used an OD_{600} of 0.8 for carrying out transformation in cotyledon of peanut variety JL-24.

According to Sarker and Islam (1999), 60 minutes was the minimum required infection time for leaflets of peanuts. In this study, similar findings were observed in case of decapitated half-embryo. With lower optical density, at least 60 minutes of infection period was required to achieve high transformation efficiency.

The previous reports on peanut variety JL-24 with cotyledonary nodes as explants showed that 72 hours was optimum for transformation (Sharma and Anjaiah, 2000; Anuradha *et al.*, 2006). Hence, the present study proves that, in case of decapitated half-embryo of BINA Chinabadam-2 variety, 24 hours is sufficient for high transformation efficiency. Long duration of co-cultivation results in bacterial overgrowth which damages the explants and therefore, percentage of positive results decreases with increasing co-cultivation duration.

Optical density varied between OD_{600} of 0.5 and 1.5. According to Rohini and Rao (2000) 1.0 is the optimum value for transformation of decapitated whole embryo of the TMV-2 variety of peanut. When this value was used to execute transformation on decapitated whole embryo of BINA Chinabadam-2 variety, 100% transformation

efficiency was achieved. However, transformation efficiency was reduced to 50% when optical density was almost halved to 0.58. Fifty percent transformation efficiency was observed with a high optical density of 1.4 as well but it was increased to 100% in case of non-pre-cultured explants. Thus, it can be concluded that an OD_{600} of 1.0 is required for 100% transformation efficiency in BINA Chinabadam-2. Lower optical density will reduce transformation efficiency, while higher optical density will give good transformation efficiency if the explants are not subjected to pre-culture.

From the above results, it is evident that the optimum conditions required for carrying out transformation in decapitated half-embryo and whole-embryo of peanut variety BINA Chinabadam-2 has been established by this study. Research is also needed to be carried out to establish *in vitro* regeneration with these explants. Once that is established with combination of the present transformation protocol and the tissue culture protocol, further work can be conducted to successfully genetically engineer this particular variety of peanut so that its genetic characteristic can be enhanced for better productivity.

Chapter V: References

5. References

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