SILVER NANOPARTICLES FOR BIOLISTICS BASED GENE DELIVERY IN PLANTS

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KERALA, INDIA

2020

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by

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(2018-11-139)

THESIS

Submitted in partial fulfilment

of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM- 695 522

KERALA, INDIA

2020

DECLARATION

I, hereby declare that this thesis entitled **"SILVER NANOPARTICLES FOR BIOLISTICS BASED GENE DELIVERY IN PLANTS"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"SILVER NANOPARTICLES FOR BIOLISTICS BASED GENE DELIVERY IN PLANTS"** is a record of bonafide research work done independently by Ms. Nitasana Rajkumari under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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ACKNOWLEDGEMENT

I bow my head before the Almighty God for the blessings and enlightenment throughout my work that enabled me to complete my thesis work successfully on time.

I feel immense pleasure to express my sincere gratitude and personal thankfulness to ***Dr. Swapna Alex****, Professor, Dept. of Plant Biotechnology, for her valuable guidance, constant encouragement, support and co-operation that she has given throughout the course of my PG work.*

I am grateful to ***Dr.K. B. Soni****, Professor and Head, Department of Plant Biotechnology, for her timely advice and her support to complete my thesis work on time.*

I convey my heartfelt thanks to ***Dr.Anith K. N.,*** *Professor, Deptpartment of Agricultural Microbiology, for his valuable suggestions in the pursuit of the work.*

I humbly express my gratitude to ***Dr. Viji M. M.****, Professor and Head, Dept. of Plant Physiology, for her valuable suggestions, encouragement and continued support in the work.*

I also express my thankfulness to ***Dr. Kiran A. G.*** *(Assistant Professor on Contract) Department of Plant Biotechnology****,*** *for his sincere co-operation, timely help and assistance during the lab work.*

Words cannot express my heartfelt thanks to ***Amala, Aswathy, Athira and Sayooj****. I would have never completed my work without their support and I am thankful for their unbound love and affection.*

I express my sincere gratitude to my seniors Nagamani Chechi, Preetam Chechi, Sachin Sir, Monisha Chechi, Athira Chechi, Arathy Chechi, Nasreena Chechi, Sowndarya Chechi and Manasa Aka for their valuable suggestion, help and support. I wish to express thanks to my juniors Anusha, Ninitha, Akshay, Vinayak, Savio and Amal.

Words cannot express my deep sense of gratitude and indebtness to my beloved ***Pabung, Mama, Benao, Nikhil, Gabriel, Dadhou, Bubok and my family*** *for their moral support and motivation.*

Finally, I thank all those who extended help and support to me in one way or another in the successful completion of this thesis work.

Nitasana R.K.

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LIST OF ABBREVIATIONS

LB	Luria Bertani medium
AgNPs	Silver nanoparticles
h	hour
min	minutes
β	beta
µg	microgram
µL	microliter
mL	milliliter
L	liter
ng	nanogram
nm	nanometer
mg	milligram
g	gram
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
BA	6-Benzylaminopurine
IAA	Indole-3-acetic acid
GA_3	Gibberellic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
TBE	Tris borate EDTA
EDTA	Ethylenediaminetetraacetic acid
%	per cent

psi	pounds per square
cm	Centimetre
et al.	Co-workers
Fig.	Figure
ppm	Parts per million
CD	Critical difference
SE	Standard Error
He	Helium
R	Replication

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Introduction

1. INTRODUCTION

Genetic transformation is a powerful tool for crop improvement. It involves the introduction of foreign DNA into plant cells or tissues followed by its integration into the nuclear genome. It can be performed by delivery of DNA through indirect method like *Agrobacterium* mediated transformation or direct methods like electroporation, chemical mediated, microinjection and biolistic transformation. However, all the techniques being used for genetic transformation have one limitation or the other.

Among the different techniques for transformation, *Agrobacterium* mediated and biolistic methods are most commonly used. However, the host range of *Agrobacterium* mediated gene transfer is relatively limited (Zhang *et al*., 2000; Carvalho *et al*., 2004). Biolistic method can be used to overcome the issues of host specificity of *Agrobacterium*. It employs high-velocity microprojectiles to deliver foreign DNA and is a rapid and simple means for transforming intact cells and tissues. It is a popular method of incorporating DNA into cells that are difficult to transfect using traditional methods.

In biolistic method, DNA is coated onto the surface of micron sized tungsten or gold particles by precipitation with calcium chloride and spermidine. The DNA elutes off from macrocarrier once it enters inside the cells. These foreign DNA reaches the nucleus and is incorporated in the host chromosomes resulting in transient expression. In this method the foreign DNA is directly delivered into intact plant cells without biological constraints or host limitations (Altpeter *et al*., 2005).

Low transformation efficiency, tissue damage and high cost of the equipment and consumables are the major disadvantages of biolistic method. Cost of consumables such as gold microcarriers is one of the major factors limiting the wide spread use of biolistic transformation (Wirth and Wahle, 2003; O'Brien and Lummis, 2011). In the current scenario, it is necessary to develop efficient gene delivery systems possessing high transformation efficiency and low cytotoxicity (Dizaj *et al*., 2014).

Nanotechnology is an applied branch of science that deals with dimensions less than 100 nm, where unique physical properties of molecules that make novel applications possible. Nanotechnology has gained great momentum in agriculture in the last decade. In nanobiotechnology, tools of nanotechnology are being applied to biological sciences to serve as probes, sensors, vehicles for gene delivery, etc. Nanoparticles have ability to bind with DNA and protect DNA from enzymatic attack and can aid in the delivery of DNA into the nucleus. Different types of nanoparticles such as mesoporous silica nanoparticles, gold nanoparticles and zinc oxide nanoparticles have been used for gene transfer into microbes and plants as carriers (Torney *et al*., 2007; Chang *et al*., 2013; Wang *et al*., 2018).

Silver nanoparticles having anti-microbial property are able to increase the permeability of the bacterial membranes *via* the generation of many pits and gaps (Li *et al*., 2010). Previous reports by Nagamani *et al*. (2019) have shown that silver nanoparticles can increase the efficiency of transformation in bacterial cells. The presence of cell wall in plant cells is the major hindrance for effective gene delivery. There are no reports on the use of silver nanoparticles for biolistic transformation of plants. Efficient use of silver nanoparticles can aid in increased transformation efficiency of plant cells with comparatively reduced cost.

The present study was undertaken with the objective to evaluate the efficacy of silver nanoparticles as carriers for gene delivery in *Nicotiana tabacum* using biolistic method.

Review of literature

2. REVIEW OF LITERATURE

2.1. TRANSFORMATION FOR CROP IMPROVEMENT

Global food production has been estimated to increase 70–100% by 2050 to meet the demand of the growing population (Tomlinson, 2013). Conventional plant breeding methods coupled with improved farm management practices has led to a significant increase in crop production. But it is not feasible with conventional plant breeding strategies alone to cope up with increase in food demand due to restriction in gene pool availability, species barrier and other biological limitations. Recent advances in plant biotechnology provides a powerful tool for genetic manipulation and improvement of crop species. Application of recombinant DNA technology and its allied disciplines holds a great promise to augment crop production.

Successful genetic transformation involves the transfer of a gene into a suitable explant, integration and expression of transgene into the host genome and regeneration of the fertile transgenic plants from the transformed tissues. The efficiency of stable transformation is strongly dependent on genotype, explant source, medium composition and transformation method. Recombinant DNA can be stably introduced into the plant genomes by different methods like *Agrobacterium* mediated, electroporation, biolistics, and microinjection (Walden and Schell, 1990). Among these techniques, *Agrobacterium* mediated and biolistic mediated are the most commonly used for transformation.

Agrobacterium mediated transformation is the most widely used for transformation of dicotyledonous plants (Budar *et. al*., 1986). *Agrobacterium tumefaciens*, is a soil dwelling bacterium that naturally infects dicots and causes tumorous growth resulting in crown gall disease. Exudation of small phenolic compounds from plant wounds stimulates the expression of *vir* genes, located on Tumour inducing (Ti) plasmid, which is responsible for the excision, transfer and integration of TDNA (a part of small independent DNA molecule outside the bacterial genome) into the plant genome. Tumour formation is a result of the incorporation of

TDNA. The natural capability of plant transformation of the organism can be manipulated by replacing the genes causing tumorous growth by genes of interest. However, *Agrobacterium* has a natural tendency to infect dicot plants only and it is limited to specific host. Low frequency of T-DNA transfer into the target genome is the major limitation. A number of factors, such as plant genotype, explant types, *Agrobacterium* strains, selection marker genes, and various tissue culture conditions are critical for transformation.

Gene transfer by biolistics with DNA coated microprojectiles has the potential to be a rapid and simple means for transforming intact cells and tissues and is used to overcome the issues of host specificity of *Agrobacterium*.

2.2. BIOLISTIC TRANSFORMATION

Sanford and colleagues at Cornell University developed the first concept of bombardment and coined the term “biolistic” (short for “biological ballistics”) (Sanford *et al*., 1987). It is also known as particle bombardment, particle gun method, particle acceleration, or microprojectile bombardment. It is generally performed with the use of Biolistic PDS-1000/He. The gene gun employs high-pressure helium released by a rupture disk to propel a macrocarrier sheet loaded with millions of DNA coated microcarriers towards the target cells. The macrocarrier will be stopped by stopping screen, and the microcarriers will penetrate towards the target cells (Kikkert, 1993). It can deliver DNA to a wide range of intact target cells of diverse host organisms. The DNA elutes off from macrocarrier once it enters inside the cells. These foreign DNA reaches the nucleus and is incorporated with the host chromosomes resulting in transient expression.

2.3. FACTORS AFFECTING BIOLISTIC TRANSFORMATION

The rate of successful genetic transformation depends on the optimization of parameters of biolistic gene gun parameters. The parameters affecting biolistic transformation includes the tissue type, type of microcarrier used, helium pressure, gap distance (the distance between the rupture disk and the macrocarrier), the distance

between macrocarrier and stopping screen, the distance between the stopping screen and target cells, amount of vacuum in the bombardment chamber etc.

2.3.1. Microcarrier

Different microcarriers are reported to be used for biolistics, and the most commonly used are made up of gold or tungsten. The gold particles are rounder and relatively uniform in size compared with tungsten particles and are also less toxic to the cells (Kikkert, 1993). Platinum and iridium are also reported to be used but yield poor transformation (Sanford *et al*., 1993). Tungsten microcarriers are heterogeneous in shape and size, toxic to cells and can subject to oxidation that can alter binding of DNA and lead to catalytic degradation of DNA bound to them (Sanford *et al*., 1993). The surfaces of gold particles are smoother than tungsten particles (Kikkert, 1993). Gold is preferred due to availability of spherical particles of narrow size range and biologically inert nature (Randolph-Anderson *et al*., 2015).

Apart from the type of microcarriers, the size of the microcarrier is also an important. Size of microcarrier penetrating to the cells is proportional to the kinetic energy of the microcarrier and materials with high density are usually preferred. According to Randolph-Anderson *et al*. (2015), size of microcarrier needs to be selected by considering the size of target cell.

Gold particles of an average size of 0.6 and 1.0 microns are commonly used as micro-carriers. Gold particles of 0.6 µm size showed increased nuclear transformation efficiency compared to gold particles of 1 µm size in maize (Randolph-Anderson *et al*., 1997), wheat (Rasco-Gaunt *et al*., 2001) and soybeans (Khalafalla *et al*., 2005). Gold particles of size 6 µm exhibited five-fold increased nuclear transformation efficiency in maize callus, ten-fold in *Saccharomyces cerevisiae* and two-and-a-half-fold increase in *Clamydomonas* compared to 1µm gold particles (Randolph-Anderson *et al*., 2015). According to Luo *et al*. (2019), gold particles of 0.6 µm size was optimum for bombardment of wheat calli.

Gold particles of size 1 µm were used for bombardment of cells from suspension culture in tobacco transformation (Beetham *et al*., 1999). According to

Randolph-Anderson *et al*. (2015) gold particles of 0.6 µm not only exhibited increased transformation efficiency but also lesser tissue damage. Gold particle of 0.6 µm is reported to be ideal for nuclear transformation in tobacco leaf disc (Yasybaeva *et al*., 2017; Gumerova *et al*., 2018).

2.3.2. Helium pressure

Helium gas pressure is required to deliver the microcarrier into the target cell and is selected according to mechanical strength of cell wall. It directly affects the velocity of microcarrier. The greater the velocity of microcarrier, the more likely that it penetrates into the cells. Helium pressure of 1000 psi is optimal for most applications since damage is markedly increased beyond 1000 psi in the biological target (Sanford *et al*., 1993). Helium pressure of 1100 psi gave highest transient expression of GUS gene in rice calli (Zuraida *et al*., 2010). The optimum helium pressure for transformation was found to be 650 psi/900 psi in maize callus (Randolph-Anderson *et al*., 2015), 900 psi in wheat calli (Ismagul *et al*., 2018), 1100 psi in *Nicotiana plumbaginifolia* (Kanwal *et al*., 2017), 1000 psi in wheat calli (Luo *et al*., 2019) and 1100 psi in somatic embryos of *Camellia sinensis* (Furukawa *et al*., 2020).

Helium pressure of 1100 and 1350 psi are reported for successful bombardment of leaves of *Nicotiana tabacum* while 900 psi is reported for bombardment of suspension culture cells of tobacco (Beetham *et al*., 1999; Briza *et al*., 2013; Okuzaki *et al*., 2013). According to Okuzaki *et al*. (2013) 0.6 µm size gold particles with 1100 psi exhibited the highest transformation in tobacco leaf bombardment.

2.3.3. Vacuum inside the chamber

Vacuum inside the chamber affects the velocity of the microcarrier as it reduces the drag on the microcarrier when it travels to the target tissue. The greater the vacuum inside the chamber, lesser is the deceleration of the microcarrier. The

vacuum pressure of 28 inch Hg yielded the highest transient GUS expression in the rice callus (Zuraida *et al*., 2010). Partial vacuum of 28 inch Hg is reported to be optimum for bombardment in maize callus (Randolph-Anderson *et al*., 2015), 27.5 inch Hg in *Citrus* (Wu *et al*., 2016), 28 mm Hg in *Nicotiana plumbaginifolia* (Kanwal *et al*., 2017), and 27 inch Hg in wheat callus (Luo *et al*., 2019).

The higher the vacuum, higher is the velocity of the microcarriers in the gene gun bombardment (Kikkert, 1993). Partial vacuum of 0.9 bar, 28 inch Hg and 711 mm Hg is reported to be optimum for bombardment during transformation of tobacco leaf (Briza *et al*., 2013; Okuzaki *et al*., 2013; Yasybaeva *et al*., 2017).

2.3.4. Target distance

Target distance means the distance between the stopping screen and target tissue and it affects the velocity of microcarrier as well as transformation efficiency. The more the target distance, the more is the spread of the microcarrier over the target tissue and lesser the helium shock striking to the cells. But long travel distance will reduce the particle speed and decrease in penetration to the target tissue. The optimum target distance to produce transient expression was found to be 5.5 cm in wheat (Rasco-Gaunt *et al*., 2001). Combination of 1100 psi helium pressure with 9 cm target distance gave the highest expression of GUS gene in rice calli (Zuraida *et al*., 2010). Target distance of 6 cm is reported to be optimum in *Citrus* (Wu *et al*., 2016), leaf tissue of *Nicotiana plumbaginifolia* (Kanwal *et al*., 2017), wheat callus (Ismagul *et al*., 2018) and somatic embryos of *Camellia sinensis* (Furukawa *et al*., 2020).

Target distance of 6 and 9 cm is reported to be optimum for bombardment of leaf in tobacco while 10 cm was optimum for bombardment of suspension cells in tobacco (Beetham *et al*., 1999; Briza *et al*., 2013; Okuzaki *et al*., 2013; Yasybaeva *et al*., 2017).

2.3.5. Gap distance

Gap is the distance between the rupture disk and microcarrier. Flight distance is the distance between the macrocarrier and stopping screen.

A gap distance of 5-10 mm is mostly used for genetic transformation. 1 cm gap distance is reported to be maintained for bombardment in tobacco cells (Kikkert *et al*., 1996; Beetham *et al*., 1999). Flight distance of 15 mm was maintained during bombardment of wheat calli (Ismagul *et al*., 2018). According to Luo *et al*. (2019) gap distance of 2.5 cm and flight distance of 0.8 mm is optimum for bombardment of wheat calli.

2.3.6. Concentration of DNA

According to Lowe *et al*. (2009) frequency of occurrence of low copy events is correlated with the amount of the DNA per shot used in the biolistic transformation. According to them, reduction of transformation frequency was observed when quantity of DNA was reduced from 25 to 2.5 ng per shot (Lowe *et al*., 2009). According to Zuraida *et al*. (2010), calcium chloride and spermidine increases the binding of DNA to microcarrier. Coating of 2.5 µg of plasmid with 50 µL of 2.5 M $CaCl_2$, 20 µL of 0.1 M spermidine, and 50 µL of 50 mg/ml gold was optimum for bombardment of wheat calli (Luo *et al*., 2019). Coating of gold particles (0.4 mg) with 2 µg of plasmid DNA for one-shot bombardment was used in somatic embryos of tea plant (*Camellia sinensis*) (Furukawa *et al*., 2020).

According to (Briza *et al*. 2013) and Okuzaki *et al*. (2013) mixing of 0.8 µg DNA with 0.5 mg gold and 4 µg DNA with 0.8 mg gold was found to be ideal in biolistic transformation of leaf discs of tobacco. Yasybaeva *et al*. (2017) used 1 µg DNA per shot for transformation of tobacco leaves.

2.3.7. Target tissue

Biolistic method can be used for both monocotyledonous and dicotyledonous plants. Different types of target tissues can also be used for bombardment. Target tissue can be embryogenic somatic tissues, immature embryo, isolated scutella, inflorescence, leaf, regenerative callus or shoot apex (Klein *et al*., 1988; Christou, 1997; Liu *et al*. 2011). Immature epicotyl explants were used for bombardment in citrus transformation (Wu *et al*., 2016). Leaves, immature embryo and calli are

reported to be used for bombardment in wheat (Ismagul *et al*., 2018; Luo *et al*., 2019). Somatic embryos induced from the seedling bud were used as target tissue in bombardment of tea plant (*Camellia sinensis*) (Furukawa *et al*., 2020).

Leaf discs and cells in suspension culture are commonly used as target tissues for transformation of *Nicotiana tabacum*. Leaf discs of size 2 cm^2 and 5 mm^2 were used as target tissue in *Nicotiana tabacum* transformation (Okuzaki *et al*., 2013; Briza *et al*., 2013; Yasybaeva *et al*., 2017). Santos-Ballardo *et al*. (2019) and Schiermeyer *et al*. (2019) used cells from suspension cultures for bombardment in tobacco transformation.

References related to biolistic transformation in *Nicotiana* sp. are given below:

Explant	**Size of gold micro carrier (µm)**	**Rupture disk (psi)**	**Target distance (cm)**	**Vacuum**	**Concentration of DNA per shot**	**Reference**
Suspension cultured cells	1.0	900	10			Beetham *et al*., 1999
Leaf disc	1.0	1100	12	28 inch Hg		Arimura *et al*., 2001
Leaf disc	0.6	1100	9	28 inch Hg	0.8µg DNA with 0.5mg gold	Briza *et al*., 2013
Leaf disc	0.3, 0.6	1100, 1350	6	711 mm Hg	4µg DNA with 0.8mg gold	Okuzaki *et al*., 2013

Leaf disc	0.6	1100			10µg DNA with 50mg gold	Wang *et al*., 2016
Leaf disc	0.6	450, 650, 900	6	0.9 bar		Yasybaeva *et al*., 2017
Suspension cultured cells		900, 1350	7, 13			Santos-Ballardo *et al*., 2019
Suspension cultured cells	0.6	650	9			Schiermeyer *et al*., 2019

2.4. PLASMID VECTORS FOR GENETIC TRANSFORMATION

A vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell where it can be replicated or expressed. Some of the commonly used vectors are binary vectors like pCAMBIA1302 and pBI121. Plasmid vectors like pUC18 and plant viral vectors like cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV) are also used (Agarie *et al*., 2020).

pBI121 is an expression vector commonly used for plant transformation. Complete sequence of pBI121 is 14758 bp. Plasmid pBI121 has the uidA (GUS) as reporter gene with the CaMV 35S promoter and NOS terminator and nptII gene under the control of a nopaline synthase (NOS) promoter and terminator. The transient expression vector carries the ampicillin resistance gene and the GUS reporter gene. It also contains kanamycin resistance gene for bacterial selection (Sharma and Anjaiah, 2000). Vector map and its restriction enzyme sites are given in Fig.1

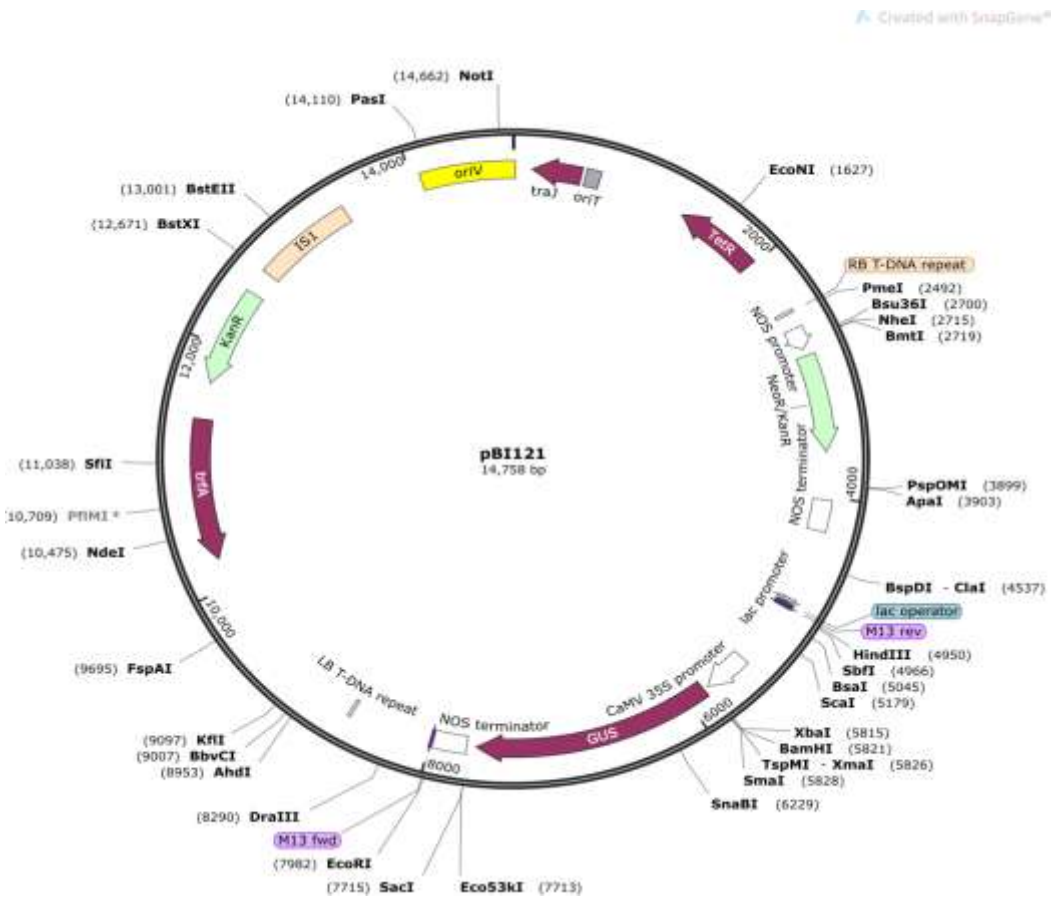


Fig: 1 pBI121 vector map

2.5. SELECTION OF TRANSFORMANTS

Selectable markers are genes or traits which can express a protein with enzymatic activity and help in selection of transformed cells/tissues artificially from non-transformed plants (Aragao and Brasileiro, 2002). Reporter genes, antibiotic and herbicides resistant marker genes are commonly used selectable markers (Ramessar *et al*., 2007).

Reporter genes are important tools for expression pattern studies of a specific gene controlled by a gene regulatory domain (Jafferson, 1987) and are generally used to optimize transformation methods. It also enables to detect the transgene localization and regulation of the expressed and tagged protein. Some of commonly

used reporter genes are chloramphenicol acetyltransferase, neomycin phosphotransferase, β-glucuronidase (GUS), green fluorescent protein (GFP) and luciferase.

2.5.1. β-glucuronidase (GUS) reporter

β-glucuronidase (GUS) is one of the widely used reporter gene for the assessment of gene activity for confirmation of transformed plants. It is a sensitive, flexible, stable and versatile gene fusion marker with wide range of available substrates and no toxic effects on plants. It is isolated from *E. coli* (Jafferson *et al*., 1987a). β-glucuronidase are glycosyl hydrolases enzymes which hydrolyse a wide variety of β-glucuronides. Its activity can be measured photometrically or fluorometrically. It can also be detected by histochemical GUS assay (Jefferson, 1987; Jefferson *et al*., 1987b). It is generally conducted after 48 hours of bombardment and blue colour spots are detected in *in situ* enzyme assay exhibiting GUS activity in transformed cells. The transient expression of GUS in biolistic bombardment is a way to evaluate the efficiency of plant transformation (Klein *et al*., 1988; Luo *et al*., 2019).

The commonly used substrate for GUS histochemical assay for plants is 5-bromo,4-chloro,3-indolyl, β-D glucuronide (X-gluc) (Jefferson, 1987; Jefferson *et al*., 1987b). GUS assay is conducted by immersing calli or leaves in the staining buffer containing 2 mM X-Gluc, 0.2% Triton X-100, 50 mM sodium phosphate buffer (pH 7.2), 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide. Blue staining is observed after incubation of 24 hours at 37°C (Luo *et al*., 2019).

The enzyme β-glucuronidase (GUS) gene expression is generally under the control of the cauliflower mosaic virus (CaMV) 35S promoter, one of the widely used promoter in studies of plant gene expression. X-gluc, a colourless soluble is hydrolysed and undergoes oxidative dimerization and formed an insoluble indigo product in presence of GUS. Potassium ferricyanide and potassium ferrocyanide accelerates oxidative dimerization and indigo blue colour crystals are visible under light microscopy (Lojda, 1970; Jefferson, 1987; Jefferson *et al*., 1987b). Optimum pH

of *E. coli* GUS is neutral and plant GUS is acidic. This optimum pH difference can restrict the endogenous GUS expression of plants with negligible interference (Alwen *et al*., 1992; Sudan *et al*., 2006).

2.5.2. Antibiotic sensitivity assay

Antibiotic resistant markers are genes which can produce proteins that offers resistance in a specific antibiotic medium. They can be broadly sub-divided into two categories: positive selection and negative selection. Positive selection benefits the growth of transformed cells while negative selection causes the death of non-transformed cells (Erikson *et al*., 2004). The mostly widely used selection agents for plant transformation are antibiotics like kanamycin, spectinomycin and hygromycin (Rashid, 2017; Kumar *et al*., 2018; Santos-Ballardo *et al*., 2019; Chen *et al*., 2020.) and herbicides like phosphinothricin (Hlozakova *et al*., 2020).

Vector pBI121 has kanamycin resistant gene and hence transformed plants can grow in medium supplemented with kanamycin. Sharma and Anjaiah, (2000) used shoot induction medium supplemented with 25 mg ml^{-1} kanamycin for selection of transformed shoots. Mohammadhassan *et al*. (2018) selected transformed shoots in a selection medium (MS with 20 gL^{-1} sucrose, 100 μgL^{-1} NAA and 2 mgL^{-1} BAP) supplemented with 50 mg/L kanamycin.

2.5.3. Regeneration of transformants

A stable micropropagation system is necessary for the selection and evaluation of the transformants after bombardment. According to Skoog and Miller (1957), an intermediate ratio of auxin and cytokinin promotes callus induction. Callus can be regenerated as a result of organogenesis stimulated by endogenously synthesized or exogenously added of auxins and cytokinins. Cytokinins induce *in vitro* organogenesis and regeneration from callus (Murashige and Skoog 1962; Taiz and Zeiger, 2010; Su *et al*., 2011). Callus initiation, multiplication and type of callus

formed depend on growth regulators and their combinations supplemented to the basal culture medium (Rakshit *et al*., 2008; Ikeuchi *et al*., 2013).

There are reports on protocols for regeneration of *Nicotiana tabacum* L. Callus induction was obtained from leaf discs in MS medium supplemented with 0.2 mg L^{-1} 2.4-D + 0.19 mg L^{-1} NAA (Opatrny and Opatbna, 1976). Klein *et al*. (1988) cultured leaf discs of tobacco in MS medium supplemented with 2.0 mgL^{-1} IAA and 0.5 mg L^{-1} BAP for callusing. According to Dixit *et al*. (2016) callus was initiated from leaf explants of tobacco in the medium containing 1.0 mgL^{-1} NAA and 1 mgL^{-1} BAP. The calli were transferred for shoot regeneration to medium supplemented with 1.0 mgL^{-1} BAP, 0.25 mgL^{-1} IAA and 0.5 mgL^{-1} GA3 (Dixit *et al*., 2016). Shoots were transferred to half strength MS basal medium supplemented with 0.1 mgL^{-1} IAA for rooting (Dixit *et al*., 2016).

2.6. NANOTECHNOLOGY

Nanotechnology plays a unique and important role in developing new methods for genetic engineering. The concepts of nanotechnology was first introduced by a famous physicist R. P. Feynman in his talk *There's Plenty of room at the bottom* (Feynman, 1995). The term 'nano-technology' was first used by Norio Taniguchi in 1974. National Nanotechnology Initiative defines nanotechnology as the manipulation of matter with at least one dimension sized from 1 to 100 nanometres. According to The European Commission "Nanotechnology is the understanding and control of matter at dimensions between approximately 1 and 100 nanometres, where unique phenomena enable novel applications. Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modelling, and manipulating matter at this length scale."

Nanotechnology is a rapidly growing branch of science and can applied in the diverse field like biotechnology, medicine, molecular biology, synthetic biology, biochemistry, agriculture and information communication sector. Its uniqueness and handling properties makes them immensely important in the present scenario. Bulk materials have constant physical properties but its properties change when their size is

reduced to nano scales. Nanoparticles have large surface area to volume ratio as compared to the bulk materials. Their surface area determines their physical properties. Nanoparticles have quantum effects due to their size which help them to acquire more optical properties.

Nanoparticles are traditionally defined as materials with at least one dimension measuring under 100 nm, whereby the small size can confer unique physical, chemical, and biological properties to the material compared to its bulk counterpart (Guston, 2007) The most commonly used nanomaterials can be grouped into: metal nanoparticles, lipids and liposomes, polymer based nanoparticles, and silica and carbon-based nanoparticles.

2.6.1. Nanobiotechnology

Nanobiotechnology is the combination of nanotechnology and biology and indicates the merger of biological research with various fields of nanotechnology. Various types of nanoparticles used are metal oxide nanoparticles, clay nanoparticles, solid lipid nanoparticles, natural polymeric nanoparticles and synthetic dendrimers nanoparticles.

Nanoparticles are reported to facilitate plant growth and overall plant health (Barrena *et al*., 2009; Arora *et al*., 2012; Salama, 2012). Advancements in plant science and agriculture indicate the usage of engineered nanoparticles serving as nanocarriers containing herbicides, fertilizers, chemicals, or genes, targeting specific plant sites prior to releasing their content (Thangavelu *et al*., 2018).

Gold nanoparticles are used for protein and nucleic acid delivery into plant and animal systems. They can protect DNA from enzymatic degradation and facilitates DNA cargo release with glutathione treatment (Han *et al*., 2005; Han *et al*., 2006). Gold nanoparticles are the most commonly used nanoparticles for biomolecule and drug delivery due to their ability to bind a wide range of organic and inorganic molecules, low toxicity, strong and tunable optical absorption (Pissuwan *et al*., 2011). According to Tian *et al*. (2013) and Demirer *et al*. (2015) gold and magnetic iron

oxide nanoparticles are the two most generally used metal nanoparticles for gene, protein, and drug delivery applications.

Metal oxide nanoparticles such as, AgO, ZnO, TiO_2 and MgO are of particular interest in biotechnology as they have physically and optically stable properties (Stoimenov *et al*., 2002; Makhluf *et al*., 2005). Photocatalytic (TiO_2, ZnO) and microbiocidal (AgO and MgO) nanoparticles are employed for pesticide degradation, detection and control of food spoilage (Makhluf *et al*., 2005; Baruah and Dutta, 2009). Metal oxides nanoparticles are used in photothermal therapy, imaging studies, delivery of biomolecules (proteins, peptides nucleic acids), biosensors, diagnostic procedures, implants, pesticide degradation (Niemeyer, 2001; Velasco-Garcia and Mottram, 2003; Oskam, 2006; Veerapandian and Yuna, 2009).

Clay nanoparticles like montmorillonite layered and double hyroxides are used for delivery of pesticides, fertilizers, plant growth promoting factors (El-Nahhal *et al*., 1999; Lakraimi *et al*., 2000). Solid lipid nanoparticles are used in delivery of DNA, xenobiotics, and pesticides, essential oils and transfection (Lai *et al*., 2006; Yu *et al*., 2009).

Natural polymeric nanoparticles like cellulose, starch, gelatin, albumin, chitin and chitosan are biocompatible, biodegradable and non-toxic for drug delivery and delivery of DNA/RNA (Liu *et al*., 2008; Puoci *et al*., 2008). Synthetic dendrimers nanoparticles are also used in delivery of therapeutic/diagnostic agents, pesticides and delivery of DNA/RNA (Liu *et al*., 2008; Puoci *et al*., 2008).

2.6.2. Nanoparticles for genetic transformation

Nanoparticles showed notable results in experiments carried out to improve the transformation efficiency due to their ability to overcome physiological barriers (Dizaj *et al*., 2014). Positively charged nanoparticles bind with the negatively charged backbones of DNA. They protect DNA from enzymatic attack and also release the foreign nucleic acids into the cell nucleus.

Nanoparticles may overcome the barrier of the cell wall and reduce the drawbacks associated with current transgene delivery systems (Mitter *et al*., 2017; Zhao *et al*., 2017; Demirer *et al*., 2018). Bacterial exposure to zinc oxide nanoparticles at sub-lethal concentrations (1 to 10 mgL^{-1}) for 24 hours produced a threefold increase in transformation efficiency of *E. coli* (Wang *et al*., 2018).

Layered double hydroxides (LDHs) have been used in gene and drug delivery to mammalian cells (Ladewig *et al*., 2010). Various nanoparticles have been popularized as gene transporters in the mammalian cells (Levy *et al*., 2010). Mesoporous silica nanoparticles assembled with polyethylenimine facilitated in delivering silenced interfering RNA in human cells (Hom *et al*., 2010).

In plant cells, nanoparticle based gene delivery system is limited. Potential nanoparticles for gene delivery in plants include calcium phosphate, carbon, silica, magnetite, strontium phosphate etc. Torney *et al*. (2007) observed green fluorescent protein (GFP) gene expression when mesoporous silica nanoparticles were used to transport DNA into isolated plant cells and leaves. Vector encapsulated with calcium phosphate nanoparticles were found more efficient in genetic transformation than *Agrobacterium tumefaciens* in plants (Naqvi *et al*., 2012). Ardekani *et al*. (2014) reported that calcium phosphate nanoparticles delivered pBI121 harbouring the GFP gene into tobacco cells.

According to Vijayakumar *et al*. (2010), carbon supported gold nanoparticles delivered DNA more efficiently into *Nicotiana tabacum*, *Oryza sativa* and *Leucaena leucocephala* compared to regular gold particles using a gene gun. The amounts of gold and plasmid present in the carbon supported gold nanoparticles were lower compared to the commercial micrometre sized gold particles. In addition, plant cell damage was minimal and therefore plant regeneration and the transformation frequency were increased.

Chang *et al*. (2013) reported that magnetic gold nanoparticles delivered plasmid DNA into the cells and protoplasts of canola and carrot. Gold nanoparticles were reported to be successful in biolistic delivery gene in rice (Mortazabi and Zohrabi, 2018).

Pasupathy *et al*. (2008) developed a novel gene delivery method in plants by using poly (amidoamine) dendrimer nanoparticles. Cationic polyamidoamine (PAMAM) dendrimers which are highly branched nanoparticles along with ultrasound (120s) and hPAMAM–DNA increased the gusA gene transfection and expression in alfalfa cells (Amani *et al*., 2018).

Nanoparticles such as nanosized alumina and TiO_2 have been reported to promote horizontal transfer of plasmid-borne antibiotic resistant genes in pure bacterial cultures in nutrient rich LB medium, by damaging cell membranes and enhancing the expression of mating pair formation and DNA transfer and replication genes (Qiu *et al*., 2012; Qiu *et al*., 2015; Ding *et al*., 2016).

Mesoporous silica nanoparticles are used in biolistic delivery to plants. They enhanced cell penetration and subsequent DNA expression. Gold mesoporous silica nanoparticles mediated biolistic delivery was successful in delivery of bovine serum albumin and enhanced green fluorescent protein (eGFP) and codelivery of protein and DNA/protein in onion epidermis cells (Martin-Ortigosa *et al*., 2012).

Calcium phosphate nanoparticles are used as gene carrier in genetic transformation of tobacco. Calcium phosphate nanoparticles successfully delivered pBI121 harbouring GFP driven by 35S promoter encoding plasmid DNA into tobacco cells (Ardekani *et al*., 2014).

2.6.3. Silver nanoparticles

Colloidal silver has been gaining more scientific attention due to its unique properties like excellent electrical conductivity, chemical stability, catalytic activity,

antibacterial activity and non-linear optical activity (Henglein, 1989; Mulvaney, 1996). Metal nanoparticles exhibit physical properties that are much different from the metal in its bulk form, like enhanced catalytic activity, which can be attributed to its morphological changes with increased surface area to volume ratio (Jose-Yacaman *et al*., 2001).

Silver nanoparticles (AgNPs) represent one of the most studied nano-particles among the metallic nanoparticles due to its distinctive optical, catalytic, sensing, antimicrobial properties and easily reduction of Ag^{+} salts to form zero valent silver (Ag) (Sharma *et al*., 2013). Silver nano-cubes with controllable dimensions can be synthesized by means of a modified polyol process that involves the reduction of silver nitrate with ethylene glycol in the presence of a capping reagent such as polyvinyl pyrollidone (Sun and Xia, 2002).

Shrivastava *et al*. (2007) reported the robust antibacterial activity and increased stability of silver (10-15 nm) against a number of drug resistant bacterial strains. According to Choi *et al*. (2008) silver nanoparticles have higher antibacterial activity than free silver ions. Sotiriou and Pratsinis (2010) reported that size of silver nanoparticles affected their antibacterial activity. Silver nanoparticles have been proven as an active bactericide against a wide range of bacteria comprising both gram-negative and gram-positive bacteria (Jones and Hoek, 2010; Nazeruddin *et al*., 2014).

Microbial properties of the AgNPs are more dependent on their shape, size, concentration and colloidal state (Pal *et al*., 2007; Gade *et al*., 2008; Rai *et al*., 2012; Nateghi and Hajimirzababa, 2014; Raza *et al*., 2016).

According to Raffi *et al*. (2008) in transmission electron microscopic analysis, bacterial membrane was completely ruptured after a few minutes on exposure to AgNPs, showing the high efficacy of silver nanoparticles and its unique property of large surface area to volume ratio. Silver nanoparticles have showed antiviral activity against a number of viruses infecting both prokaryotic and eukaryotic organisms. Viral inhibition depends on the size of AgNPs and small silver nanoparticles of 25 nm

or less, were more active in viral infectivity inhibition (Lara *et al*., 2010; De-Gusseme *et al*., 2010; Speshock *et al*., 2010; Narasimha, 2012).

Silver nanoparticles are reported to bind both double stranded as well as single stranded DNA and the binding is presumed to occur at the minor groove (An and Jin, 2012). Size of the nanoparticles used as well as their concentration is found to be critical in determining their inhibitory effects (Kim *et al*., 2012). Silver nanoparticles are found to increase the efficiency of transformation in bacterial cells (Nagamani *et al*., 2019).

Nanoparticle mediated gene delivery in plants has great importance in making plant transformation more effective and cost efficient. There are no reports on the use of silver nanoparticles for biolistic transformation in plants.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled “Silver nanoparticles for biolistics based gene delivery in plants” was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2018 to 2020. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1. STANDARDISATION OF *MEDIA FOR IN VITRO* CALLUS INDUCTION AND REGENERATION OF *NICOTIANA TABACUM.*

3.1.1. Preparation of culture medium

The basal culture medium used was Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The chemicals used for media preparation were of analytical grade (HiMedia, India; SRL, India). The glassware and tools used for the study were immersed in dilute liquid detergent overnight, washed thoroughly, dried in hot air oven (160-180^{0}C) and autoclaved at 121^{0}C and 1.06 kg cm^{-2} pressure for 20 min.

Stocks of macronutrients, micronutrients and vitamins were prepared by weighing the required quantity of chemicals using electronic weighing balance (Shimadzu, Japan) and dissolving and making up the specific volume using sterile double distilled water (Appendix I). Stocks of hormones were prepared by dissolving in the appropriate solvents and making up the volume using sterile double distilled water (Appendix II). Stock solutions were stored in refrigerator (4^{0}C). For the preparation of MS medium, double distilled water was taken in a beaker, specific volumes of stock solutions were pipetted out, and appropriate hormones combinations were added. Sucrose, inositol and MS supplement were added directly and dissolved by constant stirring. The pH of the medium was adjusted between 5.6-5.8 using 1N NaOH or 1N HCl with the help of a pH meter and the volume was made up using double distilled water. Required quantity of agar was added to the medium and melted in microwave oven. The molten medium was dispensed into sterilized culture bottles

(40 mL), closed and autoclaved at a temperature 121^0C and 1.06 kg cm^{-2} pressure for 15 min.

3.1.2. Inoculation of seeds in basal MS medium

Seeds of *Nicotiana tabacum* were surfaced sterilised with 4% sodium hypochlorite for 8 min followed by washing three times with sterile distilled water. Seeds were then soaked in sterile distilled water for 24 h. Seeds were treated with absolute ethanol for 5 min followed by washing with sterile water three times. Seeds were inoculated into the basal MS medium and maintained at a temperature of 20^0C with 16 h photoperiod.

3.1.3. Establishment of callus from leaves of Nicotiana tabacum

Young leaves were collected from the seedlings that germinated in plain MS medium using sterile blade and forceps. Leaf explants of 1 cm^2 were cut using a sterile blade. The leaf explants were inoculated in culture bottles containing MS medium with different concentrations and combinations of auxins such as Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and cytokinin such as Benzyl Adenine (BA) as described in Table 1.

Table: 1 Media composition tried for induction of callus in *Nicotiana tabacum*

Treatment	**Composition of hormones in MS medium**	**Reference**
T_1	1 mg L^{-1} NAA + 1 mg L^{-1} BA	Dixit *et al*., 2016
T_2	2 mg L^{-1} IAA + 0.5 mg L^{-1} BA	Klein *et al*., 1988
T_3	0.2 mg L^{-1} 2.4-D + 0.19 mg L^{-1} NAA	Opatrny and Opatbna, 1976

Twelve replications were maintained for each treatment. After inoculation the culture bottles were maintained at a temperature of 20^0C with 16h photoperiod.

3.1.4. Organogenesis and regeneration of Nicotiana tabacum

3.1.4.1. Shoot induction and proliferation

Two week old calli were transferred to MS medium containing 1 mg L^{-1} BA + 0.5 mg L^{-1} GA_3 + 0.25 mg L^{-1} IAA as described by Dixit *et al*. (2016) for emergence of shoots. The bottles were maintained in culture room at 20^0C temperature with a photo period of 16 h.

3.1.4.2. In vitro root induction

The *in vitro* developed shoots that emerged from the calli were transferred to half strength MS media containing 0.1 mg L^{-1} IAA as described by Dixit *et al*. (2016) for the induction of roots. The culture bottles were maintained at a temperature of 20^0C and 16 h photoperiod.

3.1.5. Hardening

The *in vitro* rooted plantlets were gently taken out from the culture bottles and washed in running tap water for removal of agar. The plants were transplanted into pro-trays containing potting mixture (autoclaved soil and vermicompost in the ratio 2:1). The pro-trays were covered with a hood made of polythene sheet containing some holes and kept in shade.

3.2. PREPARATION OF PLANT MATERIAL FOR BOMBARDMENT

3.2.1. Planting of tobacco seeds in pro-trays

Seeds of *Nicotiana tabaccum* were surfaced sterilised with 4% sodium hypochlorite for 8 min and then washed in sterile distilled water for three times. Seeds were then allowed to soak in sterile distilled water for 24 h. The seeds were planted in pro-trays filled with autoclaved vermiculite. The pro-trays containing the seeds were placed in shade for germination.

3.2.2. Preparation of leaves for bombardment

Leaves of uniform size were collected from two month old plants. The leaves were sterilised in 2% sodium hypochlorite for 2 min and washed in three changes of

sterile distilled water. Leaves were trimmed using a sharp sterile blade. The leaves/leaf discs were placed on sterile basal MS medium in petri plates for bombardment.

3.3. ISOLATION OF PLASMID VECTOR FOR BOMBARDMENT

3.3.1. Preparation of medium

Luria Bertani (LB) broth was prepared by dissolving the components in distilled water (Appendix III) and dispensed into conical flasks. The conical flask containing the medium was autoclaved for 15 min at a temperature and pressure of 121^0C and 1.06 kg cm^{-2} respectively.

LB agar medium was prepared by mixing the required quantity of agar to the LB broth (Appendix IV). Conical flasks containing the medium were autoclaved for 15 min at a temperature and pressure of 121^0C and 1.06 Kg cm^{-2} respectively.

LB medium containing rifampicin was prepared from stock solution of rifampicin. Stock solution of rifampicin at a concentration of 50 g L^{-1} was prepared by dissolving rifampicin in few drops of 10 N sodium hydroxide and making up the volume with methanol. The tubes containing stock solutions were covered with aluminium foil and stored at 4^0C. LB medium containing rifampicin was prepared by adding rifampicin to obtain a final concentration of 0.5 g L^{-1}.

LB medium containing kanamycin was prepared from stock solution of kanamycin. Stock solution of kanamycin at a concentration of 50 g L^{-1} was prepared by dissolving kanamycin sulphate in distilled water and was stored at 4^0C. LB medium containing kanamycin was prepared by adding kanamycin to obtain a final concentration of 0.25 g L^{-1}.

3.3.2. Maintenance of bacterial culture for plasmid isolation

Bacterial culture was established by transferring a loopful of bacteria from freshly streaked petri plates incubated for 24 hrs (Culture courtesy: ICAR-CTCRI, Thiruvananthapuram) to autoclaved LB medium containing rifampicin alone (0.5 g L^{-1}) or rifampicin (0.5 g L^{-1}) and kanamycin (0.25 g L^{-1}). Plain *Agrobacterium*

tumefaciens strain (EHA 105) and *Agrobacterium* strain containing plasmid vector pBI121 were inoculated in LB + rifampicin and LB + rifampicin + kanamycin respectively. The cultures were incubated in a shaker at 28^0C and 120 rpm for 48 h.

3.3.3. Isolation of plasmid

Plasmid was isolated by alkaline lysis method as described by Birnboim and Dolly (1979). The bacterial culture (*Agrobacterium* strain EHA 105 containing pBI121 plasmid vector) grown overnight was transferred to centrifuge tubes and centrifuged at 4000 rpm for 4 min. The supernatant was discarded and the bacterial pellet was resuspended in 200 µL GET (Appendix V). 300 µL of freshly prepared lysis buffer (Appendix VI) was added, contents were mixed by gentle inversion and incubated in ice for 5 mins. The solution was neutralized by adding 300 µL neutralizing buffer (Appendix VII) and mixed by inversion. It was again incubated in ice for 5 min. Cellular debris was removed by spinning at 15,000 rpm for 10 mins at room temperature. The supernatant was transferred to clean tubes and RNase A (20 µg/mL) was added. It was incubated at 37^0C for 1 h or 65^0C for 15 min. 400 µL of chloroform was added and mixed by inversion for 30 sec and centrifuged at 13,000 rpm for 1 min to separate the phases. The upper aqueous phase was collected and the chloroform extraction was repeated. The upper aqueous phase was transferred to a new tube, equal volume of 100% isopropanol was added and the contents were mixed by inversion. It was incubated at -20^0C for 20 min and centrifuged at 14,000 rpm for 10 min at room temperature. Isopropanol was removed and the pellet was washed with 70% ethanol (500 µL) and centrifuged at 10,000 rpm for 5 min. Tubes were air dried for 30 min in laminar hood and the pellet was dissolved in 60 µL sterile double distilled water. The plasmid was quantified and stored at -20ºC for further use. Plain *Agrobacterium* EHA 101 was maintained as control.

3.3.4. Quantification and quality check of plasmid DNA

Quantification and quality check of plasmid DNA was carried out using UV-visible spectrophotometer. The optical density of the DNA samples was recorded at 260 and 280 nm.

The concentration of DNA was calculated using the following formula:

Amount of DNA (µg/mL) = A_{260} x 50 x Dilution factor

Where A_{260} = absorbance at 260 nm

The quality of the DNA was judged from A_{260}/A_{280} value. A_{260}/A_{280} value range between 1.8 to 2 indicates good quality of DNA.

Quality of plasmid DNA was checked by agarose gel electrophoresis also. For making agarose gel, 1 g of agarose was added to 100 mL TBE buffer (Appendix VIII) and melted using microwave oven. When agarose solution was cooled down, 5µL ethidium bromide solution (1%) was added to it (0.5 µg per mL) and mixed well. Solution was then poured into gel tray with comb and placed in gel casting apparatus. After solidification the gel was placed in buffer tank that was completely immersed in TBE buffer and the comb was removed carefully to form wells. Gel was placed in buffer tank in such way that the wells were closer to cathode. DNA (10 µL) mixed with 2 µL of 6X gel loading dye (Appendix IX) was aadded into the wells using micropipette. In one of the wells, 1 kb DNA marker was added for comparison. The electrode in the buffer tank was connected to power pack and it was set at constant voltage of 70V. Electrophoresis was done for 1 h and 30 min. Once the dye reached $3/4^{th}$ of the gel, power-pack was switched off. The gel was taken and viewed in gel documentation unit (Gel DOC^{TM} XR^{T}, BioRad with Image Lab^{TM} software).

3.3.5. Restriction digestion of plasmid

The identity of the plasmid was confirmed by double digestion with HindIII and EcoRI to carry out plant transformation. Since pBI121 plasmid vector contains HindIII and EcoRI restriction sites, these enzymes were used for digestion.

Reaction mix (50 µL) was prepared by adding *EcoR1* (5 µL), *HindIII* (5 µL), R buffer (10X, 5 µL), plasmid DNA (20 µL) and distilled water (15 µL). The reaction mix was incubated at 37^0C for 2 h, and the reaction was stopped by keeping the mixture at 80^0C for 20 min.

The digested sample was electrophoresed on 1% agarose gel and the size of fragments were determined.

3. 4 BIOLISTIC TRANSFORMATION

3.4.1. Sterilisation of materials used for bombardment

The microcarrier launch assembly, microcarrier holder, microcarrier and stopping screen were autoclaved at 121^0C temperature and 1.06 $kgcm^{-2}$ pressure for 45 mins. The chamber inside the gene gun was wiped with 70 per cent ethanol. The rupture disks were dipped in 70 per cent isopropanol for few seconds before use. Stopping screen was autoclaved and wiped with 70 per cent ethanol before use. Leaves were surface sterilized with sodium hypochlorite (2% chlorine) for 1 min followed by washing for three times with autoclaved distilled water.

3.4.2. Preparation of gold microcarriers

Gold microcarriers of 0.6 micron size were used. 10 mg quantity of gold microcarriers was weighed in 1.5 ml tube, 1 ml of 70 per cent ethanol was added and vortexed for 5 min, and allowed to settle for 15 min. The microparticles were pelleted by spinning for 1 min in a microcentrifuge at 14,000 rpm. The supernatant

3.4.3. Coating of gold micro carriers with plasmid DNA

10 µL DNA (1 µg μL^{-1}), 50 µL 2.5 M calcium chloride and 20 µL 0.1 M spermidine were added to 50 µL of micro carrier in 1.5 ml tubes. The contents were vortexed for 2-3 min and allowed to settle for 1 min. It was centrifuged at 14,000 rpm for 1 min and the supernatant was discarded. To this 140 µL of 70 per cent ethanol was added and the supernatant was discarded. Again 140 µL of 100 per cent ethanol was added and the liquid was discarded. To this 50 µL of 100 per cent ethanol was added and the pellet was gently resuspended by tapping and vortexing at low speed for 2-3 sec.

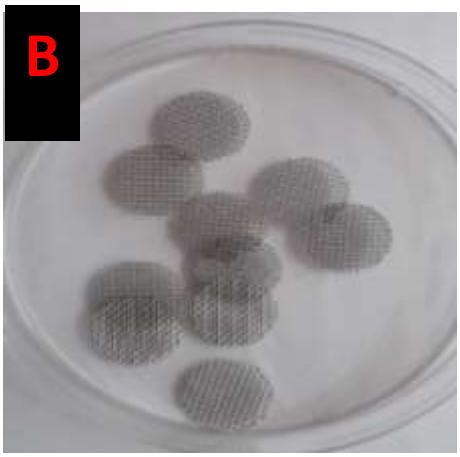


Plate: 1 Gene gun and accessories used for transformation

A-BioRad He/1000 Gene Gun, B-Stopping Screen C-Macrocarrier and holder,

D, E-Rupture disks

3.4.4. Biolistic transformation of Nicotiana tabacum with gold micro carriers

Sterile microcarrier launch assembly was fixed inside PDS-1000/He system (Bio-Rad Laboratories) and a gap of 6.35 mm was maintained between the rupture disc and microcarrier launch assembly. The DNA/gold mixture (20 µL) was added onto the macrocarrier, dried and bombarded onto leaf discs placed on sterile plain MS medium in petri plates. Bombardments were performed using rupture disks of different psi viz., 450, 650, 900 and 1100 and target distance was varied at 6 and 9 cm to optimise the parameters of biolistic transformation of *Nicotiana tabacum* for future comparison with silver nanoparticles. Vacuum pressure was maintained in the PDS-1000/He system (Bio-Rad Laboratories) at 28 inch Hg.

3.4.5. Biolistic transformation of Nicotiana tabaccum with silver nanoparticles

3.4.5.1. Coating of silver nanoparticlses with plasmid DNA

Silver nanoparticles (Sigma Aldrich, USA) of 100 nm size were used. Coating of plasmid DNA on the silver nanoparticles was carried out following the same procedure described in Section 3.4.3.

3.4.5.2. Optimisation of parameters for biolistic bombardment of silver nanoparticles

Concentration of silver nanoparticles, helium pressure and target distance were varied to determine the optimum parameters for biolistic bombardment of *Nicotiana tabacum* using silver nanoparticles.

For optimisation of concentration of AgNPs, eight different concentrations were used as shown below Table 2

Table: 2 Different concentrations of silver nanoparticles used for bombardment

Treatment	Concentration of AgNPs (mg L^{-1})
T1	0.01
T2	0.05

T3	0.50
T4	1.00
T5	2.00
T6	10
T7	20
T8	100
Control	0

20 µL of DNA AgNPs mixture was added onto the macrocarrier, dried and bombarded onto sterile leaves of *Nicotiana tabacum* placed on sterile basal MS medium in petri plates. Rupture disks of 1100 psi and target distance of 6 cm under 28 inch Hg partial vacuum was maintained in the PDS-1000/He system (Bio-Rad Laboratories, USA)

The concentration of silver nanoparticles that showed maximum transformation efficiency was selected and the procedure was repeated using different helium pressures and target distances as given in Table 3 and Table 4 respectively. The bombardments were performed under 28 inch Hg partial vacuum as described in Section 3.4.4.

Table: 3 Different Helium pressures used for bombardment

Treatment	Helium pressure (psi)
H1	450
H2	650
H3	900

H4	1100

Table: 4 Different target distances used for bombardment

Treatment	Target distance (cm)
D1	6
D2	9

3.5. ESTIMATION OF TRANSFORMATION EFFICIENCY

Transient GUS expression was assessed for determination of transformation efficiency.

3.5.1. Transient GUS expression

For GUS staining the leaves after two days of bombardment were transferred to 90 per cent acetone placed on ice. Acetone was removed from the samples and staining buffer without X-Gluc was added (Appendix IX). It was then removed and staining buffer with X- Gluc (Appendix IX) was added by keeping on ice. The leaves were incubated overnight at 37°C in dark. The leaves were then incubated in 70 per cent ethanol until the chlorophyll was removed. It was then transferred to sterile distilled water and the leaves were observed under a compound microscope for blue spots (Leica, Germany).

3.5.2. Screening for transformed calli

The leaf discs after two days of bombardment were transferred to selection medium (1 mg L^{-1} NAA + 1 mg L^{-1} BA + 100 µg ml^{-1} kanamycin) and screened for transformants. The cultures were maintained at 16 h photoperiod and observed at regular intervals.

3.5.3. Regeneration of transformed calli

Three week old calli resistant to kanamycin and surviving in the selection medium were transferred to MS medium as described in Section 3.2.1 and supplemented with kanamycin (100 µg ml^{-1}) for emergence of shoots. The bottles were maintained in culture room at 20^0C temperature and 16 h photo period.

The shoots that emerged from the calli were transferred to MS medium as described in Section 3.2.2 and supplemented with kanamycin (100 µg ml^{-1}) for rooting. The culture bottles were maintained at a temperature of 20^0C and 16 h photoperiod.

The rooted plantlets were gently taken out from the culture bottles. The plantlets were hardened and transplanted into pot trays as described in Section 3.2.3.

3.6. Statistical analysis

The numbers of GUS positive blue spots counted in the treatments were statistically compared for significance. The critical difference (CD) values at 5% level of significance were calculated using analysis of variance (ANOVA). For comparison of transformation efficiency of gold and AgNPs as carriers t-test was used at 5% level of significance.

Results

4. RESULT

The result of the study entitled “Silver nanoparticles for biolistics based gene delivery in plants” are represented in this chapter.

4.1. STANDARDISATION OF *MEDIA FOR IN VITRO* CALLUS INDUCTION AND REGENERATION *NICOTIANA TABACUM*

4.1.1. Inoculation of seeds in plain MS media

Seeds of *Nicotiana tabacum* started germination from the 22nd day onwards after inoculation in MS basal medium. Ninety five per cent germination was observed within 1 month of inoculation.

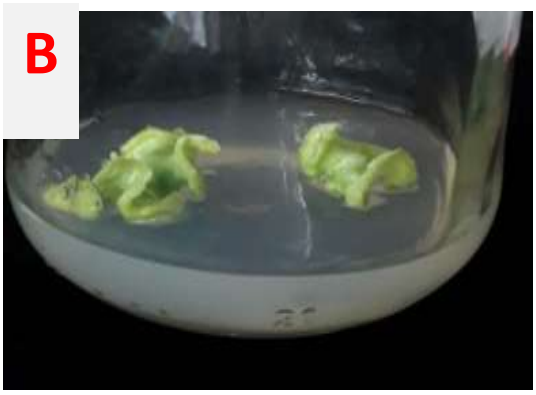
4.1.2. Establishment of callus from leaves of Nicotiana tabacum

Leaves excised from *in vitro* germinated seedlings were inoculated on MS medium containing three different concentrations and combinations of hormones for callus induction. Results for callus induction are described in Table 5.

Among the different media combinations tried, T_1 (MS + 1 mgL^{-1} NAA + 1 mgL^{-1} BA) medium showed earlier response and maximum callus induction percentage. The different stages of callus induction are shown in Plate 3. The callus was green and turned brown when maintained in the same medium for more than forty days (Plate 4).

Compared to T_1 the callus induction response was delayed in T_2 (MS + 2 mgL^{-1} IAA + 0.5 mgL^{-1} BA). Fifty per cent of leaf explants showed response by tenth day of inoculation and there was cent percent germination by 14th day. The callus was green in colour and friable. Shoot induction and regeneration was initiated from the callus within two weeks if maintained in the same medium (Plate 5).

Plate: 2 Tobacco plantlets germinated from seeds inoculated in basal MS medium



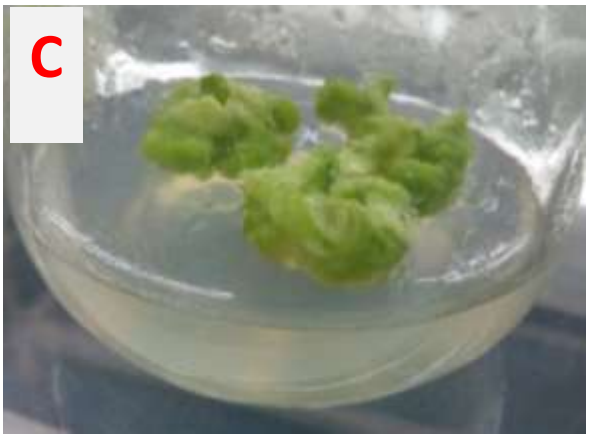


Plate:3 Stages of callus induction of *Nicotiana tabacum* in MS + 1 mgL^{-1} NAA + 1 mgL^{-1} BA medium, A: After 3 days B: After 7 days C, D: After 14 days

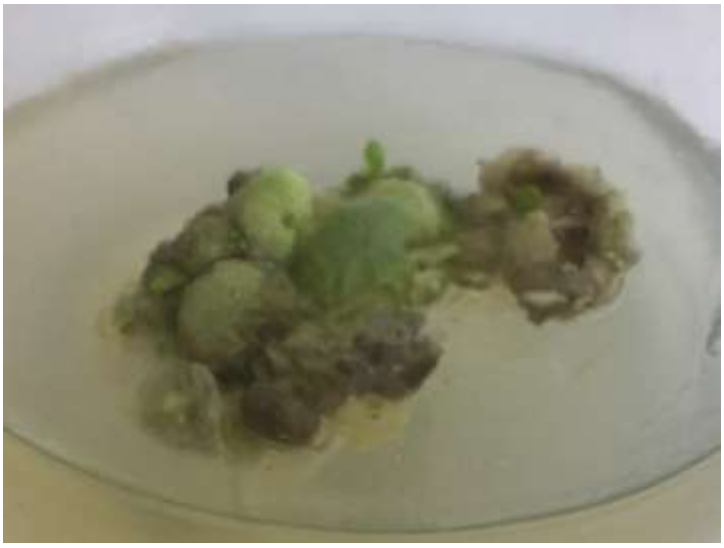


Plate: 4 Callus maintained in MS + 1 mgL^{-1} NAA + 1 mgL^{-1} BA medium (after 45 days)

Plate: 5 Shoot induction in MS + 2 mgL^{-1} IAA + 0.5 mgL^{-1} medium

Table: 5 Callus induction percentage from leaves of *Nicotiana tabacum*

Treatment	Composition of hormones in MS medium	Callus induction percentage one week after inoculation	Callus induction percentage two weeks after inoculation	Colour and nature of callus
T_1	1 mgL^{-1} NAA + 1 mgL^{-1} BA	100	100	Green
T_2	2 mgL^{-1} IAA + 0.5 mgL^{-1} BA	50	100	Green
T_3	0.2 mgL^{-1} 2,4-D + 0.19 mgL^{-1} NAA	0	0	NA

There was no callus induction in T_3 (MS + 0.2 mgL^{-1} 2, 4-D + 0.19 mgL^{-1} NAA).

4.1.3. Organogenesis and regeneration of Nicotiana tabacum

4.1.3.1. Shoot induction and proliferation

Callus established in T_1 medium was transferred to regeneration medium (MS medum + 1 mgL^{-1} BA + 0.5 mgL^{-1} GA3 + 0.25 mgL^{-1} IAA) after two weeks. The calli exhibited cent per cent response by fifth day of transfer to regeneration medium. The minimum number of shoots regenerated per callus was three and the maximum was five (Plate 6).

Plate: 6 Regeneration of shoots from callus of *Nicotiana tabacum* in MS medium + 1 mgL^{-1} BA + 0.5 mgL^{-1} GA3 + 0.25 mgL^{-1} IAA

4.1.3.2. In vitro root induction

Regenerated shoots were transferred to half strength MS medium containing 0.1 mgL^{-1} IAA. The shoots exhibited cent per cent rooting response. Root initiation was observed on 8^{th} day of transfer to rooting medium and all the shoots rooted by fourteenth day of transfer (Plate 7).

4.1.3.3. Hardening

The rooted plants showed cent per cent establishment when transferred from culture bottles into pro-trays containing potting mixture which consisted of autoclaved soil and vermicompost in the ratio 2:1.

4.2. PREPARATION OF PLANT MATERIAL FOR BOMBARDMENT

4.2.1. Sowing of tobacco seeds in pro-trays

Seeds of *Nicotiana tabacum* exhibited 95% germination after four days when sown in pot trays. Leaf explants were collected from two month-old plantlets maintained in pro-trays (Plate 8).

4.2.2. Maintenance of bacterial culture for plasmid isolation

Agrobacterium tumefaciens strain (EHA 105) with plasmid vector pBI121 and without plasmid vector pBI121 showed visible turbidity in the medium LB + rifampicin + kanamycin and LB + rifampicin respectively after 48 h of incubation in the shaker at 28^0C and 120 rpm.

4.3 ISOLATION OF PLASMID VECTOR FOR BOMBARDMENT

4.3.1. Quantification and quality check of plasmid DNA

The quantification and purity assessment of the plasmid isolated from *Agrobacterium tumefaciens* EHA 105 strain containing pBI121 vector was done using a UV-visible spectrophotometer and the results are presented in Table 6.

Plate: 7 Rooting of *in vitro* regenerated shoots in half strength MS media containing 0.1 mgL^{-1} IAA

Plate: 8 Germination of tobacco seeds in pro-tray

Table: 6 Quantification and Quality of isolated plasmid pBI121 DNA vector

Sl. No.	Sample	Absorbance at 260 nm	Absorbance at 280nm	Quality A260/A280	Concentration of DNA (μgmL^{-1})
1	Replication 1	0.0920	0.0514	1.789	2.76
2	Replication 2	0.0938	0.0521	1.800	2.81
3	Replication 3	0.1874	0.1017	1.842	2.81

Electrophoresis of the isolated plasmid vector DNA on 1% agarose gel electrophoresis exhibited good quality band corresponding to 14.6 kb indicating that the plasmid vector isolated was pBI121 (Plate 9).

4.3.2. Restriction digestion of plasmid

pBI121 as per the vector map contains one site each for *Eco*RI and *Hin*dIII. Hence, for confirmation of the isolated plasmid, double restriction digestion reactions were carried out with *Eco*RI and *Hin*dIII and the size of the fragments were observed by agarose gel electrophoresis.

Double digestion with *Hin*dIII and *Eco*R1 yielded two DNA fragments of size approx. 12-13 kb and 3 kb in agarose gel electrophoresis (Plate 10).

4. 4. BIOLISTIC TRANSFORMATION

4.4.1. Biolistic transformation of Nicotiana tabacum with gold micro-carrier

Bombardment of gold microcarriers of 0.6 µm were performed on *Nicotiana tabacum* leaves using PDS-1000/He apparatus at varied He pressure (450, 650, 900 and 1100 psi) and target distances (6 and 9 cm) to determine the optimum parameters

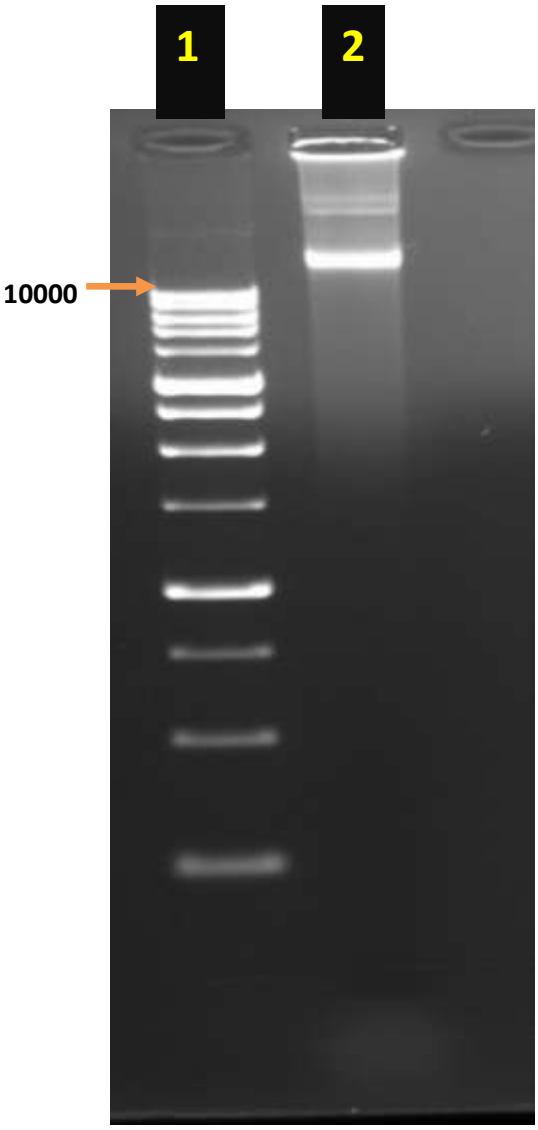


Plate: 9 Gel image of plasmid pBI121

Lane 1: 1kb DNA ladder,

Lane 2: pBI121

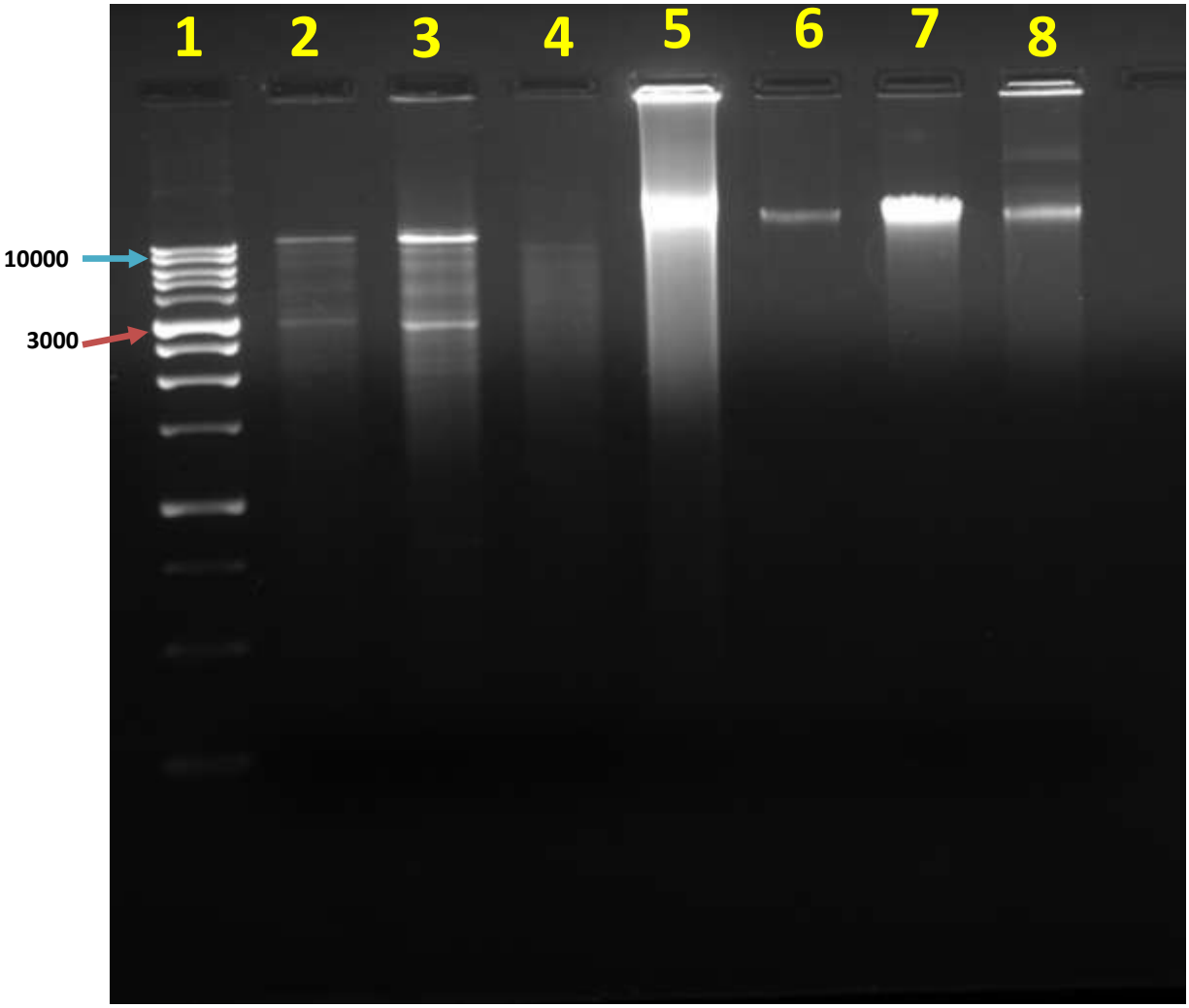


Plate: 10 Gel image of pBI121 double digested with *Eco*R1 and *Hin*dIII

Lane 1- 1kb DNA ladder

Lane 2, 3- Digested pBI121

Lane 4- Digested EHA105

Lane 5, 7, 8- Undigested pBI121

Lane 6- Undigested EHA105

of gold microcarriers and for comparison with silver nanoparticles. Vacuum pressure was maintained at 28 inch Hg.

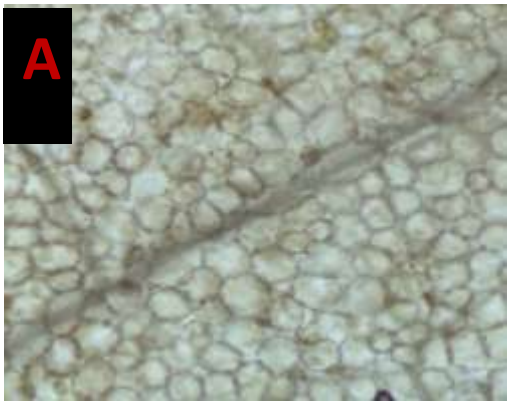
Transient expression of the GUS (uidA) gene was indicated by blue spots in all the samples except control (gold micro-carriers without DNA) and non-bombarded tissue. The number of GUS positive blue spots counted per bombardment after 48 h are presented in Table 7.

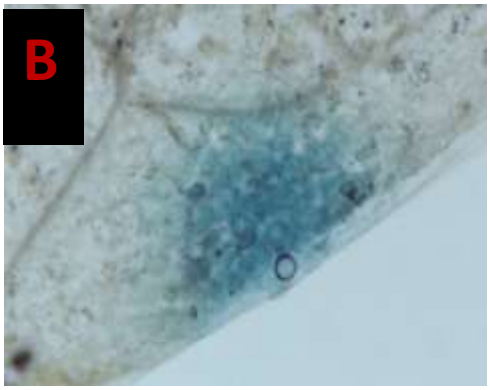
Variation in He pressure showed an effect on biolistic bombardment using gold microcarriers. Helium pressure of 650 psi exhibited highest transient expression of GUS gene followed by 450 psi, 900 psi and 1100 psi (Table 8) and was statistically significant. Target distance of 9 cm showed higher GUS gene expression than that of 6 cm (Table 8). However, it was not statistically significant.

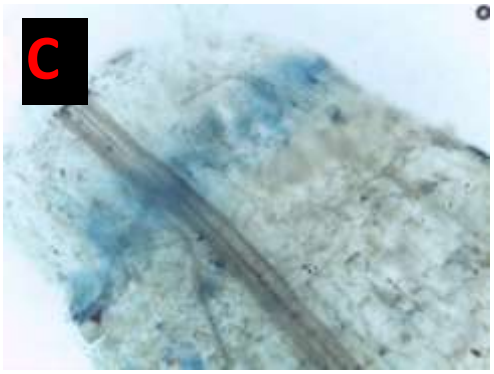
Interaction analysis of the different parameters revealed that He pressure of 650 psi and target distance of 9 cm exhibited maximum transient expression of GUS gene when gold microcarriers were used for bombardment of leaves of *Nicotiana tabacum*. The results were statistically significant when compared to other combinations (Table 9).

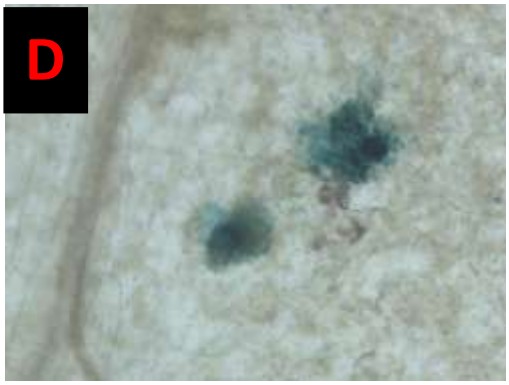
Table: 7 Number of GUS positive blue spots per bombardment in the leaves of *Nicotiana tabacum* bombarded with gold microcarriers under varied helium pressures and target distances

Average No. of GUS positive blue spots							
		Target distance					
Sl. No.	**Helium pressure**	**6 cm**			**9 cm**		
		R1	**R2**	**R3**	**R1**	**R2**	**R3**
1	**1100 psi**	5	9	8	9	14	12
2	**900 psi**	15	18	13	8	16	13
3	**650 psi**	30	28	30	65	71	67
4	**450 psi**	46	54	49	17	11	13









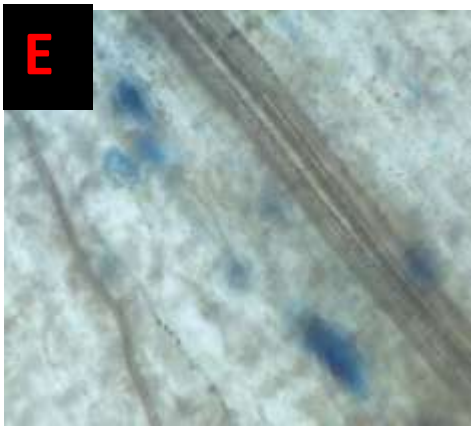


Plate: 11 GUS stained blue spots on leaves bombarded with gold microcarriers observed under microscope (Magnification 100X) A-Control; B- 650 psi & 6 cm; C- 450 psi & 6 cm; D- 650 psi & 9 cm; E- 1100 psi & 6 cm

Table: 8 Individual effect of variation of helium pressure and target distance during bombardment of leaves of *Nicotiana tabacum* with gold microcarriers

Helium Pressure	**Average No. of GUS positive blue spots**
1100 psi	9.500
900 psi	13.833
650 psi	48.500
450 psi	31.667
SE(m)	1.205
CD (0.05)	3.643
Target Distance	**Average No. of GUS positive blue spots**
6cm	25.417
9cm	26.333
SE(m)	0.852
CD (0.05)	N/A

Table: 9 Interaction effect of variation of helium pressure and target distance during bombardment of leaves of *Nicotiana tabacum* with gold microcarriers

He pressure & distance	**Average No. of GUS positive blue spots**
1100 psi, 6 cm	7.333
900 psi, 6 cm	15.333
650 psi, 6 cm	29.333
450 psi, 6 cm	49.667
1100 psi, 9 cm	11.667
900 psi, 9 cm	12.333
650 psi, 9 cm	67.667
450 psi, 9 cm	13.667
SE(m)	1.704
CD (0.05)	5.152

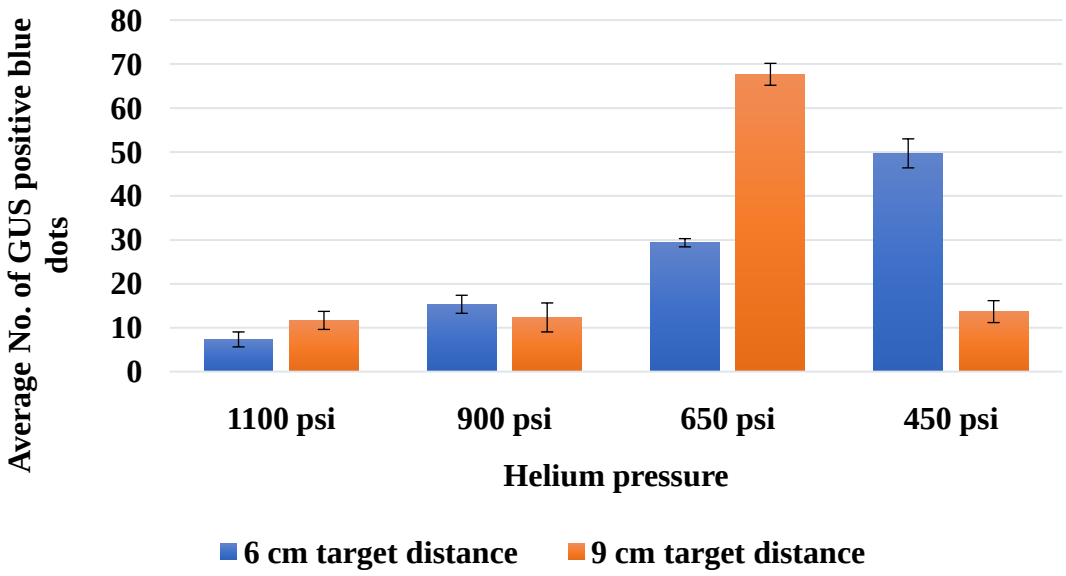


Fig 2: Effect of transient GUS expression with varied helium pressures and target distances for gold microcarriers

4.3.2. Biolistic transformation of Nicotiana tabacum with silver nanoparticles

4.3.2.1. Optimisation of parameters for biolistic bombardment of silver nanoparticles

Bombardments were performed on *Nicotiana tabacum* leaves using PDS-1000/He apparatus and silver nanoparticles of size 100 nm by varying the concentration of silver nanoparticles (0, 0.01, 0.05, 0.5, 1, 2, 10, 20, 100 mgL^{-1}), He pressure (450, 650, 900 and 1100 psi) and target distance (6 and 9 cm) to test the efficacy of silver nanoparticles as carriers for biolistic bombardment of *Nicotiana tabacum*. Vacuum pressure was maintained at 28 inch Hg.

Transient expression of the GUS (uidA) gene was indicated by blue spots in all the samples except control (silver nanoparticles without DNA coating) and non-bombarded tissue. The numbers of GUS positive blue spots counted per bombardment are presented in Table 10.

Among the different concentrations of AgNPs tried as carriers for bombardment, T6 (10 mg L^{-1}) showed the maximum number of GUS positive blue spots (Fig 3) and was statistically significant compared to other treatments.

Table: 10 Comparison of GUS positive blue spots in the leaves of *Nicotiana tabacum* bombarded with different concentrations of AgNPs

Treatment	R1	R2	R3	Average No. of GUS positive blue spots	CD
T1	3	4	5	4.000	cd
T2	1	0	1	0.667	e
T3	1	2	1	1.333	de
T4	2	4	2	2.667	cde
T5	4	3	6	4.333	c
T6	29	32	30	30.333	a
T7	13	12	9	11.333	b
T8	5	8	2	5.000	c
CD (0.05) = 2.804					

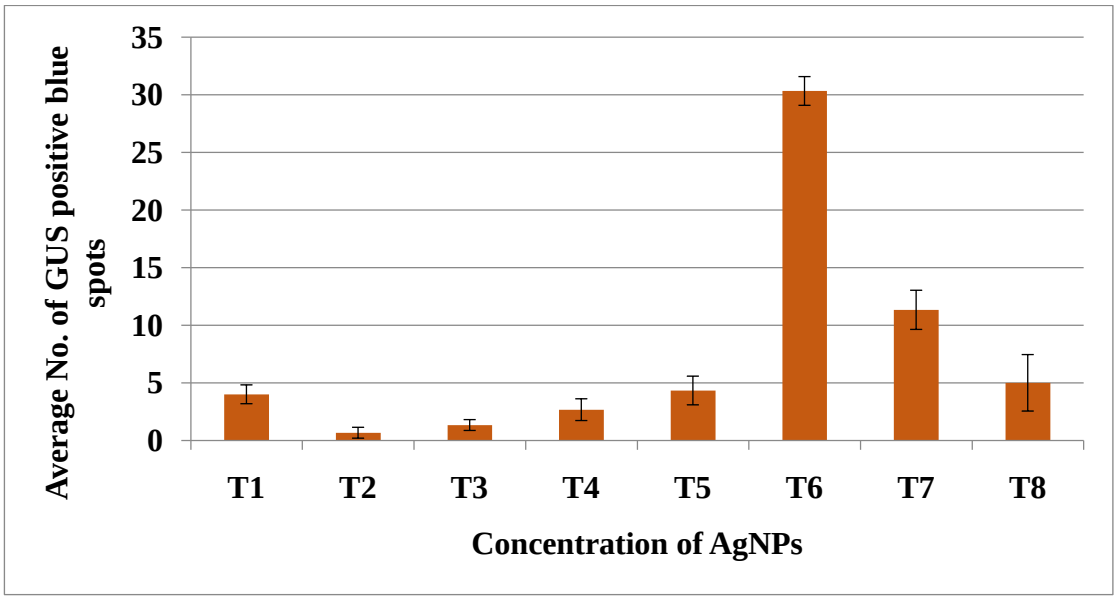


Fig: 3 Effect of different concentrations of AgNPs in bombardment of tobacco leaves

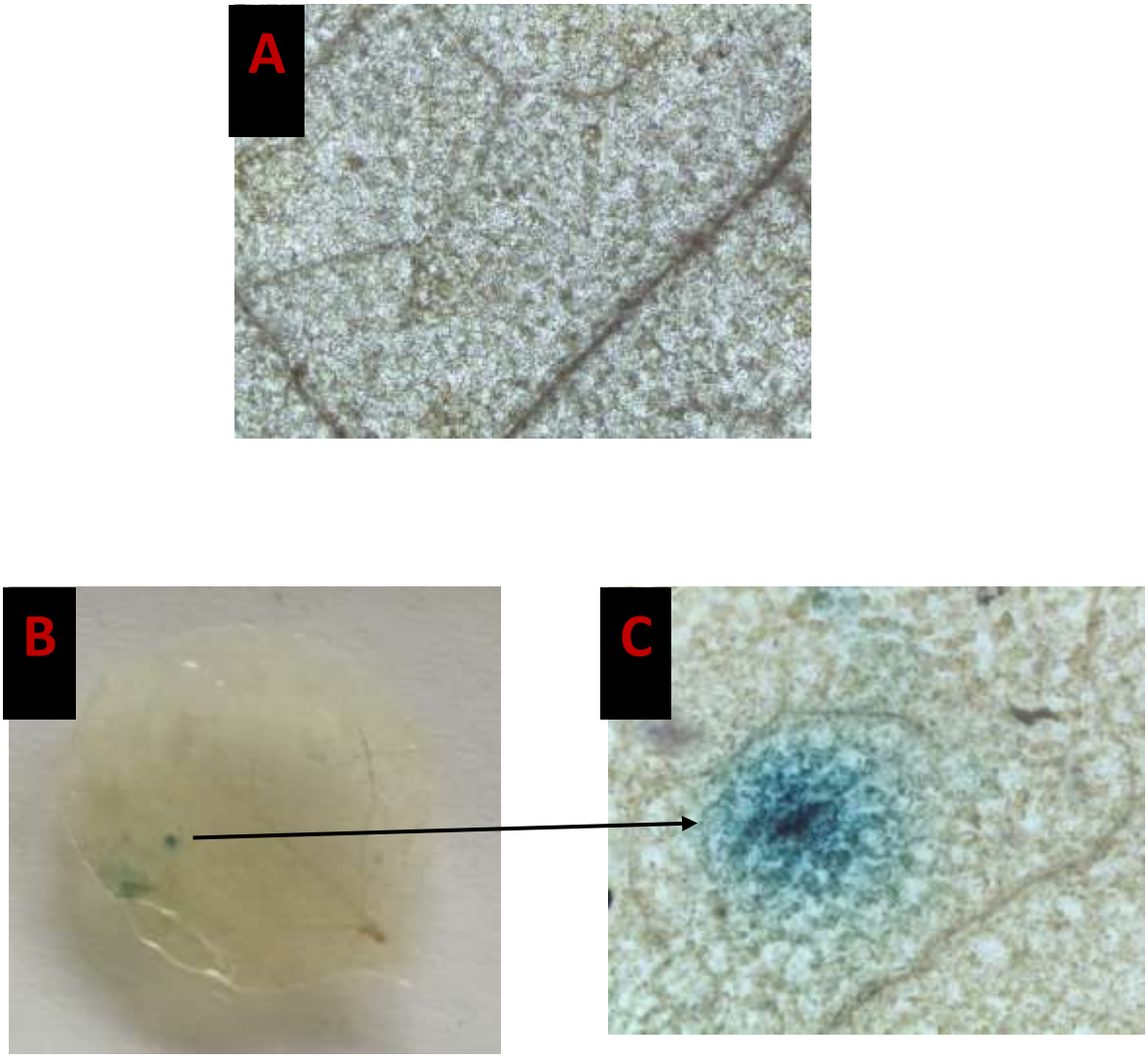


Plate: 12 GUS stained blue spots on leaves bombarded with AgNPs

A-Control (100X) (without pBI121) B- Leaf bombarded with10 mg/L AgNPs with pBI121 C- 10 X magnified view of B

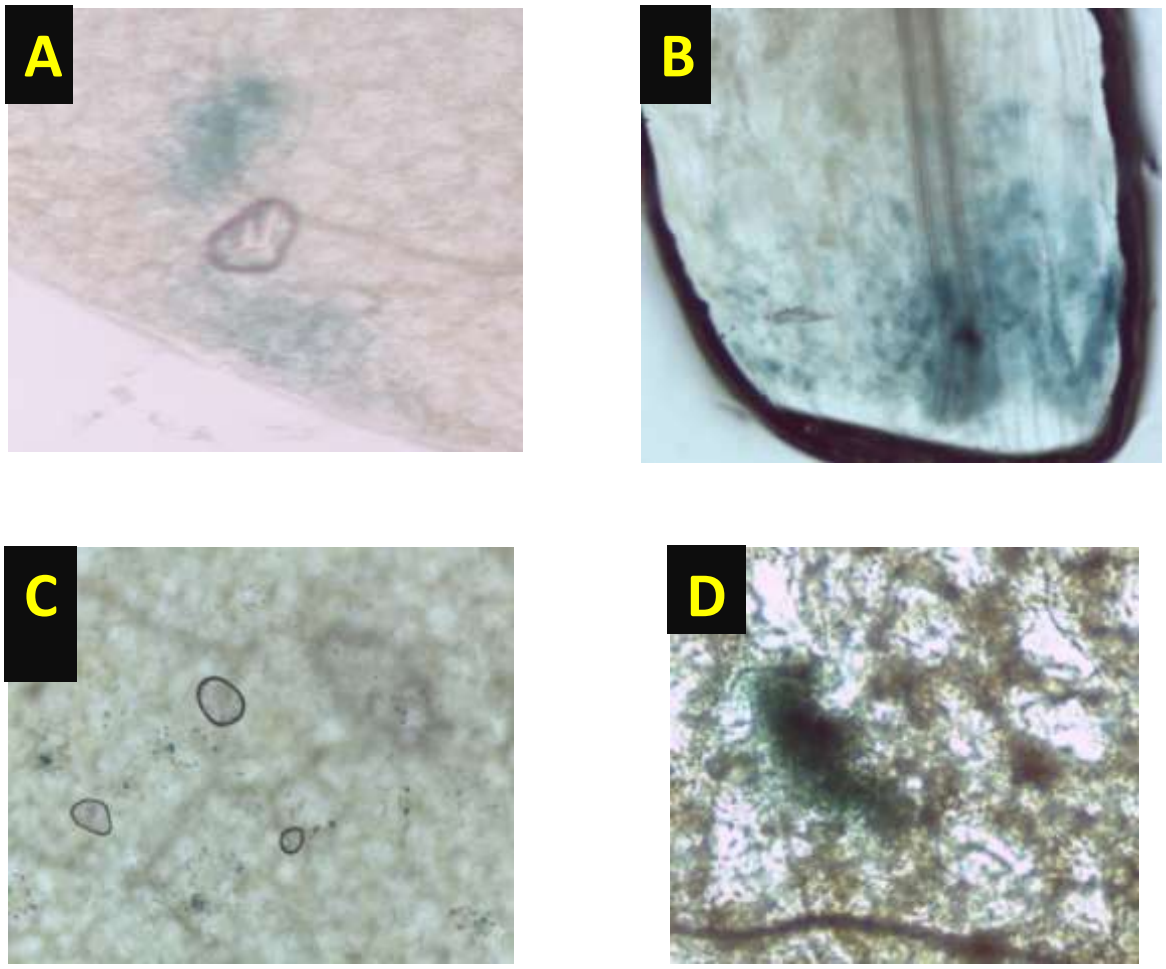


Plate: 13 GUS stained blue spots on leaves bombarded with different concentration of AgNPs observed under microscope 100X

A-0.01 mg/L of AgNPs; B-2 mg/L of AgNPs; C-10 mg/L of AgNPS;

D-20 mg/L AgNPs

Difference in transient expression of GUS positive cells was observed under varying He pressure and target distance, while using silver nanoparticles as carriers of bombardment. Among the varying helium pressures tried, 900 psi exhibited maximum number of GUS positive cells followed by 1100, 650 and 450 psi (Fig 4). The results were statistically significant. Variation in target distance also showed significant difference in the number of GUS positive cells (Table 12). Target distance of 6 cm showed higher GUS gene expression than target distance of 9 cm and was statistically significant (Table 12).

Table: 11 Number of GUS positive blue spots in the leaves of *Nicotiana tabacum* bombarded with AgNPs under varied helium pressures and target distances

Average No. of GUS positive of AgNPs bombardment of leaves							
		Target distance					
Sl. No.	**Helium pressure**	**6 cm**			**9 cm**		
		R1	**R2**	**R3**	**R1**	**R2**	**R3**
1	**1100 psi**	29	32	30	19	4	11
2	**900 psi**	75	87	81	11	15	12
3	**650 psi**	3	13	8	5	3	3
4	**450 psi**	37	12	18	13	13	11

Table: 12 Individual effect of variation of Helium pressure and target distance during bombardment of leaves of *Nicotiana tabacum* with AgNPs

Helium pressure	**Average No. of GUS positive blue spots**
1100 psi	20.833
900 psi	46.833
650 psi	5.833

450 psi	17.333
SE(m)	2.487
CD (0.05)	7.522
Target distance	**Average No. of GUS positive blue spots**
6cm	35.417
9cm	10.000
SE(m)	1.759
CD (0.05)	5.319

Interaction effects of variation of helium pressure and target distance while using AgNPs as carriers for biolistic bombardment revealed that combination of 900 psi and 6 cm yielded the highest transient GUS expression (Table 13) among all the combinations tried and the result was statistically significant.

Table: 13 Interaction effect of variation of Helium pressure and target distance during bombardment of leaves of *Nicotiana tabacum* with AgNPs

He Pressure and distance	**Average No. of GUS positive blue spots**
1100 psi, 6 cm	30.333
900 psi, 6 cm	81.000
650 psi, 6 cm	8.000
450 psi, 6 cm	22.333
1100 psi, 9 cm	11.333
900 psi, 9 cm	12.667
650 psi, 9 cm	3.667
450 psi, 9 cm	12.333
SE(m)	3.518
CD (0.05)	10.637

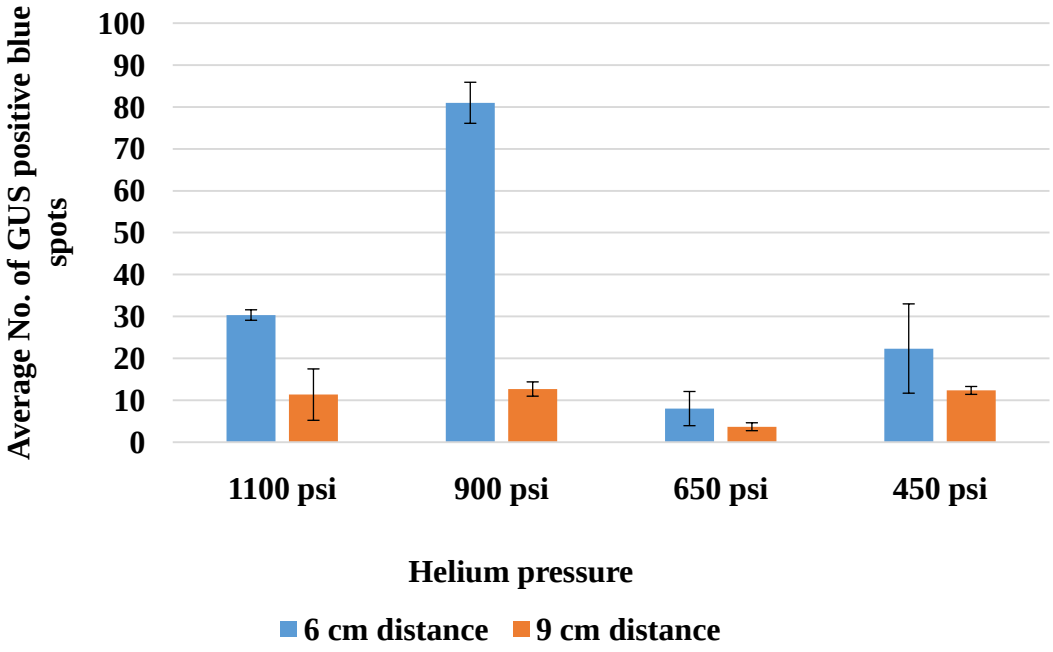
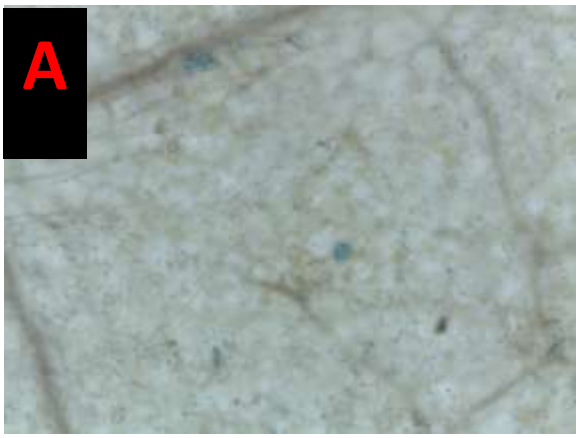
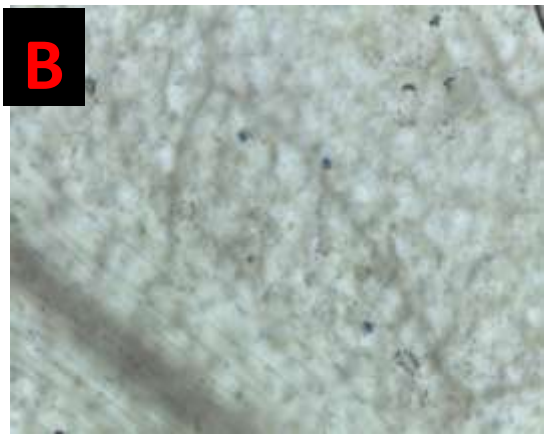


Fig: 4 Effect of transient GUS expression with variation in Helium pressure and target distance using AgNPs as carriers





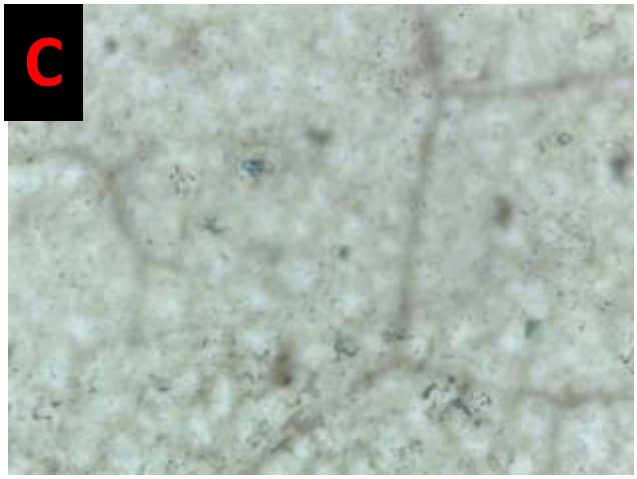


Plate: 14 GUS stained leaves bombarded with different helium pressure observed under microscope (Magnification 100X)

A- Bombarded at 450 psi, 6 cm
B- Bombarded at 900 psi, 6 cm
C- Bombarded at 1100 psi, 6 cm

4.4. COMPARISON OF TRANSFORMATION EFFICIENCY WHILE USING GOLD MICROPARTICLES AND AGNPs AS CARRIERS OF DNA FOR BIOLISTIC BOMBARDMENT

Comparison of gold microparticles and AgNPs as carriers of DNA during biolistic bombardment of leaves indicated that AgNPs at 900 psi and target distance of 6 cm exhibited more transient GUS expression than all othercombinations of gold microparticles under varying He pressure and target distance. The result of transient expression using gold microparticles for bombardment and replacement with silver nanoparticles was statistically significant (Table 14).

Table: 14 Comparison of efficiency of gold particles and AgNPs as carriers during biolistic bombardment into *Nicotiana tabacum* leaves

	Gold microparticles	**AgNPs**	**T-test**
Average	67.667	81.000	**3.430**
Standard Deviation	3.055	6.000	T - Table (0.05) 2.776
Variance	9.333	36.000	

3.5. SCREENING FOR TRANSFORMED CALLI

The bombarded calli (from the treatments of gold and silver showing maximum transformation efficiency) along with control were screened for transformants by transferred to MS medium supplemented with selection agent kanamycin.

The control calli started drying within one week of transfer to selection medium whereas the calli bombarded with gold/silver as carriers survived in the selection medium and remained green (Plate 15 & 16). Percentage of callus induction

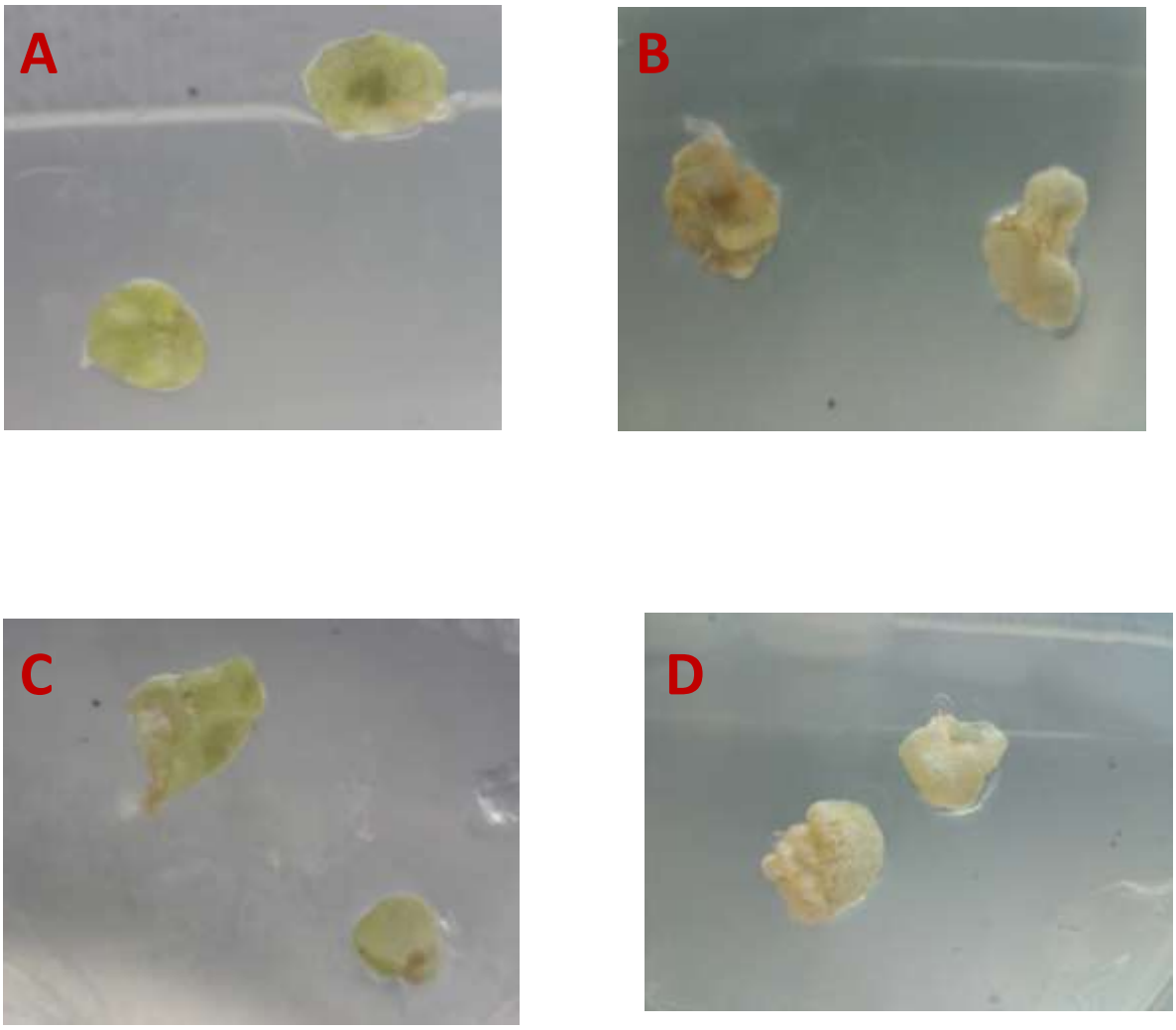


Plate 15: Leaves bombarded with gold microcarriers and transferred to callus induction medium with kanamycin

Bombarded with gold microcarriers without pBI121 A- after 48 hours of inoculation; B- After 5 days

Bombarded with gold microcarriers with pBI121 C- after 48 hours of inoculation; D- After 5 days

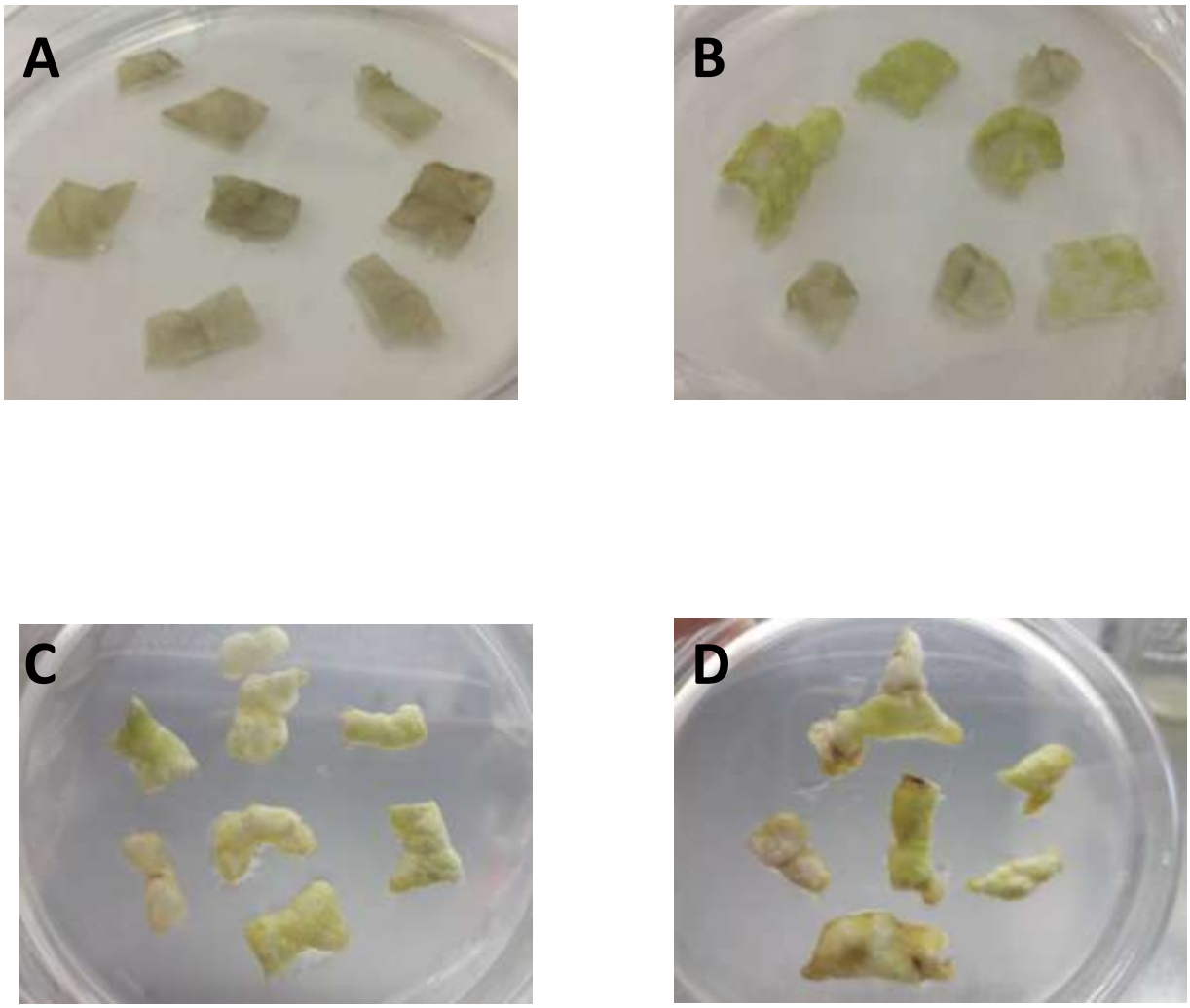


Plate: 16 Leaves bombarded with AgNPs and transferred to callus induction medium with selection agent.

A-Control (Bombarded with AgNPs without pBI121); after 3 days
B - Bombarded with AgNPs and pBI121; after 3 days

C- After 5 days of B,

D- After 7 days of B

of the leaf discs bombarded with DNA using gold microparticles and with silver nanoparticles as carriers exhibited 65% and 70% respectively.

The green calli were transferred to regeneration medium after two weeks for induction of shoots and further rooting and hardening.

Discussion

5. DISCUSSION

In biolistic transformation, foreign DNA is directly delivered into intact plant cells without biological constraints or host limitations (Altpeter *et al*., 2005). It is a popular method of incorporating DNA into cells that are difficult to transfect using traditional methods. In this method the DNA is usually coated onto the surface of micron sized carriers and it elutes off from the microcarrier once it enters inside the cell. This foreign DNA reaches the nucleus and is incorporated in the host chromosomes resulting in transient expression. Cost of consumables such as gold microcarriers is one of the major factors limiting the wide spread use of biolistic transformation (Wirth and Wahle, 2003; O'Brien and Lummis, 2011).

Many researchers have reported nanoparticles mediated gene delivery through biolistic in plants. Vijayakumar *et al*. (2000) reported carbon supported gold nanoparticles delivered DNA more efficiently into *Nicotiana tabacum*, *Oryza sativa* and *Leucaena leucocephala* compared to regular gold particles using a gene gun. Gold nanoparticles were reported to be successful in biolistic delivery gene in rice (Mortazabi and Zohrabi, 2018). Torney *et al*. (2007) observed green fluorescent protein (GFP) gene expression when mesoporous silica nanoparticles were used to transport DNA into isolated plant cells and leaves. There are no reports on the use of silver nanoparticles for biolistic transformation of plants. Efficient use of silver nanoparticles can aid in increased transformation efficiency of plant cells with comparatively reduced cost. Hence, the present study was undertaken with the objective to evaluate the efficacy of silver nanoparticles as carriers for gene delivery in *Nicotiana tabacum* using biolistic method.

Success of plant transformation systems is based on the ability of totipotency and regeneration. In order to produce stable and viable transgenic plants, many different explants like leaf discs, shoot apices, stem segments, cells suspension, hypocotyls, epicotyls, immature embryos, anther and mature intact seeds, have been tested for their regeneration capacity (Ruma *et al*., 2009; Zuraida *et al*., 2010; Liu *et al*., 2011; Wu *et al*., 2016; Yasybaeva *et al*., 2017; Santos-Ballardo *et al*., 2019 Furukawa *et al*., 2020; Rustgi *et al*., 2020).

In the present study, biolistic transformation was carried out in leaf discs of *Nicotiana tabacum* belonging to Family Solanaceae as it is considered as the fruit fly of plant kingdom and is reported to be widely used for biolistic based genetic transformation and regeneration from callus to whole plantlets (Briza *et al*., 2013; Okuzaki *et al*., 2013; Wang *et al*., 2016; Yasybaeva *et al*., 2017). In accordance with previous reports (Dixit *et al*., 2016) cent percent callus induction was observed in *Nicotiana tabacaum* in the present study in MS medium supplemented with 1 mg L^{-1} NAA and 1 mg L^{-1} BA. The medium MS + 2 mg L^{-1} IAA + 0.5 mg L^{-1} BA reported by Klein *et al*. (1988) also showed callus induction in the present study. However, the medium MS + 0.2 mg L^{-1} 2, 4-D + 0.19 mg L^{-1} NAA reported by Opatrny and Opatbna (1976) did not show callus induction in the present study. The variation from previous reports may be due to the variation in the endogenous level of hormones in the cultured tissues. According to Duclercq *et al*. (2011) the combinations of endogenous (produced in the tissues) and exogenous (supplemented in the medium) hormones defines tissue response.

Cent percent shoot induction was obtained in the medium (MS media + 1 mg L^{-1} BA + 0.5 mg L^{-1} GA_3 + 0.25 mg L^{-1} IAA) reported by Dixit *et al* (2016). Dixit *et al*. (2016) have reported 82 per cent shoot induction in their study. Callus mediated shoot generation is reported in hormonal combinations with higher concentration of cytokinin and lower concentrations of auxins in tobacco (Fajkus *et al*., 1998). Dhaliwal *et al*. (2004) have also reported that shoot induction was successful in tobacco in medium supplemented with benzyl adenine. According to Ali *et al*. (2018) also, maximum number of shoot induction was obtained in medium with higher cytokinin auxin ratio.

Cent percent root initiation was observed from the regenerated shoots within two weeks in half strength MS medium supplemented with 0.1 mg L^{-1} IAA. Dixit *et al*. (2016) observed 99% root induction in the same medium within two weeks. *In vitro* rooting from callus cell cultures were observed in MS medium supplemented with 2 mg L^{-1} IAA and 0.2 mg L^{-1} kinetin in tobacco by Peters *et al*. (1974). According to Xu *et al*. (2008) rate of root induction in *Malus zumi* increased by

increasing the concentration of IAA or IBA from 60 to 120 mg L^{-1} and reduced on further increasing the concentration to 240 mg L^{-1}.

Choice of an appropriate vector is important in biolistic transformation. The vector must possess appropriate reporter or selectable marker genes with suitable promoters. Plasmid vector pBI121 of size 14.6 kb is reported to be widely used for plant transformation and hence was selected for the present study. It is reported for biolistic transformation in *Scoparia dulcis* L. (Srinivas *et al*., 2016), mothbean *Vigna aconitifolia* (Kamble *et al*., 2003) and *Paulownia elongata* (Castellanos-Hernandez *et al*., 2009).

Plasmid pBI121 was isolated by alkaline lysis method as described by Birnboim and Dolly (1979). Bennett *et al*. (1986) and Chowdhury and Akaike (2005) have reported good yield and quality of plasmid by following this method. In the present study also quantity and quality of plasmid was good. In order to confirm the vector pBI121, the plasmid was double-digested with *Hin*dIII and *Eco*RI. Double digestion with *Hin*dIII and *Eco*R1 produced two DNA fragments of size approx. 12-13 kb and 3 kb. As *Hin*dIII and *Eco*R1 restriction enzyme have only one recognition site each within the plasmid, the result indicates the good quality of the isolated plasmid. The 3.0 kb fragment carries the cauliflower mosaic virus (CaMV) 35S promoter (835 bp), GUS coding region (1812 bp) and nopaline synthase (NOS) terminator (253 bp) and the 12–13 kb fragment contains the neomycin phosphotransferase II (NPTII) selection marker, kanamycin resistant gene, T-DNA left and right border (Jefferson *et al*. 1987b; Chen *et al*., 2003).

Reporter genes are powerful tools to estimate the efficiency of genetic transformation. pBI121 has *gusA* gene which is one of the most widely used reporter genes which encodes the enzyme ß-glucuronidase. Optimum pH of *E. coli* GUS is neutral and plant GUS is acidic. This optimum pH difference can restrict the endogenous GUS expression of plants with negligible interference (Alwen *et al*., 1992; Sudan *et al*., 2006). The unbombarded leaf discs did not show any GUS

activity, whereas blue spots were observed in all the bombarded tissues with gold microcarriers/silver nanoparticles indicating transient expression of the gene.

Optimization of biolistic parameters is a pre-requisite for developing an efficient and reliable genetic transformation for any crop species (Birch and Bower, 1994; Heiser, 1995). Gold and tungsten microcarriers are usually used for biolistic transformation. Tungsten is reported to be toxic to the cells (Russell *et al*., 1992; Adamakis *et al*., 2012). Tungsten causes catalytic attack to the DNA bound to it and subsequently can degrade the DNA. Toxicity of the tungsten is the major limitation for use of tungsten as microcarriers as it produces cell injury. Stable transformants are reported to be threefold higher using gold microcarriers than tungsten particles (Russell *et al*., 1992). Gold is non-toxic to cells. But its disadvantage is that it is highly expensive. Moreover, gold is unstable in sterile aqueous suspensions and agglomerates irreversibly over a period of time (Sanford *et al*., 1993). Hence, in the present study gold microcarriers were used as a positive control for comparison of transformation efficiency of silver nanoparticles.

For successful gene delivery microcarriers carrying the DNA must effectively penetrate into the target tissue. Some of the common parameters optimized for gene delivery into plants are size of micro-carrier, helium pressure, target distance, vacuum inside the chamber, the concentration of DNA used per bombardment, types of target tissue, osmotic treatment prior to transformation and explant age (Ingram *et al*., 1999; Kadir and Parveez, 2000; Vidal *et al*., 2003; Ivic-Haymes and Smigocki, 2005; Indurker *et al*. 2007).

Size of gold microcarrier is generally chosen based on the size of the target cells and is approximately one-tenth the diameter of the cell (Sanford *et al*., 1993). Gold particles of 0.6 µm size is reported to show increased nuclear transformation efficiency compared to gold particles of 1 µm size in maize (Randolph-Anderson *et al*., 1997), wheat (Rasco-Gaunt *et al*., 2001) and soybeans (Khalafalla *et al*., 2005). Hence, gold microcarriers of 0.6 micron size were used in the present study and they exhibited successful transformation. Gold particles of size 0.6 µm are reported to

exhibit five-fold increased nuclear transformation efficiency in maize callus, ten-fold in *S. cerevisiae* and two-and-a-half-fold increase in *Clamydomonas* compared to 1µm gold particles (Randolph-Anderson *et al*., 2015). According to Bespalhok Filho *et al.* (2003) large gold particles of 1.5-3.0 µm size exhibited poor transient expression of GUS gene.

Helium pressure and target distance are reported to affect the relative velocity of the microcarrier and penetration capacity into the target tissue (Southgate *et al*., 1995). In the present study varied helium pressure and target distances were tried and parameters were optimised for gold microparticles for being used as a positive control for comparison with silver nanoparticles as carriers.

Helium is a light gas and expands at faster rate which can also impart higher velocities to lightweight macro-carriers (Sanford *et al*., 1993). In the present study, different helium pressures *viz*., 450, 650, 900 and 1100 psi were tried for bombardment using gold micro particles. Helium pressure of 650 psi exhibited maximum transient expression with gold microcarriers followed by 450, 900 and 1100 psi in the present study. According to Yasybaeva *et al*. (2017) also the highest transformation efficiency was found at 650 psi in nuclear transformation in tobacco leaf. However, in a hardwood tree (*Paulownia elongata*), 450 psi was found to be the best helium pressure compared to 900 and 1350 psi when bombarded on leaf surface (Castellanos-Hernandez *et al*., 2009).

Higher helium pressures are reported to cause more injury to target cells due to acoustic and gas shock and reduction in transformation efficiency. According to Russell *et al*. (1992) efficiency of transformation does not correlate linearly with increase in Helium pressure. According to them high pressures beyond 1100 psi may cause irreparable damage to the target cells and then lead to death prior to GUS staining. Kirkkert (1993) observed that the ability to penetrate the microparticles into different cell layers or tissue is mostly depended on the propelling of helium force. Damage is markedly increased beyond 1000 psi in biological target (Sanford *et al*., 1993). High pressure of 1550 psi, was demonstrated to cause damage to the target

tissues in sorghum (Tadesse *et al*., 2003). In the present study also there was significant reduction in transient GUS expression at 1100 psi compared to 650 psi while using gold microcarriers for bombardment.

Helium pressures of higher psi are also reported to be successful in some cases. Yao *et al*. (1996) observed that there was no significant difference between Helium pressures of 1100, 1350, or 1550 psi in bombardment of tangelo embryogenic suspension cultures. Helium pressure 1550 psi gave the highest expression of transgene compared to 1100 and 1350 psi (Bespalhok Filho *et al*., 2003). However, in hop (*Humulus lupulus* L.) 1350 psi helium pressure was found to be optimum (Batista *et al*., 2008). According to Zuraida *et al*. (2010) in rice calli there was reduction in transient GUS expression during bombardment at lower (450 psi) and higher helium pressure (1300 psi and 1550 psi) and the optimum was 1100 psi. Lower expression at lower pressure may be due to poor penetration capability of the microcarrier towards the target tissue and higher pressure bombardment leads to injuries to target tissue.

Effect of different target distances (distance between the macrocarrier and the target tissue in the bombardment chamber) in transient GUS expression while using gold microcarriers for bombardment were analysed. Target distance of 9 cm showed higher GUS gene expression than target distance of 6 cm. Reduction in cell damage due to increase in target distance may be a factor for increased expression 9 cm observed in the present study. Target distance of 9 cm is reported to be optimum in garden balsam (*Impatiens balsamina*) (Taha *et al*., 2009). It is also reported to show significantly higher transient expression than other distances in wheat, castor, cowpea, mothbean and olive (Kamble *et al*., 2003; Ikea *et al*., 2003; Odake, 2004; Sailaja *et al*., 2008; Perez-Barranco *et al*., 2009). However, target distance of 7 cm was found optimum for sugarcane and hardwood tree (Paulownia elongata) (Jain *et al*., 2007; Castellanos-Hernandez *et al*., 2009). According to Batista *et al*. (2008) target distance of 12 cm was optimum in hop (Humulus lu pulus L). Tissue dislocation and no GUS expression is reported in extremely reduced target distance of 2. 5 cm in all types of explants of tomato *viz*., shoot tips, hypocotyls and cotyledons (Ruma *et al*., 2009).

From the interaction study, it is evident that distance and helium pressure shows a significant relation with transient GUS expression in the present study. Maximum transient GUS expression was observed at 9 cm for 650 psi and at 6 cm for 450 psi. Longer distances were observed to be optimum for higher pressures whereas shorter distances were optimum for lower pressures. The expression level of GUS gene is reported to be maximum at 10 cm for 5 atm, 12 cm for 6 atm and 14 cm for 8 atm by Fadeev *et al*. (2006) in wheat. Target distance of 12 cm is reported to be optimal for 1350 psi helium pressure in hop (*Humulus lupulus* L.) (Batista *et al*., 2008). The highest GUS expression was observed at 9 cm for 900 psi and 12 cm for 1300 psi in rice calli (Zuraida *et al*., 2010). In few cases short distances are reported to be favourable at higher pressure for bombardment. According to Bespalhok Filho *et al*. (2003) helium pressure 1550 psi at a target distance of 6 cm exhibited highest expression of transgene compared to 1100 and 1350 psi in *Citrus*.

Cellular uptake of nanoparticles is generally dependent on its size, charge and surface properties (Hillaireau and Couvreur, 2009; Mailander and Landfester, 2009). Sizes of AgNPs range from 1-100 nm. AgNPs possess special and unique physical and chemical features due to their small size (Nowack and Bucheli, 2007). While attempting silver nanoparticles as carriers of DNA for biolistic bombardment size of AgNPs is very critical. It is reported that toxicity increases with decrease in size of AgNPs (Kim *et al*., 2012). Concentration of 0.4 nM AgNPs of size 9 nm is reported to inhibit the growth of *E. coli* by destabilizing the outer membrane (Lok *et al*., 2006). AgNPs of size 20-30 nm was found to be effective in inhibiting the growth of fungi *Bipolaris sorokiniana and Magnaporthe grisea* (Jo *et al*., 2009). AgNPs of 8 ± 2 nm size exhibited the highest antibacterial activity level compared to 47 ± 7 nm and 45 ± 5 nm in bacteria (Syu *et al*., 2014). AgNPs of size 8 ± 2 nm caused the maximum inhibition in cotyledon growth in Arabidopsis with maximum oxidative stress whereas particles of size 45 ± 5 nm exhibited growth promotion with the lowest induction of oxidative stress (Syu *et al*., 2014). Since AgNPs with smaller size (lesser than 50 nm) are reported to be toxic, AgNPs of 100 nm were chosen for the present study to be used as carriers of DNA into the cell during biolistic transformation.

AgNPs of different concentration ranging from 0.01-100 mgL^{-1} were tried as carriers of DNA for biolistic bombardment of *Nicotiana tabacum*. While using AgNPs for bombardment, T6 (10 mg L^{-1}) showed the maximum transient GUS expression. Reduction in GUS expression was observed under higher concentrations. Toxicity may be increased with increase in concentration of AgNPs due to increase in oxidative stress response. There are reports that increased concentration of AgNPs cause inhibitory effect on cells in most cases. AgNPs of 200 ppm (size 20-30 nm) are reported to be the most effective in inhibition of fungi *Bipolaris sorokiniana and Magnaporthe grisea* (Jo *et al*., 2009). Absolute inhibition (100%) was observed in 100 ppm concentration of AgNPs (7-25nm) and lowest level of inhibition at 10 ppm concentration in most of the fungi (Kim *et al*., 2012). According to Jaiswal *et al*. (2015) cell viability significantly reduced from 81% to 26% with increase in concentration of AgNPs from 4 to 33 ppm at 48 h exposure time. Lower GUS expression at lower concentration (0.01, 0.05, 0.5 and 1 mgL^{-1}) of AgNPs observed in the present study may be due to inadequate amount of AgNPs to coat plasmid DNA.

Effect of varying helium pressure while using AgNPs for bombardment was analysed. Helium pressure of 900 psi exhibited maximum transient expression with silver nanoparticles of size 100 nm. In the present study, reduction in size of carriers to nanoscale resulted in higher transient expression at higher helium pressure compared to gold microparticles. Bombardment of gold nanoparticles of size 50-100 nm is reported to be successful at 1100 psi Helium pressure in rice calli (Mortazavi1 and Zohrabi, 2018). Bombardment of mesoporous silica nanoparticles was successful at 650 psi in tobacco leaves and maize immature embryo (Torney *et al*., 2007).

In the present study, different target distances in transient GUS expression for AgNPs were analysed. Target distance of 6 cm showed higher GUS gene expression than target distance of 9 cm and was statistically significant. Target distance of 6 cm is reported to yield maximum transient GUS expression in broccoli (Puddephat *et al*., 1999), in cauliflower stem explants (Heiser, 1995) and in embryogenic axis of *Vigna anguiculata* (Ikea *et al*., 2003). Target distance of 6 cm yielded higher GUS expression than 9 and 12 cm in watermelon (Suratman, *et al*., 2010). Bombardment of gold nanoparticles of size 50-100 nm are reported to be successful at 5 cm target

distance in rice calli (Mortazavi1 and Zohrabi, 2018) and mesoporous silica nanoparticles was successful at 9 cm in tobacco leaves and maize immature embryo (Torney *et al*., 2007).

Transient GUS expression was reduced at 9 cm compared to 6 cm for AgNPs bombardment. This may be due to reduction in velocity of the carriers and increased flight distance resulting in reduction in penetration force thereby resulting in fewer cells receiving the plasmid DNA (Oard *et al*., 1990; Parveez *et al*., 1997). Marchant *et al*. (1998) reported that transient GUS expression was not detected when the target distance was beyond 10 cm and tissues with stronger cell wall require shorter target distances which enhance penetration. Sreeramanan *et al*. (2005) reported longer flight distance resulted in reduced expression of transient gene. GUS expression is reported to be significantly declined at 10 cm target distance in shoot tips, hypocotyls and cotyledons explants (Ruma *et al*., 2009).

The vacuum inside the biolistic chamber must be reduced as it affects residual vapour pressure in the biological sample (Sanford *et al*., 1993). Partial vacuum is a must because gas inside the chamber deaccelerates incoming microprojectiles. Partial vacuum of 28-29 inch Hg (about 710-740 mm Hg) is reported to be commonly used for biolistic transformation in tobacco (Arimura *et al*., 2001; Briza *et al*., 2013; Okuzaki *et al*., 2013), *Citrus* (Wu *et al*., 2016). Zuraida *et al*., 2010 observed the highest transient GUS expression in the rice callus under vacuum pressure of 28 inch Hg compared to 26 and 27 inch Hg vacuum level. In the present study also, vacuum pressure level inside the PDS-1000/He system maintained at 28-inch Hg, yielded successful transformation when gold microparticles or silver nanoparticles were used for transformation.

Optimum parameters for AgNPs and gold bombardment were different. There was hundred times reduction of size 100 nm in the nanoparticles used compared to the gold microparticles. The size of microcarrier correlated with helium pressure and it was observed that higher acceleration pressure was required for smaller carriers. Nanoparticles like gold nanorods and gold capped with mesoporous silica nanoparticles are reported to exhibit significantly increased transient expression at

higher pressures of 1350 and 1550 psi and lesser target distance of 4 cm in onion epidermis (Martin-Ortigosa *et al*., 2012). Small sized particles are suitable at higher pressure while larger sized particles are suitable at lower pressure. According to Okuzaki *et al*. (2013) transient expression using 1 and 0.6 micron sized gold particles was higher in 1100 psi than 1350 psi whereas transient expression using 0.07 micron was similar at both 1100 and 1350 psi in tobacco.

Transformation efficiency of gold microparticles and AgNPs as carriers of DNA for biolistic bombardment were compared at optimum parameters. Transient expression was significantly higher when AgNPs was used compared to gold microparticles as carriers for biolistic transformation of leaf discs of *Nicotiana tabacum*. One of the major drawbacks of biolistic transfection is cell/ tissue damage (O'Brien *et al*., 2001; O'Brien and Lummis, 2006). One possible way to reduce tissue damage is to reduce the size of the micro-carrier carrying the DNA. Mostly used micro-carriers are 0.6 and 1.0 μm diameter projectiles (Randolph-Anderson *et al*., 2015; O'Brien and Lummis, 2006) but smaller sized particles of less than 100 nm are also reported to be used (Torney *et al*., 2007; O'Brien and Lummis, 2011). O'Brien and Lummis (2011) compared the tissue damage between 1 μm gold and nanoparticles carriers and observed cell damage of 9 ± 2% in nanoparticles and 22 ± 3% in microparticles. The use of nanometer sized particles has the potential advantage of increasing efficiency in transfection of smaller cells, organelles and specific cellular regions with reduced cell damage (O'Brien and Lummis, 2011). In the present study higher transformation efficiency and high callus induction percentage from the transformed leaf discs using silver nanoparticles also indicates that there is no significant tissue damage when silver nanoparticles are used for biolistic bombardment.

Lesser transient expression is reported when DNA coated with nano sized gold mesoporous silica nanoparticles were used for bombardment (Torney *et al*., 2007). According to them, approximately 32 GFP-fluorescent foci per cotyledon were observed whereas gold microcarriers of size 0.6 μm produced approximately 73 GFP-fluorescent foci per cotyledon. Mortazavi and Zohrabi (2018) reported plasmid delivery through biolistic method by gold nanoparticles of size 50, 100, 600, and 1000

nm. According to them similar levels of transgene integration was observed with varying sizes of nanoparticles when used for bombardment of rice embryogenic calli. However, according to Okuzaki *et al*. (2013), gold nanoparticles of size 300 nm produced more effective transient expression than 600 nm gold nanoparticles and slightly lesser than 70 nm gold particles in tobacco plastid transformation.

Selection of transformed tissue is essential since only a small proportion of cells/tissues will be transformed. The selection of transformed cells is based on the expression of a marker gene which produces an enzyme aid resistance to a cytotoxic substance (Wilmink and Dons, 1993; Angenon *et al*., 1994). Selection and regeneration is carried out under the appropriate selective regime (Twyman *et al*., 2002; Oneto *et al*., 2010). Selections of transformed cells minimize the escapes of non-transformed cells and increase the chance to screen only transformed cells to obtain transgenic plants (Sahrawat *et al*., 2003). In this study, pBI121 has kanamycin resistant gene and hence kanamycin at a concentration of 100 µg mL^{-1} was used as selection agent for callus induction and regeneration from the bombarded leaf discs. Kanamycin is reported as selection agent after plant transformation in rose (Marchant *et al*., 1998) peanut (Sharma and Anjaiah, 2000), almond (Ramesh, *et al*., 2006) and tobacco (Mohammadhassan *et al*., 2018).

Callus induction percentage of 65% was observed from the bombarded tissue when gold microcarriers were used and 70% when silver nanoparticles were used as carriers for bombardment. Inhibitory (Stampoulis *et al*., 2009) and stimulatory effects (Rezvani *et al*., 2012) are reported for AgNPs in plants. Treatment with AgNPs is reported to increase the expression of ABA signalling gene like NCED3 and RD22 by 2-fold and 3.5-fold respectively in Arabidopsis (Syu *et al*., 2014). However, no inhibitory effects of silver nanoparticles were observed during callus induction in the bombarded leaf discs of *Nicotiana tabacum.*

It is necessary to develop efficient and cost effective gene delivery systems possessing high transformation efficiency and low cytotoxicity (Dizaj *et al*., 2014). There are no previous reports on use of silver nanoparticles for biolistic bombardment

in any plant species. Comparison of cost of consumables indicates that there is 37.5 fold reduction in cost on substitution of gold microparticles with silver nanoparticles under optimum conditions in *Nicotiana tabacum*. The results of the present study indicate that silver nanoparticles can be used as a cost effective substitute of gold microcarriers for biolistic bombardment of *Nicotiana tabacum* without affecting the transformation efficiency. This is the first report on the use of silver nanoparticles as carriers for gene delivery for plant transformation.

There are reports that biolistic gene delivery systems tend to result in a multiple-copy integration of the transgenes in plants (Kohli *et al*., 1998; Dai *et al*., 2001). Hence, confirmation of presence of gene by polymerase chain reaction and determination of copy number of integrated genes by Southern hybridisation in the regenerated plants needs to be taken up as a future line of work.

Summary

SUMMARY

The study entitled “Silver nanoparticles for biolistics based gene delivery in plants” was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2018 to 2020. The objective of the study was to evaluate the efficacy of silver nanoparticles as carriers for gene delivery in *Nicotiana tabacum* using biolistic method.

The study envisaged efficient biolistic transformation in the leaf of *Nicotiana tabacum* with silver nanoparticles compared to gold microcarriers. The transformation efficiency was compared by counting the number of GUS positive blue spots. The transformed leaf discs were screened in a selection medium supplemented with antibiotic kanamycin for callus induction. The surviving calli were transferred to regeneration medium for shoot induction and rooting.

Standardisation of *in vitro* callus induction and regeneration medium of *Nicotiana tabacum* was carried out for screening of transformants. Leaf explants from *in vitro* grown seedlings were inoculated in basal Murashige and Skoog (MS) medium with varied concentrations of cytokinin and auxin for callus induction. MS medium with 1 mg L^{-1} NAA + 1 mg L^{-1} BA medium showed cent percent callus induction within two weeks. The medium MS + 2 mg L^{-1} IAA + 0.5 mg L^{-1} showed only fifty per cent callus induction by tenth day of inoculation. The medium MS + 0.2 mg L^{-1} 2, 4-D + 0.19 mg L^{-1} NAA did not show any response. The calli from MS medium with 1 mg L^{-1} NAA + 1 mg L^{-1} BA exhibited cent percent shoot induction on transfer to MS medium with 1 mg L^{-1} BA + 0.5 mg L^{-1} GA_3 + 0.25 mg L^{-1} IAA and the shoots exhibited cent percent rooting on transfer to half strength MS medium containing 0.1 mg L^{-1} IAA.

For biolistic transformation plasmid vector pBI121 was isolated from freshly cultured *Agrobacterium tumifaciens* EHA105 harbouring pBI121 by alkali lysis method and the quantity and quality were checked using UV visible spectrophotometer and agarose gel electrophoresis. Sufficient amount of good quality plasmid ranging from 2.7-2.8 µg mL^{-1} of predicted size was obtained (14.6 kb). For confirmation restriction digestion of the plasmid was carried out. Double restriction

digestion of pBI121 with *Eco*RI and *Hin*dIII produced two bands of size 12-13 kb and 3 kb in agarose gel electrophoresis as predicted.

Bombardments were performed using PDS-1000/He apparatus (Bio-Rad). Vacuum level inside the chamber was maintained at 28 inch Hg. Efficiency of biolistic transformation using silver nanoparticles was compared with gold microcarriers by GUS assay after 24 h. Uniform leaves collected from two month old plants of *Nicotiana tabacum* grown in pro-trays were used for biolistic bombardment. Surface sterilization was carried out using 4% sodium hypochlorite for 2 minutes.

Optimum parameters for gold microcarriers of 0.6 µm size were determined by varying the He pressure and target distance for comparison with silver nanoparticles as carriers. Helium pressure were varied at 450, 650, 900 and 1100 psi and target distance at 6 and 9 cm. Coating of plasmid DNA (1µg/µL) onto gold microparticles (60 mg/mL) were carried out by precipitating with spermidine and calcium chloride. No GUS expression was observed in the unbombarded leaf discs or leaf discs bombarded with microcarriers alone without DNA coating. Helium pressure of 650 psi exhibited significantly high transient expression of GUS gene followed by 450 psi, 900 psi and 1100 psi. Target distance of 9 cm showed higher GUS gene expression compared to 6 cm, however, was not statistically significant. Interaction analysis of the different parameters tried, revealed that He pressure of 650 psi and target distance of 9 cm was the optimum for gold microcarriers.

To test the efficacy of silver nanoparticles for biolistic transformation, different concentrations of silver nanoparticles *viz*., 0.01, 0.05, 0.5, 1, 2, 10, 20 and 100 mgL^{-1} of 100 nm size (Sigma Aldrich) were tried at 1100 psi He pressure and 6 cm target distance. Coating of plasmid DNA (1µg/µL) onto silver nanoparticles was carried out by precipitating with spermidine and calcium chloride. Among the different concentrations of silver nanoparticles tried as carriers for bombardment, 10 mg L^{-1} showed maximum GUS positive blue spots followed by 20, 100, 2, 0.01, 1, 0.5 and 0.05 mg L^{-1} and results were statistically significant. No GUS expression was observed in leaf discs bombarded with silver nanoparticles alone without DNA.

For optimization of silver nanoparticles as carrier in biolistic transformation of *Nicotiana tabacum*, bombardment of 10 mg L^{-1} silver nanoparticles was carried out by varying the Helium pressure (450 psi, 650 psi, 900 psi, 1100 psi) and target distance (6 cm and 9 cm). Helium pressure of 900 psi exhibited maximum number of GUS positive cells followed by 1100, 650 and 450 psi. The results were statistically significant. Target distance of 6 cm showed higher GUS gene expression than target distance of 9 cm and was statistically significant. Interaction effect of variation of helium pressure and target distance revealed that combination of 900 psi and 6 cm yielded significantly high transient GUS expression with silver nanoparticles.

Comparison of transformation efficiency between gold microparticles and silver nanoparticles as carriers of DNA during biolistic bombardment leaves were carried out. Silver nanoparticles at 900 psi and target distance of 6 cm were compared with gold microcarriers at 650 psi and 9 cm at 28 inch Hg vacuum level. Silver nanoparticles as carriers exhibited higher transient GUS expression compared to gold microparticles. The difference was statistically significant by Student T-test at 5% level of significance.

The bombarded leaf discs from the treatments of gold and silver showing maximum transformation efficiency and unbombarded leaf discs were screened in MS medium supplemented with 100 mg L^{-1} selection agent kanamycin. All the unbombarded leaf discs started drying within one week of transfer to selection medium whereas 65% of the leaf discs bombarded with DNA using gold microparticles exhibited callus induction and 70% of leaf discs bombarded with DNA using silver nanoparticles as carriers showed resistance to the selection agent kanamycin and exhibited callus induction. The green calli were transferred to regeneration medium after two weeks for induction of shoots and further rooting.

To conclude, the results of the study indicated that silver nanoparticles at a concentration of 10 mg L^{-1}, He pressure of 900 psi and target distance of 6 cm resulted in successful biolistic transformation of leaves of *Nicotiana tabacum*. The transformation efficiency was significantly higher using silver nanoparticles than 0.6 µm gold microparticles as carriers and there was a reduction in cost of consumables

by 37.5 fold. This is the first report of efficient use for silver nanoparticles for biolistics based gene delivery in plants.

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Appendices

APPENDIX 1

Composition of MS medium

MS medium						
Stock	**Chemical**	**Quantity for 1L of MS (mg)**	**Conc. of stock**	**Volume of stock (mL)**	**Quantity for preparing stock (mg)**	**Volume for 1L MS media (mL)**
Macro nutrients						
A	NH_4NO_3	1650	40X	100 mL	6600	25 mL
	KNO_3	1900			7600	
	$MgSO_4.7H_2O$	370			1480	
	KH_2PO_4	170			680	
B	$CaCl_2$. 2 H_2O	440	200X	100 mL	8800	5 mL
Micro nutrients						
C	KI	0.83	1000X	100 mL	83	1 mL
	H_3BO_3	6.2			620	
	$MnSO_4$. H_2O	16.9			1690	
	$ZnSO_4$. 7 H_2O	8.6			860	
	Na_2MoO_4. 2 H_2O	0.25			25	
D	$FeSo_4$. 7 H_2O	27.8	200X	100 mL	556	5 mL
	Na_2 EDTA. 2 H_2O	37.3			746	
E	$CuSO_4$. 5 H_2O	0.025	5000X	100 mL	12.5	0.2 mL
	$CoCl_2$. 6 H_2O	0.025			12.5	
Organic supplements						
F	Nicotinic acid	0.5	1000X	100 mL	50	1 mL
	Pyridoxine – HCl	0.5			50	
	Thiamine – HCl	0.1			10	
	Glycine	2			200	
Components directly added						
	MS supplement	3.3g				
	Myoinositol	100 mg				
	Sucrose	30g				
	Agar	6g				

APPENDIX II

Preparation of hormone stocks

(A) Preparation of NAA (1000 ppm): Stocks of NAA were prepared by weighing 0.1 g of NAA and dissolving in few drops of 1N NaOH or ethanol and making up volume to 100 mL with double distilled water.

(B) Preparation of BA (1000 ppm): Stocks of BA were prepared by weighing 0.1 g of BA and dissolving in few drops of 1N NaOH or ethanol and making up volume to 100 mL with double distilled water.

(C) Preparation of IAA (1000 ppm): Stocks of IAA were prepared by weighing 0.1 g of IAA and dissolved in few drops of 1N NaOH or ethanol and making up volume to 100 mL.

(D) Preparation of GA_3 (1000 ppm): Stocks of GA_3 were prepared by weighing 0.1 g of GA_3 and dissolved in drops mL of water and made up volume to 100 mL double distilled water.

(E) Preparation of 2.4-D (1000 ppm): Stocks of 2,4-D were prepared by weighing 0.1 g of 2,4-D and dissolved in few drops of 1N NaOH or ethanol and making up volume to 100 mL with double distilled water.

APPENDIX III

Composition of LB broth

Casien hydrolysate : 10 g

Yeast extract : 5 g

Sodium chlorite : 10 g

Dissolved in 1 L sterile double distilled water

APPENDIX IV

Composition of LB agar medium

Casien hydrolysate : 10 g

Yeast extract : 5 g

Sodium chlorite : 10 g

Agar : 15 g

Dissolved in 1 L sterile double distilled water

APPENDIX V

GET (Glucose EDTA) buffer for plasmid isolation

Glucose : 90.1 mg

EDTA (0.5 M) : 200 µL

Tris- Cl (0.5 M) : 500 µL

The pH was adjusted to 8.0 and volume made upto 10 mL, autoclaved and stored at $4^{o}C$

APPENDIX VI

Lysis buffer for plasmid isolation

1N NaOH (1N) : 2 mL

SDS : 0.1 g

Volume made upto 10 mL with sterile double distilled water

Prepared fresh (Not autoclaved)

APPENDIX VII

Neutralizing solution for plasmid isolation

Potassium acetate (5M) : 6 mL

Glacial acetic acid : 1.15 mL

Water : 2.85 mL

Not autoclaved; stored at room temperature

APPENDIX VIII

TBE (Tris borate EDTA) buffer

Tris base	:	10.8 g
Boric acid	:	5.5 g
EDTA (0.5 M)	:	4 mL

Volume made upto 1L with double distilled water

APPENDIX IX

Gel loading dye

Sucrose	:	10 g (40%)
Bromophenol blue	:	62.5 mg (0.25%)
EDTA (0.5 M, pH 8)	:	1 mL (20 mM)

APPENDIX X

Preparation of GUS staining buffer

(A) 100 mM Potassium Phosphate Buffer (pH 7.2, 20 mL):

KH_2PO_4 (0.2 M) : 2.8 mL

K_2HPO_4 (0.2 M) : 7.2 mL

Double distilled water : 10 mL

(B) 100 mM X-Gluc CHA salt:

100 mg of X-Gluc was weighed and dissolved in 1.9 mL of DMF (N, N-dimethylformamide).

(C) GUS staining buffer preparation

GUS Staining Buffer Preparation (5 ml)		Final Concentration
5 mL 0.1 M Phospahte buffer	:	50 mM
0.2 mL 10 per cent Triton X	:	0.2 per cent
0.2 mL 100 mM Potassium ferri/ferro cyanide	:	2 mM

4.6 mL double distilled water

100 µL X-Gluc (100 mM) was added to 5 mL staining buffer

SILVER NANOPARTICLES FOR BIOLISTICS BASED GENE DELIVERY IN PLANTS

by

NITASANA RAJKUMARI

(2018-12-026)

ABSTRACT

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

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KERALA, INDIA

2020

ABSTRACT

The study entitled “Silver nanoparticles for biolistics based gene delivery in plants” was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2018 to 2020. The objective of the study was to evaluate the efficacy of silver nanoparticles as carriers for gene delivery in *Nicotiana tabacum* using biolistic method.

Standardisation of *in vitro* callus induction and regeneration medium of *Nicotiana tabacum* was carried out for screening of transformants. Leaf explants from *in vitro* grown seedlings were inoculated in basal Murashige and Skoog (MS) medium with varried concentrations of cytokinin and auxin for callus induction. MS medium with 1 mgL^{-1} NAA + 1 mgL^{-1} BA showed cent per cent callus induction. Calli exhibited cent per cent shoot induction on transfer to MS medium supplemented with 1 mgL^{-1} BA + 0.5 mgL^{-1} GA_3 + 0.25 mgL^{-1} IAA and the shoots exhibited cent per cent rooting on transfer to half strength MS medium containing 0.1 mgL^{-1} IAA.

For biolistic transformation, plasmid vector pBI121 (14.6 kb) was isolated from freshly cultured *Agrobacterium tumefaciens* EHA105 by alkaline lysis method and the quality of DNA was confirmed by double restriction digestion with *Eco*RI and *Hin*dIII producing bands of predicted size (12-13 kb and 3 kb).

Biolistic bombardments were performed using PDS-1000/He apparatus (Bio-Rad) using gold microcarriers (size 0.6 micron at a concentration of 60 mgL^{-1}) or silver nanoparticles (size 100 nm, varying concentrations *viz*., 0.01, 0.05, 0.5, 1, 2, 10, 20 and 100 mgL^{-1}) by maintaining a vacuum pressure of 28 inch in the chamber. Helium pressures (450 psi, 650 psi, 900 psi, 1100 psi) and target distances (6 cm and 9 cm) were optimised to determine the maximum transformation efficiency of gold microcarriers and silver nanoparticles for comparison. Leaf discs of *Nicotiana tabacum* surface sterilized using 4% sodium hypochlorite for 2 min were used for bombardment. Transformation efficiency was determined by counting the number of blue spots on GUS assay.

Helium pressure of 650 psi and target distance of 9 cm exhibited maximum transformation efficiency for *Nicotiana tabacum* using gold microcarriers. Among the different concentrations of silver nanoparticles tried as carriers for bombardment, 10 mgL^{-1} exhibited significantly higher transformation efficiency. Interaction effect of variation of helium pressure and target distance revealed that combination of 900 psi and 6 cm exhibited maximum transformation efficiency of silver nanoparticles.

Comparison of gold microparticles and silver nanoparticles as carriers of DNA during biolistic bombardment indicated that transformation efficiency of silver nanoparticles was significantly higher than gold microparticles (81.0 vs 67.7). The bombarded leaf discs from the treatments of gold and silver carriers showing maximum transformation efficiency along with un-bombarded leaf discs were screened in MS medium supplemented with 100 mgL^{-1} selection agent kanamycin. All the un-bombarded leaf discs started drying within one week of transfer to selection medium whereas 65% of the leaf discs bombarded with DNA using gold microparticles and 70% of leaf discs bombarded with silver nanoparticles as carriers exhibited callus induction. The green calli were transferred to regeneration medium after two weeks for induction of shoots and further rooting and hardening.

To conclude, the results of the study indicated that silver nanoparticles at a concentration of 10 mg L^{-1}, He pressure of 900 psi and target distance of 6 cm exhibited maximum efficiency in leaves of *Nicotiana tabacum* without cytotoxic effect on callus induction. The maximum transformation efficiency of silver nanoparticles was sixteen per cent higher compared to maximum transformation efficiency of gold microcarriers and exhibited 37.5 fold cost reduction of consumables. This is the first report of efficient use for silver nanoparticles for biolistics based gene with delivery in plants.