SILVER NANOPARTICLES FOR AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION

AMALA BENNY

(2018-11-098)

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM- 695 522

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SILVER NANOPARTICLES FOR AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION

by

AMALA BENNY

(2018-11-098)

THESIS

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2020

DECLARATION

I, hereby declare that this thesis entitled **"SILVER NANOPARTICLES FOR AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION"** 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.

Vellayani AMALA BENNY

Date: (2018-11-098)

CERTIFICATE

Certified that this thesis entitled **"SILVER NANOPARTICLES FOR AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION"** is a record of bonafide research work done independently by Ms. Amala Benny 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.

Vellayani

Date: 23/10/20

Dr. Swapna Alex
(Major advisor, Advisory committee)
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Amala Benny (2018-11-098)** a candidate for the degree of Master of Science in Agriculture, with major in Plant Biotechnology, agree that the thesis entitled **"SILVER NANOPARTICLES FOR AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION"** may be submitted by **Ms. Amala Benny** in partial fulfillment of the requirement for the degree.

Dr. Swapna Alex
(Major advisor, Advisory committee)
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram

Dr. Soni K. B.
(Member, Advisory committee)
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram

Dr. Anith K. N.
(Member, Advisory committee)
Professor
Department of Agricultural Microbiology
College of Agriculture, Vellayani
Thiruvananthapuram

Dr. M. M. Viji
(Member, Advisory committee)
Professor and Head
Department of Plant Physiology
College of Agriculture, Vellayani
Thiruvananthapuram

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LIST OF ABBREVIATIONS

A. tumefaciens	*Agrobacterium tumefaciens*
E. coli	*Escherichia coli*
LB medium	Luria Bertani medium
Ag NPs	Silver nanoparticles
EC_{50}	Half maximal effective concentration
min	Minutes
h	Hour
cfu	Colony Forming Units
µg	Microgram
mg	Milligram
mL	Millilitre
L	Litre
ng	Nanogram
%	per cent
OD	Optical Density
CD	Critical Difference

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Introduction

1. INTRODUCTION

Genetic engineering is an efficient method for developing plants with desirable traits. Transformation dates back to 1928 when Frederick Griffith discovered that heat killed cells of a pathogenic bacterium could transform the non-pathogenic cells into pathogenic type. *Agrobacterium tumefaciens*, a Gram negative soil bacterium has been harnessed as a tool for plant genetic transformation since the early 1980s. It is a natural genetic engineer and plant cells can be stably transformed by *Agrobacterium* and regenerated into fertile plants with inheritable features. In comparison to other methods of transformation such as particle bombardment, *Agrobacterium* mediated transformation has several advantages. These include the ability to transfer large segments of DNA with minimal rearrangement, fewer copy gene insertion, higher efficiency and minimal cost (Perez-Pineiro *et al*., 2012).

The most commonly used methods for the transformation of *Agrobacterium* are electroporation, freeze/thaw transformation and triparental mating (Wise *et al*., 2006). Among them, the most efficient method is electroporation as the recovery of transformants is high compared to other methods (10^6 transformants per µg circular plasmid DNA). However, the major drawback of electroporation is potential cell damage and the high expense involved. Triparental mating is time consuming. Freeze-thaw is a comparatively cost effective and easy method. However, low transformation efficiency is the major limitation, and hence methods to improve the transformation efficiency of *Agrobacterium* are needed.

In the recent years, nanoparticle mediated transformation represents a promising approach for both animal and plant genetic engineering. Liu *et al*. (2008) reported that nanoparticles play an important role as a gene carrier in biotransformation process, as well as protection of DNA damage from ultrasound. Polymeric, calcium phosphate, chitosan, liposomes, metallic (gold, iron oxide), carbon-based and silicon-based nanoparticles are commonly used. Nanoparticles can help in overcoming the hindrance of physiological barriers such as cell wall and thus increase the transformation efficiency.

Silver is utilized as an antimicrobial agent in food preservation and production of potable water for more than thousand years (Vimbela *et al*., 2017). The electrostatic attraction between positively charged silver nanoparticles and negatively charged bacterial cells plays an important role in antimicrobial activity (Abbaszadegan *et al*., 2015). Silver nanoparticles can affect the integrity of biological membranes. Toxicity of silver nanoparticles is size dependent and toxicity was found to decrease with increase in the size of the particle from 10 nm to 80 nm (Ivask *et al*., 2014).

Studies on the use of silver nanoparticles for genetic transformation of plants and microbes are very limited. Previous studies from the Department of Plant Biotechnology, College of Agriculture, Vellayani have shown that silver nanoparticles can aid in transformation of *Escherichia coli* cells if used in optimum concentration (Nagamani *et al*., 2019). Efficient use of sliver nanoparticles at concentrations below toxic levels for *Agrobacterium* can cause interactions with the biological membrane that aid in the uptake of foreign DNA.

The present study was undertaken with the objective to evaluate the efficacy of silver nanoparticles in improving the transformation efficiency of *Agrobacterium tumefaciens*.

Review of Literature

2. REVIEW OF LITERATURE

Genetic engineering is rapidly replacing conventional plant breeding techniques as it has reduced the time required for the development of new commercial varieties of crops. Genetic transformation has paved the way for developing crop varieties with improved growth characteristics, disease as well as pest resistance, environmental stress tolerance, and enhanced nutritional value. The utilization of genetic engineering to improve the agriculture in developing countries, might help to circumvent expensive, high input crop production as well as move the traditional agriculture towards low input sustainable practices (Odium, 1989).

Genetic transformation is a powerful mechanism of horizontal gene transfer in natural bacterial populations. Discovery of transformation in Pneumococcus by Griffith in 1928 has led to extensive studies of the phenomenon in different organisms. Bacteria such as *Bacillus, Micrococcus, Hemophilus* etc., have the ability to uptake free DNA due to presence of specific proteins on their exterior surface that binds to DNA and transport it inside the cell. The bacteria such as *Escherichia coli* and *Agrobacterium tumefaciens* that naturally lack competency can be made competent by various physical and chemical methods. Effective bacterial transformation is necessary for recombinant DNA research, gene cloning, complementation studies, quantification of gene expression and tagging with reporter proteins. According to Kotnik *et al*. (2015) translocation of foreign genetic material across bio membranes plays an important role in recombinant DNA technology, since natural genetic transformation is not present in majority of bacteria.

2.1 *Agrobacterium tumefaciens* - A NATURAL GENETIC ENGINEER

Agrobacterium tumefaciens was identified as the causative agent of crown gall disease in plants over 100 years ago (Smith and Townsend, 1907). The molecular basis of crown gall disease is the transfer and stable integration of T-DNA into the plant genome. Braun (1947) reported that a tumour inducing principle (TIP) i.e. T-DNA, determines the pathogenicity of *Agrobacterium*. *A. tumefaciens* has a unique virulence mechanism that help in the delivery of T-DNA into host cell. The genes involved in the pathogenicity such as virulence genes and T-DNA are located within

Ti plasmid (200-500 kbp) of *Agrobacterium*. The replacement of T-DNA with gene of interest has been a major breakthrough in the genetic engineering of plants. Klumper and Qaim (2014) reported that *Agrobacterium tumefaciens* mediated genetic transformation has boosted agricultural biotechnology, which has led substantially to improved quality and yield of crops.

Agrobacterium can transfer its T-DNA far beyond its natural plant host including fungi (Bundock *et al*., 1995), human cells (Kunik *et al*., 2001) and algae (Kumar *et al*., 2004).

The different strains of *Agrobacterium tumefaciens* used in transformation studies are GV3101, EHA105, AGL1 and MP90. Chetty *et al*. (2013) reported that *Agrobacterium* strain EHA105 has higher transformation efficiency and fewer transgene insertions in plants such as tomato.

2.1.1 Molecular mechanism of gene transfer

The ability of *Agrobacterium* to transfer its genetic material to host cell makes it a powerful transgenic vector. The transformation is facilitated by different genes present on the tumour inducing (Ti) plasmid. *A. tumefaciens* uses a unique virulence mechanism to induce tumours: it delivers DNA fragments (transferred DNA or T-DNA) to host cells where the T-DNA ultimately integrates into the genome. Host signal induced by wounding activates the virulence mechanism, T-DNA region is nicked from the Ti- plasmid and transferred to the host cell, and genes in T-DNA can be integrated and expressed in the host cell. The vir region of Ti-plasmid encodes approximately 35 proteins of which about 20 are required for tumour formation (Nester, 2015).

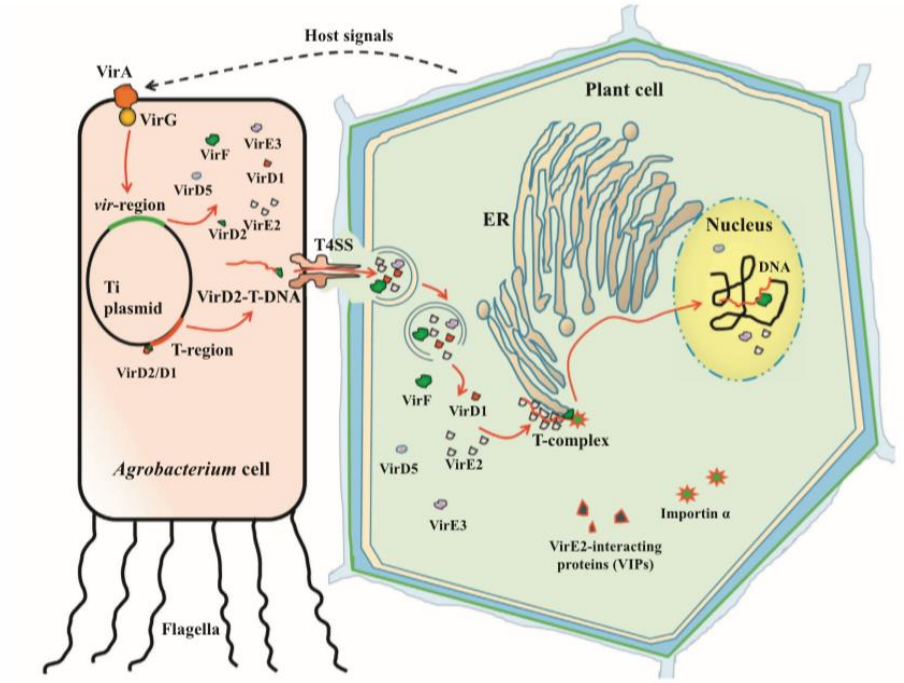


Fig.1 Molecular mechanism of *Agrobacterium* mediated T-DNA transfer process (Guo *et al*., 2019)

The virulence genes for T-DNA transfer and their corresponding functions are given below (Gelvin, 2000):

Virulence protein	**Function**
*Vir*A	Phenolic sensor of a two-component regulatory system
*Vir*G	Phenolic response regulator of a two-component regulatory system
*Vir*B1- B11	Synthesis and assembly of T-pilus
*Vir*C1	Putative “overdrive” protein; enhancement of T-DNA transfer
*Vir*D1	Required for T-DNA processing *in vivo*, and for double strand T-DNA border nicking *in vitro*
*Vir*D2	• T- DNA border specific endonuclease • Putative “pilot protein” that leads the T-strand through the transfer apparatus and into the plant
*Vir*E1	• Required for VirE2 export from *Agrobacterium* • Chaperone for VirE2

*Vir*E2	• Single strand binding protein (SSBP) • Binds to T-DNA during its transfer
*Vir*F	Presumed to mark some host plant proteins for proteolysis
*Vir*H	Detoxification of the phenolics produced by plant cells at the wound site

2.1.2 Binary vectors

In 1983 two prominent groups of scientists made a major breakthrough that allowed the use of *Agrobacterium* for gene transfer by splitting the vir and T-DNA regions of Ti-plasmid onto two separate replicons resulting in T-DNA binary system (Hoekema *et al.,* 1983). Binary vectors are most commonly used for plant transformation and contains the T-DNA region flanked by 25bp repeat sequences of right and left borders, and multiple cloning site in which gene of interest can be inserted. It has the ability to replicate in both *Escherichia coli* and *Agrobacterium tumefaciens*. *A. tumefaciens* containing a helper Ti-plasmid provides trans components for integration of T-DNA into the plant genome (Lin, 1994). Binary vectors *viz*., pBIN, pBI, pCAMBIA, pART and pGA are commonly used for transformation.

pART27 is reported for transformation in economically important crops such as broccoli (Gapper *et al*., 2002), grape vine (Lizamore and Winefield, 2015). It carries the RK2 minimal replicon for maintenance in *Agrobacterium,* the ColE1 origin of replication to maintain high copy number in *E. coli* and the Tn7 spectinomycin resistance gene as a bacterial selectable marker (Gleave, 1992). The T-DNA of pART27 carries the chimeric kanamycin resistance gene (nopaline synthase promoter-neomycin phosphotransferase-nopaline synthase terminator) distal to the right border. Wang and Pijut (2014) reported that *Agrobacterium* with pART27-PsAGRNAi construct showed higher transformation efficiency (21.7%) in black cherry than with pBI121-MDL4 or pBI121-PsTFL1 (5% each), indicating that the vector have a strong impact on transformation**.**

2.2 METHODS OF TRANSFORMATION OF *AGROBACTERIUM*

Transformation is a physicochemical process in which chemical modification is involved in induction of competency and physical process is involved in the uptake of foreign DNA (Asif *et al*., 2017). Competent cells are bacterial cells that can accept extrachromosomal DNA fragments or plasmids (naked DNA) from the environment. Artificial competency can be induced by various physical and chemical methods. The efficient introduction of vector into *A. tumefaciens* is of great practical importance in plant transformation. The different methods for transformation of *Agrobacterium* include triparental mating, electroporation and freeze-thaw method.

2.2.1 Triparental mating

Triparental mating is an effective method for moving a nonconjugative, but mobilizable plasmid into *Agrobacterium* (Wise *et al*., 2006). It is a tedious process as it makes use of two *E. coli* strains for the transformation of *Agrobacterium*. The *E. coli* strain carrying helper plasmid, encoding protein for formation of mating bridge, gets transferred itself to another *E. coli* strain carrying mobilizable plasmid. The helper plasmid assists in the transfer of mobilizable plasmid to *Agrobacterium* through mating bridge.

2.2.2 Electroporation

Electroporation is the most efficient method of transformation of *Agrobacterium* and can result in 10^6 transformants per µg of circular plasmid DNA (Wise *et al*., 2006). It uses a short electric pulse to create aqueous pores in the lipid membrane of the bacteria which allows DNA molecules to enter the cell. Transformation efficiency depends on electric field strength, pulse length, DNA quality and cell density (Rodriguez *et al*., 2007). According to Mahmood *et al*. (2008), transformation efficiency of *Agrobacterium* strains LBA4404 and EHA105 increased with increase in number of electric pulses.

Dower *et al*. (1988) reported that maximum transformation efficiency in *E. coli* was obtained with 20 msec pulsed at 7 kV/cm. The success of the method depends on regulating the strength of the electric field and the duration of the pulse.

Rhee *et al*. (2007) reported that electroporation is more efficient compared to chemical method ($CaCl_2$) in *Bacillus coagulans*. Although electroporation has several advantages over the other methods of transformation, its application is limited to few species.

2.2.3 Freeze-thaw method

Freeze-thaw method is the simplest and fastest method of transformation. Dityatkin *et al*. (1972) reported that DNA molecules penetrate into the frozen-thawed cells by passive diffusion through temporary lesions in the cell wall and membrane. Maximum transformation was observed after freezing of bacteria at -70 °C and thawing at 37 °C.

According to Wise *et al*., (2006), the transformation efficiency of freeze-thaw method ranges from 10^2 to 10^3 transformants per µg DNA in *Agrobacterium tumefaciens*.

The freeze-thaw technique in *Agrobacterium* involves competent cell preparation using divalent cations. According to Tsai *et al*. (1989), among the different cations tested *viz*., Ca^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+}, Na^{+} and Rb^{+}, Ca^{2+} showed maximum transformation efficiency. The chemical method of transformation involves opening gated membrane channels by the action of polyvalent cations (Sword, 2003).

The low temperatures used in transformation protocols congeals the lipid moiety and consequently restricts the fluidity of the cell membrane which strengthens calcium-cell surface interaction. Thus, the calcium ions bound with cell surface as well as the foreign DNA, brings the DNA to the cell (Asif *et al*., 2017).

Jyothishwaran *et al*. (2007) reported that transformation efficiency of *Agrobacterium tumefaciens* strain LBA4404 was increased by modified freeze-thaw technique in which competent *Agrobacterium* cells were prepared by treating with 100 mM magnesium chloride and 20 mM calcium chloride at early logarithmic phase.

According to Mandel and Higa (1970) uptake of DNA by competent bacterial cells relies on damaging the cell wall by exposure to divalent cations and rapid changes in temperature that alter the fluidity of the cell membrane. Panja *et al*. (2008)

reported that transformation technique involves increase in pore size of cell surface by loss of protein and lipid due to the subsequent lowering of temperature and heat shock process. Thus, the membrane becomes depolarised and reduces the repulsion between negatively charged DNA molecule and cell membrane.

The limitations of conventional DNA delivery methods such as, low transformation efficiency, tissue damage, uncontrolled DNA integration into the host genome can be overcome by the application of nanotechnology. Gene transformation with nanoparticles is a novel method in plant biotechnology. The advancement in the nanotechnology may open the way for further development of efficient transformation systems.

2.3 NANOTECHNOLOGY

The word nanotechnology was first quoted by the renowned scientist Richard Phillips Feynman in his lecture entitled "there's plenty of room at the bottom" at an American Physical Society meeting at Caltech on December 29, 1959. The term nanotechnology was coined in 1974 by Norio Taniguichi. It is a novel, innovative, interdisciplinary scientific approach that involves designing, development and application of materials as well as devices at molecular level in nanometre scale (Ali *et al*., 2014). It plays an important role in development of newer and improved materials that are robust and economic sources of renewable energy and pollution-free environment, thus upgrading the current world to a better and easier habitation (Punjabi *et al*., 2018). It has evolved as a key transformational tool to address delivery challenges and enhance the utility of plant genetic engineering (Cunnigham *et al*., 2018).

2.3.1 Properties and applications of nanoparticles

Particles of size 1-100 nm are considered as nanoparticles. They possess unique physical, chemical, optical and biological properties compared to their bulk equivalent due to the increase in the number of atoms on the surface. Wijnhoven *et al*. (2009) reported that improved chemical activity of metallic nanoparticles is due to their crystallographic surface structure and the higher surface area per mass, allowing a larger number of atoms to interact with their surroundings.

The properties of nanoparticles such as tiny size, easy penetration to the cuticle, non-volatile nature and being able to release their active ingredients slowly, has aided in their extensive use in agriculture (Sarmast *et al.,* 2016).

Nanotechnology plays a key role in revamping agriculture and food production to fulfil the demands in an efficient and cost-effective way (Scrinis and Lyons, 2007). The application of nanotechnology in agriculture enhanced food values, reduced agricultural inputs, improved nutrient contents and longer shelf life (Ali *et al*., 2014).

Nanoparticles are promising materials for biomolecule delivery, owing to their ability to traverse plant cell walls without external force and highly tuneable physicochemical properties for diverse cargo conjugation and broad host range applicability (Abbaszadegan *et al*., 2015).

The application of physicochemical and biological properties of nanostructures in the field of medicine and agriculture gave rise to new era in the field of science known as nanobiotechnology (Asghari *et al*., 2016).

2.3.2 Nanobiotechnology

Nanobiotechnology refers to the use of nanotechnology to manipulate living organisms, as well as to enable the consolidation of biological and non-biological materials (Scrinis and Lyons, 2007). It is the amalgamation of engineering and molecular biology that is leading to a new class of multifunctional devices and systems for biological and chemical analysis with better sensitivity, specificity, and a higher rate of recognition (Morais *et al*., 2014).

2.3.3 Genetic transformation using nanoparticles

Genetic transformation in plants are hindered mainly by the thick cell wall, having size exclusion limit of 5-20 nm**.** The plant scientists are constantly exploring efficient tools to increase the transformation frequency. In the past decade nanoparticles has gained popularity for biomolecule delivery into plants. The nanoparticles such as calcium phosphate, carbon-based silica, gold, magnetite, strontium phosphate, magnesium phosphate and manganese phosphate can be used as

a vector for gene transfer (Potrykus, 1991). The nanoparticle mediated transformation has some advantages over other methods as they are not subject to microbial attack, can be easily synthesized, are less toxic and exhibit a good stability (Hoffmann-Tsay *et al*., 1994).

Nanoparticle mediated gene transfer methods have the potential to directly transfer DNA into the cells, achieving stable integration and rapid expression of the transgene (Rai *et al*., 2015).

The mechanism of nanoparticle mediated gene delivery involves recognition and adsorption of DNA coated nanoparticles on the cell membrane followed by internalization of the particle by endocytosis.

Torney *et al*. (2007) reported that DNA coated Type-II mesoporous silica nanoparticles (MSNs) can serve as an efficient delivery system compared to PEG mediated transformation of tobacco protoplast. Transient transformation of 7% of the cells was achieved when 10^6 cells were incubated with 1 µg DNA coated on 10 µg Type-II MSNs.

Calcium phosphate nanoparticle based genetic transformation of plants was reported by Naqvi *et al*. (2012) with transformation efficiency of 80.7% compared to 54.4% by *Agrobacterium tumefaciens* and 4% using naked DNA. Positively charged nanoparticles can be linked with negatively charged backbones of nucleic acid and directed into the plant cells (Zhao *et al*., 2017; Cunningham *et al*., 2018).

Kwak *et al*. (2019) demonstrated that chitosan based positively charged single walled carbon nanotube (SWNT) carriers can be used to protect and deliver plasmid DNA to the chloroplasts without external biolistic or chemical aid.

Chatterjee and Sarkar (2014) reported that glutathione functionalised gold nanoparticles mediated transformation of plasmid DNA (pUC19) into *E. coli* DH5α have higher transformation efficiency (4.2×10^7/µg DNA) compared to conventional calcium chloride mediated method (2.3×10^5/µg). According to He *et al*. (2003) nanoparticles protect the embedded DNA from nucleases within the cell due to their small size with greater surface area and pore structure.

Nagamani *et al*. (2019) reported that *E. coli* cells treated with silver nanoparticles (100 nm) at a concentration of 1 mgL^{-1} exhibited higher transformation (7.9 x 10^4 cfu/ng of DNA) compared to calcium chloride mediated method (2.3 x 10^3 cfu/ng of DNA).

Deshmukh *et al*. (2019) demonstrated that arginine-glucose functionalized hydroxyapatite nanoparticles (R-G-HAp NPs) can serve as an efficient plasmid delivery vehicle for both Gram positive and Gram negative bacterial transformation. R-G-HAp NPs mediated transformation in *E. coli* exhibited 10^2 and 10^3 fold higher transformation efficiency as compared to calcium chloride and electroporation, respectively. The transformation efficiency of *Staphylococcus aureus* using R-G-HAp NPs was 10^2 fold higher than electroporation.

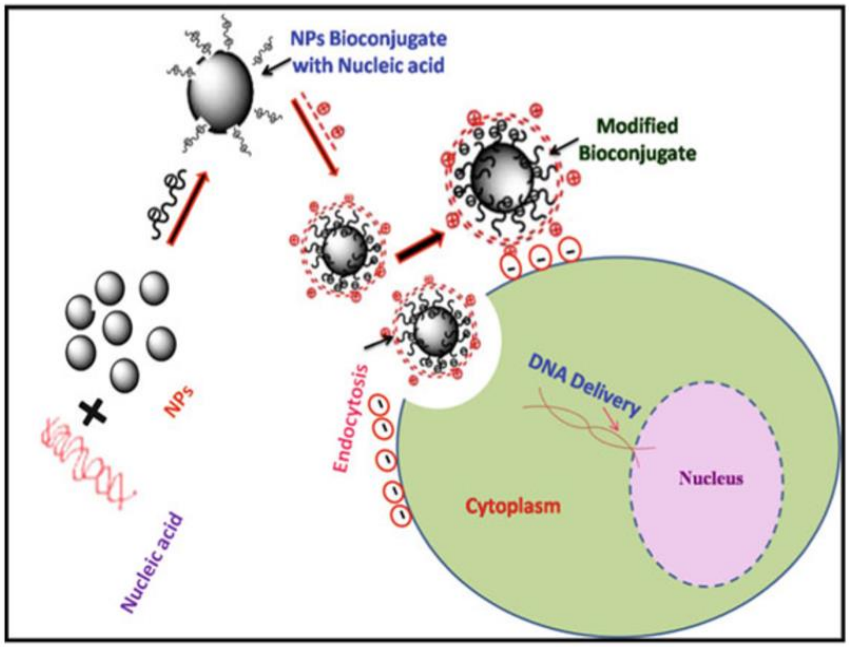


Fig.2 Nanoparticle mediated gene delivery into plant cell (Rai *et al*., 2015)

2.3.4 Antibacterial properties of nanoparticles

The increased concern for antibiotic resistance of bacteria has led to the adoption of nanoparticles as a promising alternative to the antibiotics because of their wide range of antibacterial property. Paul Ehrlich pioneered the development of nanoparticle as a drug delivery system in medical field (Kreuter, 2007). Nanoparticles

are widely used in healthcare products such as burn dressings, scaffold, water purification systems and medical devices (Thomas *et al*., 2007). Metallic nanoparticles like silver, gold, copper, titanium have wider applications in biomedical field (Vimbela *et al*., 2017) due to their high surface area-to-volume ratio and unique physio-chemical properties (Liao *et al*., 2019).

The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials (Ravishankar and Jamuna, 2011).

Among the variety of nanoparticles with antibacterial property, silver nanoparticle is widely explored due to its broad-spectrum antimicrobial properties and robust antimicrobial effectiveness against various bacteria, viruses, and fungi (Tang and Zheng, 2018).

2.3.5 Silver nanoparticles

Silver nanoparticles have received great emphasis among the noble metals due to their strong toxicity that silver exhibits in various chemical forms to a wide range of microorganisms (Hollady *et al*., 2011). The non-toxic property of silver nanoparticles to mammalian tissues and environmentally friendly nature when used in relatively low concentrations (Baruwati *et al*., 2013) led to its wider application in health care, textile fibres, food packaging and antibacterial fields.

The most important application of silver nanoparticles in the field of agriculture involves the antimicrobial and nutritional action (Sarmast and Salehi, 2016).

Abdi *et al*. (2008) reported that silver nanoparticles of size 35 nm were used to suppress the bacterial contamination in plant tissue culture techniques of *Valeriana officinalis* without any adverse effects on the growth characteristics of regenerated plantlets. Sarmast and Salehi (2016) reported that addition silver nanoparticles in the MS medium of *Tecomella undulata* improved survival percentage of explants and increased the mean number and length of shoots by the inhibition of ethylene by silver ions.

2.3.5.1 *Toxicity of silver nanoparticles*

The toxic properties of silver on bacteria have been examined for more than 60 years (Khalandi *et al*., 2017).

Kim *et al*. (2007) observed that silver nanoparticles at a concentration 3.3 to 33 nM exhibited antimicrobial activity against yeast, *E. coli* and *Staphylococcus aureus* grown on Mueller Hinton Agar (MHA) plates. The growth inhibition effect was concentration dependent.

Li *et al*. (2010) reported that silver nanoparticles of size 5 nm at a concentration of 10 μg/ml could inhibited the growth of 10^7 cfu/ml *E. coli* cells in liquid Mueller–Hinton medium due to leakage of reducing sugars and proteins and inactivation of respiratory dehydrogenase enzyme.

Ayala-Nunez *et al*. (2009) reported that silver nanoparticles of size 10 nm inhibited *in vitro* growth of methicillin-resistant *Staphylococcus aureus* (MRSA) at noncytotoxic concentrations due to larger surface area to volume ratio.

According to Amin *et al*. (2009) silver nanoparticles exhibited higher toxicity against both Gram positive (*Staphylococcus capitis*) and Gram negative (*E. coli*) bacteria compared to gold nanoparticles. Inhibitory effect on Gram negative bacteria was higher compared to Gram positive bacteria owing to the presence of lipopolysaccharide on the membrane surface of Gram negative bacteria.

Kalishwaral *et al*. (2010) reported that silver nanoparticles exhibited antibiofilm activity when tested *in vitro* on biofilms formed by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* during 24 h treatment.

Asharani *et al*. (2011) reported that silver nanoparticles (5-35 nm) showed toxicity to zebrafish embryos whereas gold nanoparticles of same size were non-toxic.

Sarmast *et al*. (2010) demonstrated that nano silver (18.5 nm) at concentration of 10-20 μg/ml in LB medium impeded the growth of *Agrobacterium tumefaciens*

strain LBA4404. Bacterial growth was suppressed in concentrations more than 15 µg/ml.

Gurunathan *et al*. (2014) reported that green synthesised silver nanoparticles from leaf extract of *Allophylu scobbe* exhibited toxicity at a concentration of 0.1 to 0.6 µg/ml against both Gram negative (*Pseudomonas aeruginosa*, *Shigella flexneri*) and Gram positive (*Staphylococcus aureus*, *Streptococcus pneumoniae*) bacteria.

Gurunathan (2015) reported that silver nanoparticles of size 8 nm at concentration of 1.4 µg/ml inhibited the growth of *Escherichia coli* and *Klebsiella pneumoniae*.

Anthony *et al*. (2014) evaluated the toxicity of 40 nm silver nanoparticles prepared from culture supernatant of *Bacillus marisflavi* and reported 10 mg/mL to be the minimum inhibitory concentration against *Pseudomonas aeruginosa*.

Velumurugan *et al*. (2014) reported that green synthesised silver nanoparticles (10-20 nm) from the root extract of *Zingiber officinalis* showed effective bactericidal property against food pathogens such as S*taphylococcus* spp. and *Listeria* spp., and the minimum inhibitory concentration was 30 ± 14.3 µg/ml and 20 ± 12.8 µg/mL respectively.

According to Ivask *et al*. (2014) toxicity of citrate coated silver nanoparticles increased with decrease in particle size of 20-80 nm due to the release of Ag ions. *In vitro* studies revealed that crustaceans are the most sensitive organisms to silver nanoparticles followed by algae, *E. coli*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae* and mammalian fibroblasts.

Jagielski *et al*. (2018) reported that silver nanoparticles are strong inhibitors of Prototheca algae, and the minimal algicidal concentration (MAC) values matched the corresponding minimum inhibitory concentration (MIC) values of other species.

Liao *et al*. (2019) reported that silver nanoparticle of size range 5-10 nm exhibits high bactericidal effect against multidrug resistant *Pseudomonas aeruginosa* with the minimal inhibitory concentration (MIC) range of 1.406 - 5.625 µg/mL and the minimal bactericidal concentration (MBC) range of 2.813 - 5.625 µg/mL.

Dong *et al*. (2019) reported that silver nanoparticles of size 10 ± 5 nm can completely inhibit *Vibrio natriegens* at a lower minimal inhibitory concentration (1.0 µg/ml), whereas a particle size of about 90 ± 5 nm can inhibit bacteria at a higher concentrations (11.5 µg/ml) indicating that smaller the particle size, the better the bactericidal effect.

2.3.5.2 *Mode of action of silver nanoparticles*

The mode of action of silver nanoparticles is still unclear but many scientists have given different theories related to antibacterial property. The wider application of silver nanoparticles as bactericides is due to their enormously great surface area, which provides good interaction with microbes (Rai *et al*., 2009).

The impact of silver nanoparticles on the bacteria involves the detachment of cytoplasm membrane from the cell wall as well as the loss of ability of DNA to replicate upon the infiltration of silver ions and binding to the phosphorous moieties of DNA (Jeong *et al*., 2005).

The mechanism of antibacterial activity of silver nanoparticles involves disruption of membrane functionality from an interaction between released Ag+ ions and the cell membrane and formation of reactive oxygen species (ROS) ultimately causing damage to the cell due to oxidative stress (Vimbela *et al*., 2017).

Electron spin resonance spectroscopy studies by Kim *et al*. (2007) suggested that free radicals generated by the silver nanoparticles on interaction with bacteria have the capacity to disrupt the cell membrane resulting in cell death.

Transmission electron microscopy (TEM) and Energy dispersive X-ray analysis (EDAX) studies by Sondi and Salopek-sondi (2004) confirmed the incorporation of silver nanoparticles on the membrane of *E. coli*, which was recognized by the formation of pits on the membrane.

Dakal *et al*. (2016) reported that Gram negative bacteria are highly susceptible to silver nanoparticles compared to Gram positive bacteria as the amount of negatively charged peptidoglycan layer is high in Gram positive bacteria.

Silver ion released by silver nanoparticles possess high affinity to electron donating groups such as sulfhydryl, amino, imidazole, phosphate and carbonyl groups that are extensively present on membrane or proteins. Silver ions can bind to thiol groups (ASH) of the protein forming stable AS-Ag bonds, which can alter the 3D structure of proteins and block active binding sites (Tang and Zheng, 2018).

The study conducted by Dong e*t al*. (2019) on the antibacterial activity of silver nanoparticles of different particle size against *Vibrio natriegens* (Gram negative) revealed that Ag NPs can enter the bacterial cell by disrupting the cell membrane as well as cause DNA damage by increased production of reactive oxygen species.

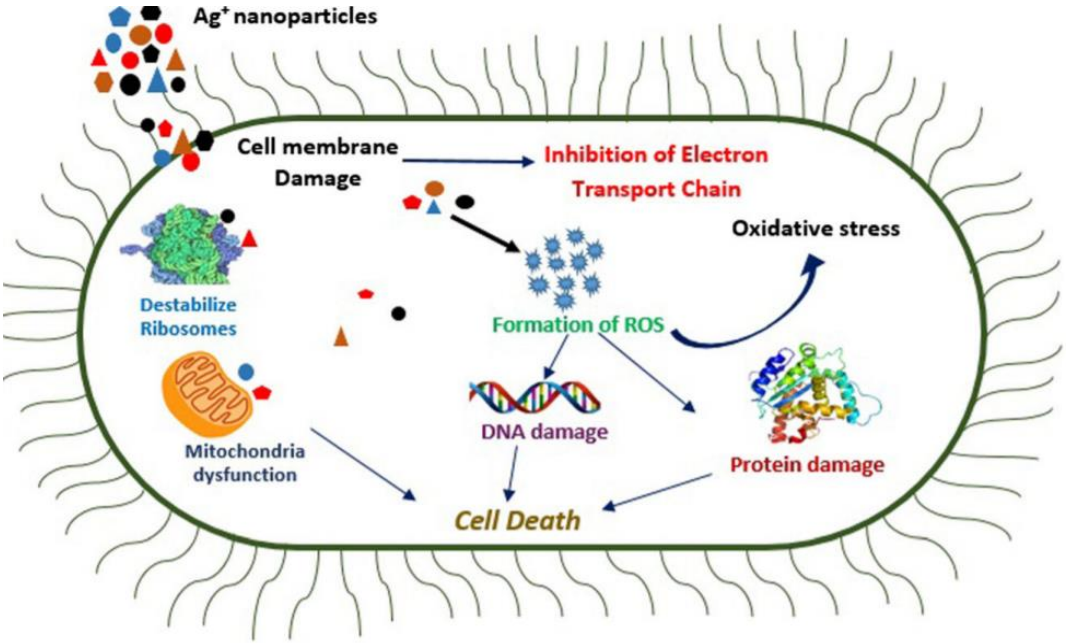


Fig.3 Mechanism of action of silver nanoparticles on bacteria (Rahman *et al*., 2019)

There are no reports on the toxicity effects of silver nanoparticles of size 100 nm on *Agrobacterium tumefaciens* or the use of silver nanoparticles for induction of competency and transformation in *Agrobacterium*.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Silver nanoparticles for *Agrobacterium* mediated transformation" was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2018 to 2020. Details regarding the experimental materials used and the methodology adopted for various experiments are presented in this chapter.

3.1 TOXICITY ASSAY OF SILVER NANOPARTICLES ON *Agrobacterium tumefacien CELLS*

Agrobacterium tumefaciens strain EHA105 (Culture courtesy: ICAR-CTCRI, Thiruvananthapuram) in the exponential phase of its growth was exposed to different concentrations of silver nanoparticles (100 nm size) and the colony forming ability of the treated cells was checked using the protocol of Ivask *et al*. (2014). The half maximal effective concentration (EC_{50}) value was estimated by using untreated bacterial cells as control.

3.1.1 Culture Medium

3.1.1.1 *Chemicals*

The chemicals used for the preparation of the culture medium were of analytical grade and procured from Sisco Research laboratories (SRL), India. The antibiotics were purchased from Himedia Laboratories, Mumbai, India. Silver nanoparticles of 100 nm particle size were from Sigma Aldrich, USA. They were diluted into different concentrations using sterile double distilled water for further studies.

3.1.1.2 *Composition and preparation of medium*

Luria Bertani (LB) broth and LB Agar were used for microbial experiments (Appendix I). Media were sterilized by autoclaving at a pressure of 1.06 kg cm^{-2} at a temperature of 121 ^{o}C for 20 min. The medium was stored at 25 ± 2 ^{o}C for further use.

3.1.2 Growth curve of *Agrobacterium* strain EHA105

Single colony of *A. tumefaciens* from freshly streaked plate was inoculated in 10 mL LB broth containing rifampicin (100 µg/mL) (Appendix II) and incubated overnight at 28 ^{o}C on a rotary shaker at 140 rpm. After 24 h the optical density (OD) at 600 nm was measured using a spectrophotometer and the value was adjusted to 1 by adding sterile LB broth. From the diluted culture 100 µL was added to 50 ml LB broth and OD_{600} was measured at 4 h interval for 48 h. Growth curve was plotted as Absorbance *vs* Time.

3.1.3 Estimation of EC_{50} of silver nanoparticles on *Agrobacterium tumefaciens*

Agrobacterium strain EHA105 in the exponential phase of its growth was exposed to different concentrations of silver nanoparticles (Table 1) and the colony forming ability of the treated cells was checked to find out the EC_{50}. Overnight culture of *Agrobacterium* was added to fresh LB broth containing rifampicin (100 µg/mL) in 1:50 ratio and cultured till exponential phase (OD_{600}=1). Bacterial cells were pelleted out at 5000 rpm for 5 min and re-suspended in 1 mL of sterile double distilled water. 200 µl of bacterial suspension was mixed with 200 µl each of different concentrations of silver nanoparticles in sterile tubes and incubated at 28 ^{o}C for 4 h. After incubation, serial dilutions of the cultures were made and 100 µl was spread on LB agar plates containing rifampicin (100 µg/mL). Colonies formed on the different treatment plates were counted. Two replications were maintained. EC_{50} value was estimated by using the statistical package SPSS.

Table 1. Concentrations of silver nanoparticles used for toxicity assay

Treatments	Concentration of silver nanoparticles (mg L^{-1})
T_1	0
T_2	0.01
T_3	1
T_4	5
T_5	10
T_6	20

3.2 ISOLATION OF PLASMID (pART27) FROM *E. coli*

Escherichia coli cells harbouring pART27 was inoculated in LB broth supplemented with spectinomycin (100 µg/mL) (Appendix II). The culture was incubated overnight at 37 ºC on a rotary shaker at 140 rpm. Plasmid was isolated by alkaline lysis method (Bimboim and Doly, 1979). Bacterial culture (2 mL) was taken in sterile microcentrifuge tubes and the cells were pelleted at 4000 rpm for 5 min at room temperature. The supernatant was discarded and the bacterial pellet obtained was re-suspended completely in 200 µl of GET buffer (Appendix III) by inverting the tubes. 300 µl of lysis buffer (Appendix III) was added, mixed gently by inversion and kept on ice for 5 min. To this 300 µl of neutralizing solution (Appendix III) was added and mixed by inversion and kept on ice for 5 min. Cell debris were separated from the supernatant by centrifuging at 15,000 rpm for 10 min. The supernatant was transferred to a fresh tube and treated with RNAase (20 µg/mL). It was incubated at 65 ºC for 20 min. 400 µl of chloroform was added to the tubes and mixed by inversion for 30 seconds. The mixture was centrifuged at 13,000 rpm for 1 min and the upper aqueous phase was separated and washed again with chloroform. The upper aqueous phase was transferred to a fresh tube and an equal volume of 100% isopropanol was added, mixed by inversion and incubated at -20 ºC for 20 min. It was centrifuged at 14,000 rpm for 10 min and the pellet obtained was washed in 500 µl of 70% ethanol. After centrifugation at 10,000 rpm for 5 min at room temperature, the supernatant was discarded and the pellet was thoroughly air dried by placing in a laminar hood and later dissolved in sterile double distilled water. The plasmid was quantified and stored at -20 ºC for further use.

3.2.1 Quantification of plasmid DNA

Quantification of DNA was carried out using UV-visible spectrophotometer. The optical density of the DNA samples was recorded at both 260 and 280 nm. The concentration of DNA was calculated using the following formula:

Amount of DNA (ng/µL) = 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} values between 1.8 to 2 indicate good quality DNA.

3.3 INDUCTION OF COMPETENCY AND TRANSFORMATION OF BACTERIAL CELLS

As calcium chloride freeze-thaw technique is the conventional method for induction of competency and transformation, the efficiency of different treatments using silver nanoparticles were compared with calcium chloride freeze-thaw technique (positive control). The different treatments tried were replacement of calcium chloride and freeze-thaw with silver nanoparticles (ie., silver nanoparticles alone were used) or replacement of freeze-thaw (ie., silver nanoparticles with 20 mM calcium chloride) or replacement of calcium chloride (ie., silver nanoparticles with freeze-thaw) in the conventional technique. Efficacy of addition of silver nanoparticles to conventional calcium chloride freeze-thaw technique for improvement of efficiency was also assessed.

Agrobacterium (strain EHA105) cells in the exponential phase were used for giving treatments. For conventional calcium chloride freeze-thaw technique, protocol by Weigel and Glazebrook (2006) was used. The competent cells without pART27 was taken as the negative control. Concentrations below EC_{50} were used for all treatments with silver nanoparticles. Two replications were maintained for all the treatments used for induction of competency and transformation.

3.3.1 Induction of competency and transformation by silver nanoparticles

Agrobacterium tumefaciens EHA105 was grown in 10 mL LB broth (Appendix I) containing rifampicin (100 µg/mL) at 28 ºC on rotary incubator at 140 rpm. 1000 µl overnight grown culture was transferred to 50 ml LB broth and incubated at 28 ºC at 140 rpm for 3 h to attain an OD_{600} of 0.3 to 0.4 (early log phase). The culture was kept on ice for 30 min. 1.5 mL of chilled culture was transferred to sterile 2 mL tubes. The cells were pelleted down at 4000 rpm for 10 min at 4 ºC. The supernatant was poured off and bacterial cells were resuspended in 200 µl of six different concentrations of silver nanoparticles (0.01 mgL^{-1}, 0.5 mgL^{-1}, 1 mgL^{-1}, 2 mgL^{-1} 4 mgL^{-1}, 6 mgL^{-1}) which caused minimal toxicity and incubated 60 min on a

rotary shaker at 28 ºC and 140 rpm. After incubation 1 µg of plasmid (pART27) was added to the bacterial suspension, gently shaken and incubated on ice for 15 min. To the bacterial suspension 1 mL of LB broth as added and incubated at 28 ºC for 3-4 h with shaking. The cells were plated on LB agar containing rifampicin (100 µg/mL) and spectinomycin (100 µg/mL) (Appendix II) as selectable marker.

3.3.2 Induction of competency and transformation by silver nanoparticles and calcium chloride

Agrobacterium tumefaciens EHA105 was grown in 10 mL LB broth containing rifampicin (100 µg/mL) at 28 ºC on a rotary shaker at 140 rpm. Overnight grown culture (1000 µL) was transferred to 50 mL of fresh LB broth and cultured till early exponential phase (OD_{600}=0.3 to 0.4). The culture was kept on ice for 30 min and 1.5 mL of chilled culture was transferred to sterile 2 mL microcentrifuge tubes. The cells were pelleted at 4000 rpm for 10 min at 4 ºC. The supernatant was decanted off. The pellet was re-suspended in 1 mL of 20 mM $CaCl_2$ solution and again pelleted at 4000 rpm for 5 min. The pellet was re-suspended in 100 µL of 20 mM $CaCl_2$ and 100 µL silver nanoparticles of four different concentrations (0.01 mgL^{-1}, 0.5 mgL^{-1}, 1 mgL^{-1}, 2 mgL^{-1}) separately, which caused minimal toxicity and incubated for 30 min on ice. 1 µg of the plasmid DNA was added to each tube, mixed gently and incubated on ice for 15 min. To the bacterial suspension 1 mL LB broth was added and incubated at 28 ºC for 3-4 h with shaking. The cells were plated on LB agar amended with rifampicin (100 µg/mL) and spectinomycin (100 µg/mL).

Table 2. Different treatments for induction of competency and transformation of *Agrobacterium tumefaciens* EHA105

Treatment	Methods for induction of competency
T_1	0.01 mgL^{-1} AgNPs
T_2	0.5 mgL^{-1} AgNPs
T_3	1 mgL^{-1} AgNPs
T_4	2 mgL^{-1} AgNPs
T_5	4 mgL^{-1} AgNPs
T_6	6 mgL^{-1} AgNPs
T_7	0.01 mgL^{-1} AgNPs + 20 mM $CaCl_2$
T_8	0.5 mgL^{-1} AgNPs + 20 mM $CaCl_2$
T_9	1 mgL^{-1} AgNPs + 20 mM $CaCl_2$
T_{10}	2 mgL^{-1} AgNPs + 20 mM $CaCl_2$
T_{11}	0.01 mgL^{-1} AgNPs + Freeze-thaw
T_{12}	0.5 mgL^{-1} AgNPs + Freeze-thaw
T_{13}	1 mgL^{-1} AgNPs + Freeze-thaw
T_{14}	2 mgL^{-1} AgNPs + Freeze-thaw
T_{15}	0.01 mgL^{-1} AgNPs + 20 mM $CaCl_2$ + Freeze-thaw
T_{16}	0.5 mgL^{-1} AgNPs + 20 mM $CaCl_2$ + Freeze-thaw
T_{17}	1 mgL^{-1} AgNPs + 20 mM $CaCl_2$+ Freeze-thaw
T_{18}	2 mgL^{-1} AgNPs + 20 mM $CaCl_2$+ Freeze-thaw
T_{19}	20 mM $CaCl_2$+ Freeze-thaw
T_{20}	Control

3.3.3 Induction of competency and transformation by silver nanoparticles and freeze-thaw method

Agrobacterium tumefaciens EHA105 was grown in 10 mL LB broth containing rifampicin (100 mgL^{-1}) at 28 ºC on a rotary shaker at 140 rpm. Overnight grown culture (1000 µl) was transferred to 50 ml LB broth and incubated at 28 ºC at 140 rpm for 3 h. The culture (OD_{600}=0.3-0.4) kept on ice for 30 min. Then the culture was transferred to fresh 2 mL tubes. The cells were pelleted at 4000 rpm for 10 min at 4 ºC. The supernatant was decanted off and bacterial cells were resuspended in 200 µl of four different concentrations of silver nanoparticles (0.01 mgL^{-1}, 0.5 mgL^{-1}, 1 mgL^{-1}, 2 mgL^{-1}) which caused minimal toxicity. 1µg of plasmid DNA (pART27) was added to the competent cells and shaken gently. It was incubated on ice for 15 min. The cells were frozen for 5 min in liquid nitrogen and thawed for 5 min at 37 ºC. After incubation the tubes were quickly plunged on ice bath and chilled for 5 min. 1 mL LB broth was added to the tube and incubated at 28 ºC for 3-4 h with shaking. The cells plated on LB agar modified with rifampicin (100 µg/mL) and spectinomycin (100 µg/mL).

3.3.4 Induction of competency and transformation using silver nanoparticles and calcium chloride by freeze-thaw method

Competent cells prepared using combination of silver nanoparticles and calcium chloride were transformed using pART27 by freeze-thaw method. *Agrobacterium tumefaciens* EHA105 were grown in 10 mL LB broth containing rifampicin (100 µg/mL) at 28 ºC on rotary shaker at 140 rpm. Overnight grown culture (1000 µL) was transferred to 50 mL of fresh LB broth cultured till early exponential phase (OD_{600}=0.3 to 0.4). The culture was kept on ice for 30 min, 1.5 mL of chilled culture was transferred to sterile 2 mL microcentrifuge tubes. The cells were pelleted at 4000 rpm for 10 min at 4 ºC. The supernatant was decanted off. The pellet was resuspended in 1 mL of 20 mM $CaCl_2$ solution and again pelleted at 4000 rpm for 5 min. The pellet was resuspended in 100 µL of 20 mM $CaCl_2$ and 100 µL silver nanoparticles of four different concentrations (0.01 mgL^{-1}, 0.5 mgL^{-1}, 1 mgL^{-1}, 2 mgL^{-1}) which caused minimal toxicity and incubated for 30 min on ice. 1 µg of the plasmid DNA was added to each tube, mixed gently and incubated on ice for 15

min. The cells were frozen for 5 min in liquid nitrogen and thawed for 5 min at 37 ºC. After incubation the tubes were quickly plunged in ice bath and chilled for 5 min. 1 mL LB broth was added to the tube and incubated at 28 ºC for 3-4 h with shaking. The cells plated on LB agar modified with rifampicin (100 µg/mL) and spectinomycin (100 µg/mL).

3.3.5 Induction of competency and transformation by calcium chloride and freeze-thaw technique

The *Agrobacterium* cells were grown in 10 ml of the Luria Bertani (LB) broth for overnight at 28 ºC on rotary incubator at 140 rpm. Overnight grown culture (1000 µl) was transferred to 50 ml LB broth and incubated at 28 ºC at 140 rpm for 3 h to attain OD_{600} of 0.3 to 0.4. The culture was kept on ice for 30 min and later transferred to sterile 2 ml chilled microcentrifuge tubes. The cells were pelleted at 4000 rpm for 10 min at 4 ºC. The supernatant was decanted off. The pellet was resuspended in 1 mL of 20 mM $CaCl_2$ solution and pelleted at 4000 rpm for 5 min. 200 µl of 20 mM $CaCl_2$ was added to the pellet and resuspended in it. The competent cells were stored in ice and transformed with pART27 vector. To 200 µL of the competent cells, 1 µL (1 µg) of the plasmid DNA was added and mixed gently. The tubes were kept on ice for 15 min and then frozen for 5 min in liquid nitrogen and incubated at 37 ºC for 5 min. After incubation the tubes were quickly plunged in ice bath and chilled for 5 min. LB broth (1 mL) was added to the tube and incubated at 28 ºC for 3-4 h with shaking. The cells were plated on LB agar modified with rifampicin (100 µg/mL) and spectinomycin (100 µg/mL).

3.4 CHECKING FOR TRANSFORMATION

Transformation of *Agrobacterium* with pART27 using different treatments was checked by culturing the transformed cells on Luria Bertani media containing 100 µg/mL rifampicin and 100 µg/mL spectinomycin as selectable markers and the colonies were counted.

3.4.1 Comparison of transformation efficiency

Transformation efficiency is defined as the number of transformants *i.e.*, colony forming units (cfu) obtained per µg of plasmid DNA and the transformation efficiency of different treatments were calculated as follows.

$$\text{Transformant (cfu)} = \frac{(number\ of\ bacterial\ colonies\ x\ dilution\ ratio\ x\ original\ transformation\ volume)}{plated\ volume}$$

$$\text{Transformation efficiency} = \frac{\text{transformant (cfu)}}{\text{plasmid DNA (µg)}}$$

3.5 CONFIRMATION OF TRANSFORMATION

3.5.1 Isolation of plasmid

For confirmation of transformation, plasmid was isolated by alkaline lysis method (Bimboim and Doly, 1979) from the bacterial colonies from plates that showed higher transformation efficiency among the different treatments.

The quality of isolated plasmid was checked by agarose gel electrophoresis (Appendix IV). One percent agarose with ethidium bromide incorporated in it was prepared, melted and gel was cast. Electrophoresis was done at a constant voltage of 70 V. Together with the plasmid DNA, 1 kb DNA marker was also run. The gel was taken and viewed in gel documentation unit (Gel DOC^{TM} XR^{T}, Bio-Rad with Image Lab^{TM} software).

3.5.2 Colony PCR

Transformants were tested for the presence of the binary vector, pART27 in *A. tumefaciens* by colony PCR analysis with the gene specific primer for *npt*II. The sequence of primers used for *npt*II gene are given below:

Primer	Length	GC per cent	Sequence (5'-3')
*npt*IIF	18	55	GGTGCCCTGAATGAACTG
*npt*IIR	18	50	TAGCCAACGCTATGTCCT

The PCR reactions were carried out in 20 µl volume reaction mixtures containing 200 µM dNTPs, 10 pM of each primer, 1U of Taq polymerase and 1X Taq polymerase buffer. A single transformed bacterial colony was added to the reaction mix. The cycling conditions consisted of a denaturing step at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, annealing for 30 s at 55 ^{o}C, and elongation for 30 s at 72 °C; followed by a final extension at 72 °C for 5 min. The annealing temperature was determined based on the melting temperature value of the primer. The PCR products were separated on 1.2 percent (w/v) agarose gel containing 0.5 µg/mL ethidium bromide by horizontal gel electrophoresis. The PCR products were visualized in gel documentation unit (Gel DOC^{TM} XR^{T}, Bio-Rad with Image Lab^{TM} software.

Result

4. RESULTS

The results of the present study on "Silver nanoparticles for *Agrobacterium* mediated transformation" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2018 - 2020 are presented below.

4.1 TOXICITY ASSAY OF SILVER NANOPARTICLES ON *Agrobacterium tumefaciens* CELLS

4.1.1 Growth kinetics of *Agrobacterium tumefaciens* strain EHA105

Growth kinetics of *Agrobacterium tumefaciens* strain EHA105 was determined by recording the absorbance (OD_{600}) at four hour interval (Table 3). Growth curve depicted the different phases of bacterial growth *viz*., lag phase, log phase and stationary phase (Fig.4). Lag phase was observed for the initial 12 h followed by log phase for nearly 18 h and stationary phase 42 h after inoculation.

Table 3 Optical density at 600 nm of *Agrobacterium tumefaciens* EHA105 at different time intervals

Time period (hours)	OD at 600nm
0	0.00
4	0.02
8	0.05
12	0.08
16	0.25
20	0.69
24	1.18
28	1.57
32	1.69
36	1.72
40	1.75
44	1.83
48	1.79

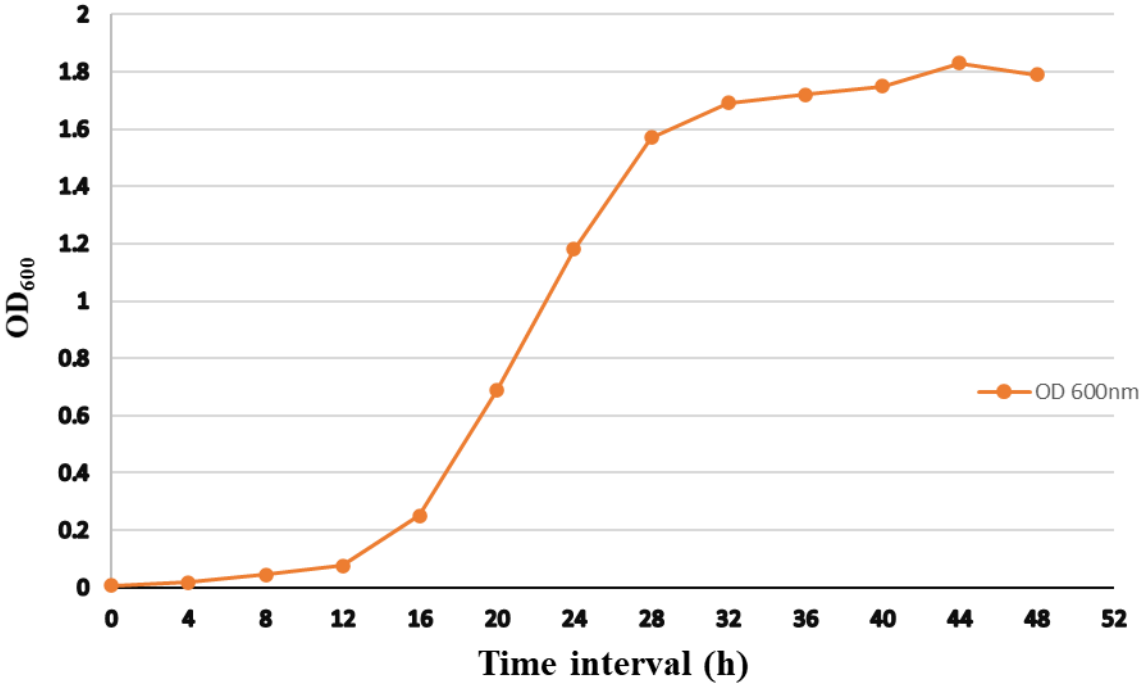


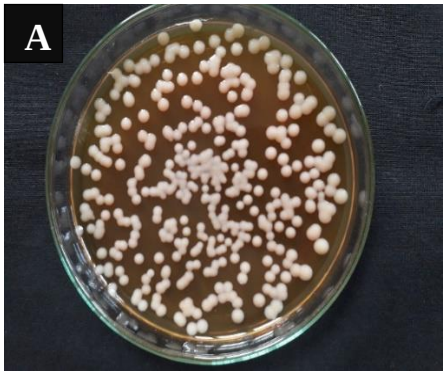
Fig.4 Growth curve of *Agrobacterium tumefaciens* EHA105

4.1.2. Estimation of EC_{50} of silver nanoparticles for *Agrobacterium tumefaciens*

Agrobacterium tumefaciens EHA105 cells showed dose dependent response to different treatments of silver nanoparticles (Table 4). Among the treatments, maximum inhibition (91%) was observed in treatment T_6 (20 mgL^{-1}). Minimum inhibition (3.3%) was observed in treatment T_2 (0.01 mgL^{-1}). Probit analysis of toxicity assay estimated the half maximal effective concentration (EC_{50}) of silver nanoparticles as 8.707 mgL^{-1}.

Table 4 Response of *Agrobacterium tumefaciens* EHA105 to different concentrations of silver nanoparticles

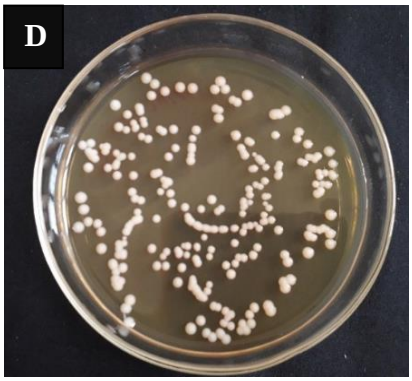
Treatment	Concentration of AgNPs (mgL^{-1})	cfu/mL	Inhibition (%)
Control	0	3.0×10^9	0
T_1	0.01	2.9×10^9	3.3
T_2	1	2.0×10^9	33
T_3	5	1.8×10^9	40
T_4	10	1.5×10^9	50
T_5	20	2.7×10^8	91



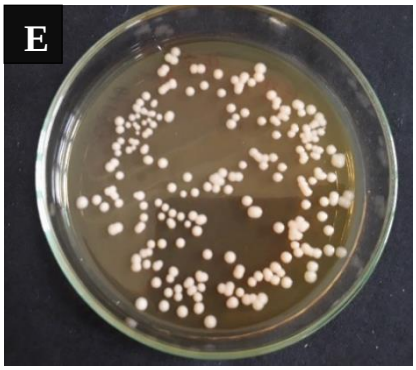
Control

0.01 mgL^{-1} AgNPs

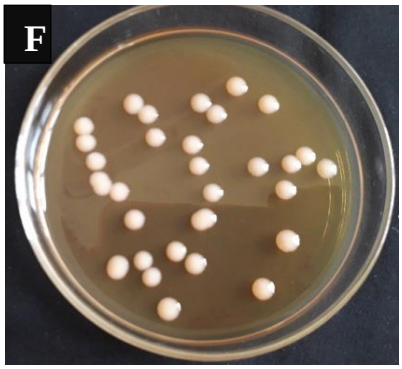
1 mgL^{-1} AgNPs



5 mgL^{-1} AgNPs



10 mgL^{-1} AgNPs



20 mgL^{-1} AgNPs

Plate 1. Toxicity assay of silver nanoparticles on *Agrobacterium tumefaciens* EHA105

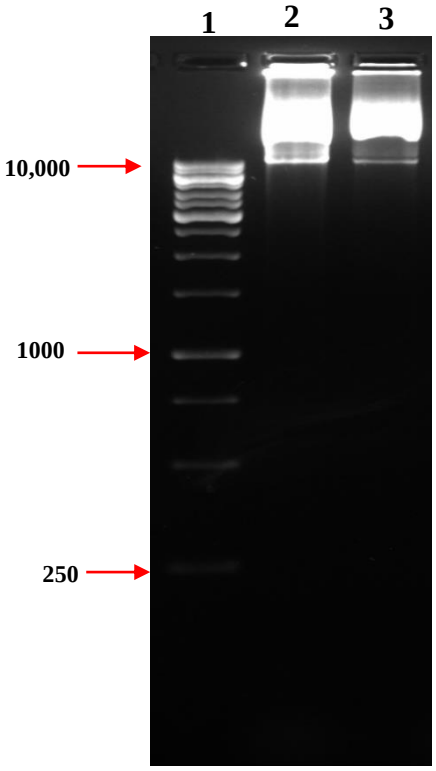
4.2 Isolation of plasmid DNA

Plasmid vector pART27 was isolated from *E. coli* cells. The absorbance at 260 nm and 280 nm are presented in Table 5.

Table 5 Quantity and quality of isolated plasmid

Vector	Replication	A_{260}	A_{280}	A_{260}/ A_{280}	Quantity (ng/µL)
pART27	R1	0.1874	0.1017	1.842	2811
	R2	0.0938	0.0521	1.800	2814
	R3	0.0921	0.0514	1.789	2760

Agarose gel electrophoresis of the isolated plasmid exhibited good quality band of predicted size (11.6 kb) (Plate 2).



Lane 1- 1 kb DNA ladder

Lane 2,3- Isolated pART27

Plate 2. Gel image of plasmid isolated from *E. coli*

4.3 Induction of competency and transformation of bacterial cells

The competent bacterial cells transformed with pART27 were screened by plating in LB agar plates supplemented with rifampicin (100 µg/mL) and spectinomycin (100 µg/mL). In treatments where conventional calcium chloride freeze-thaw technique (T_{19}), silver nanoparticles with calcium chloride technique (T_7 - T_{10}) and silver nanoparticles with calcium chloride freeze-thaw technique (T_{15} - T_{18}) were used, colonies appeared 48 h after plating whereas, in treatments using silver nanoparticles alone (T_1 - T_6) and silver nanoparticles with freeze-thaw technique (T_{11} - T_{14}), transformed colonies were observed only after 60 h of plating. The colonies that emerged from cells transformed using treatments with silver nanoparticles were comparatively smaller compared to that of conventional technique. No colonies were observed in negative control plates.

4.4 Transformation efficiency

The transformation efficiency of different treatments was assessed by counting the colonies that emerged from transformed cells in the selection medium. The efficiency varied significantly among the treatments (Table 6).

All the six treatments using silver nanoparticles alone (T_1 - T_6) showed significantly higher transformation efficiency ranging from 2.74 to 2.91 log cfu/µg of DNA compared to conventional technique (2.31 log cfu/µg of DNA). The best four concentrations selected for combining with calcium chloride and freeze-thaw were 0.01, 0.5, 1 and 2 mgL^{-1}.

All treatments with silver nanoparticles along with calcium chloride showed significantly higher transformation efficiency compared to conventional technique. Among all the treatments tried, maximum transformation efficiency was exhibited by 0.5 mgL^{-1} silver nanoparticles with 20 mM calcium chloride (3.34 log cfu/µg of DNA) which was on par with 0.01 mgL^{-1} silver nanoparticles with 20 mM calcium chloride (3.33 log cfu/ µg of DNA).

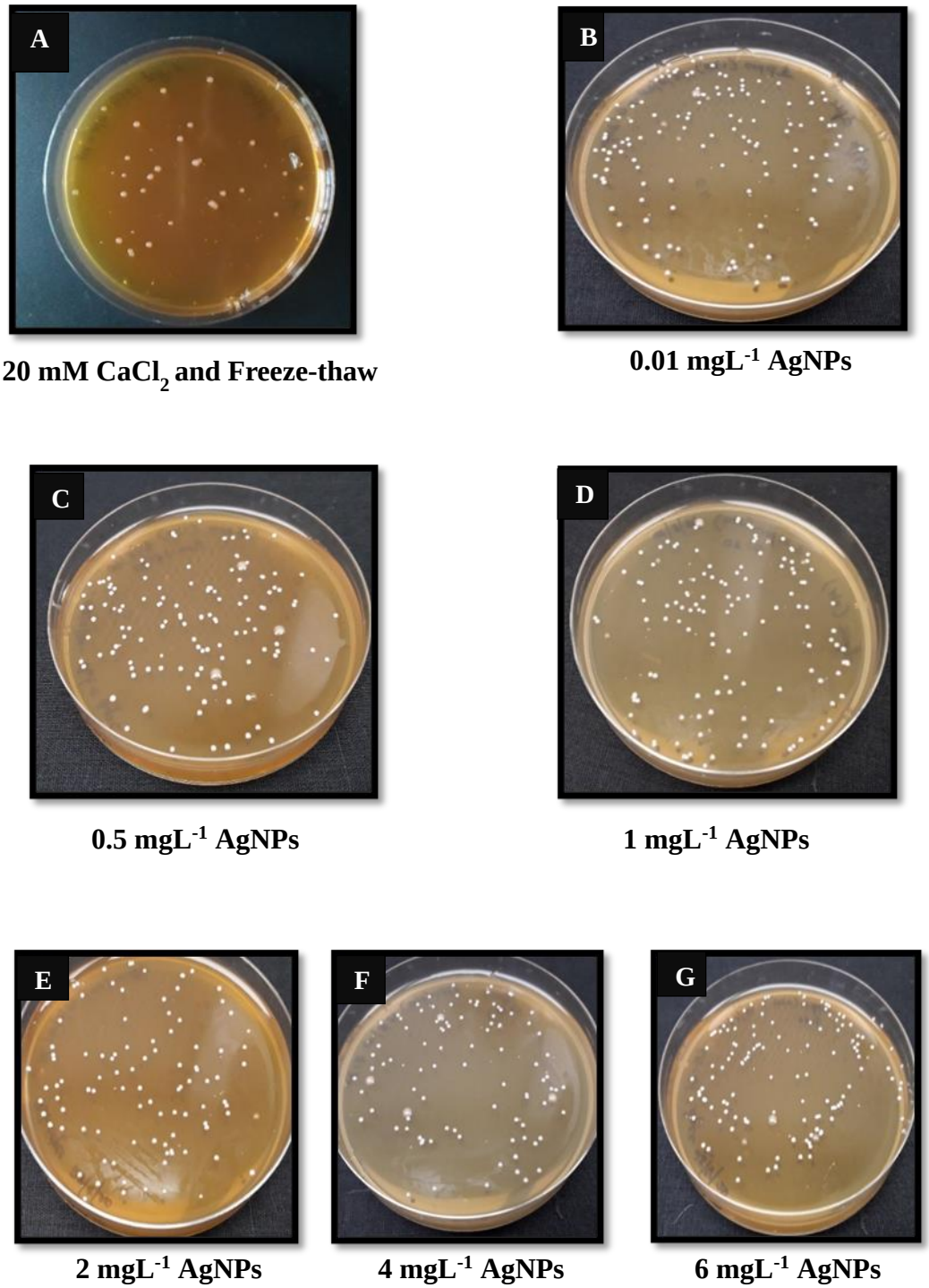
The freeze-thaw method in combination with silver nanoparticles and calcium chloride showed significantly higher transformation efficiency compared to the conventional calcium chloride freeze-thaw technique. However, it was lower

compared to treatment where silver nanoparticles with calcium chloride was used. Transformation efficiency of silver nanoparticles with calcium chloride along with freeze-thaw technique were lower or comparable to lower concentrations of silver nanoparticles alone. However, it was higher compared to higher concentrations of silver nanoparticles alone (4 mgL^{-1}, 6 mgL^{-1}).

In treatments with silver nanoparticles with freeze-thaw there was no significant increase in transformation efficiency compared to conventional technique.

Table 6 Transformation efficiency of *A. tumefaciens* EHA105 cells made competent with different treatments

Treatment	Transformation efficiency (log cfu/µg of DNA)
T_1	2.86^{d}
T_2	2.86^{d}
T_3	2.91^{c}
T_4	2.78^{f}
T_5	2.75^{g}
T_6	2.74^{g}
T_7	3.33^{a}
T_8	3.34^{a}
T_9	3.27^{b}
T_{10}	3.25^{b}
T_{11}	2.32^{h}
T_{12}	2.30^{h}
T_{13}	2.18^{i}
T_{14}	2.10^{j}
T_{15}	2.81^{e}
T_{16}	2.84^{d}
T_{17}	2.79^{ef}
T_{18}	2.78^{f}
T_{19}	2.31^{h}
T_{20} (Control)	0
CD (0.05) = 0.027	



20 mM $CaCl_2$ and Freeze-thaw

0.01 mgL^{-1} AgNPs

0.5 mgL^{-1} AgNPs

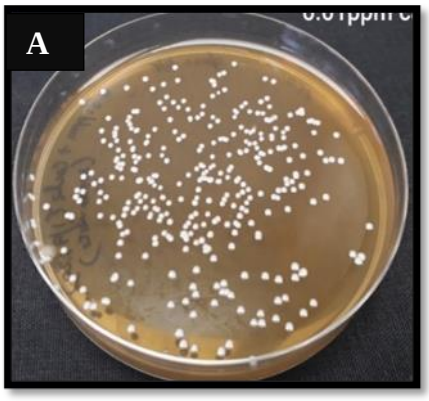
1 mgL^{-1} AgNPs

2 mgL^{-1} AgNPs

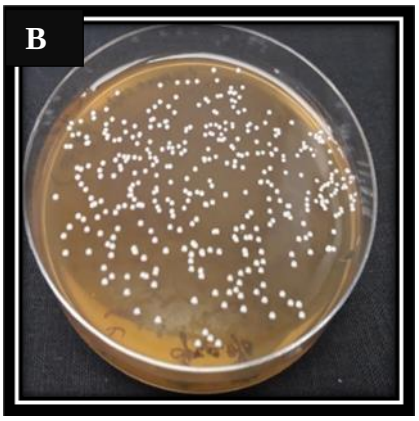
4 mgL^{-1} AgNPs

6 mgL^{-1} AgNPs

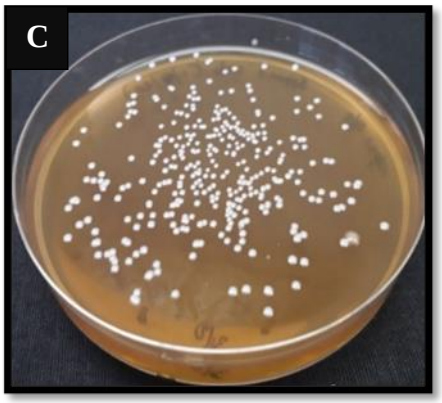
Plate 3. Colonies of *A. tumefaciens* EHA105 from cells transformed using conventional technique (Calcium chloride with freeze-thaw) and different concentrations of silver nanoparticles alone



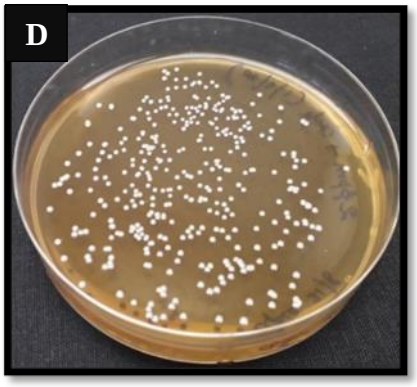
0.01 mgL^{-1} AgNPs + 20 mM CaCl$_2$



0.5 mgL^{-1} AgNPs + 20 mM CaCl$_2$

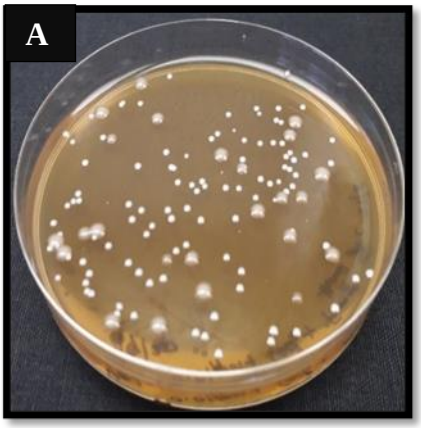


1 mgL^{-1} AgNPs + 20 mM CaCl$_2$

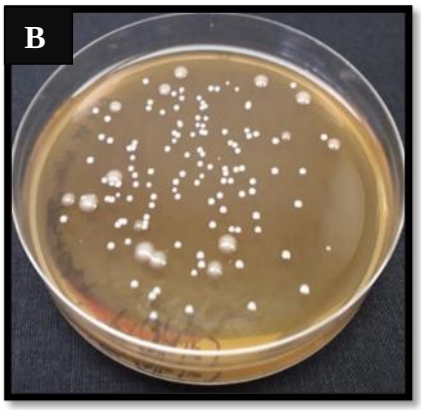


2 mgL^{-1} AgNPs + 20 mM CaCl$_2$

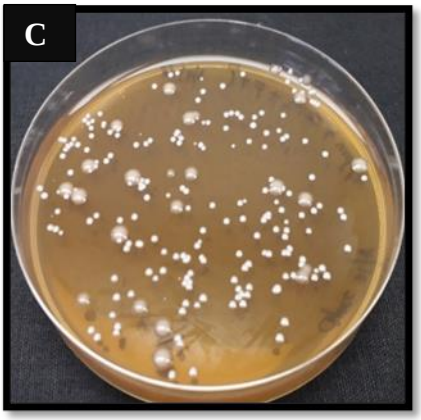
Plate 4. Colonies of *A. tumefaciens* EHA105 from cells transformed using different concentrations of silver nanoparticles along with 20 mM calcium chloride



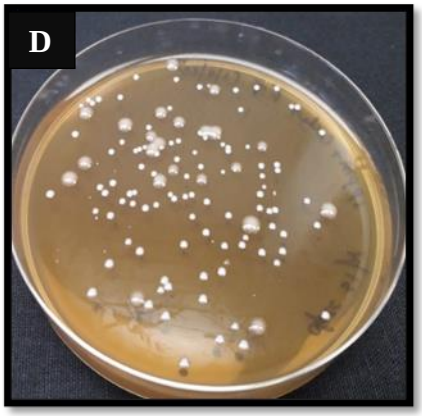
0.01 mgL^{-1} AgNPs + 20 mM $CaCl_2$ + Freeze-thaw



0.5 mgL^{-1} AgNPs + 20 mM $CaCl_2$ + Freeze-thaw

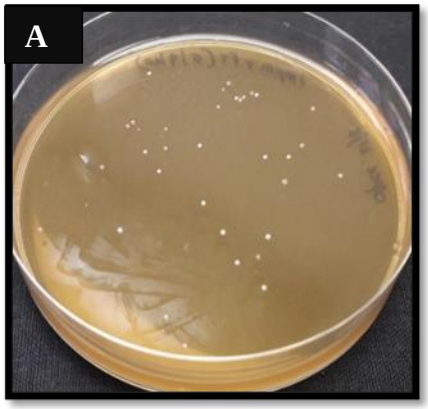


1 mgL^{-1} AgNPs + 20 mM $CaCl_2$ + Freeze-thaw

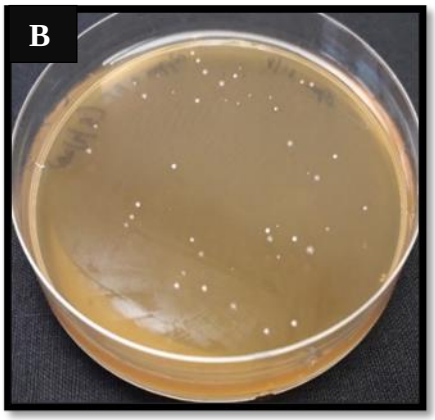


2 mgL^{-1} AgNPs + 20 mM $CaCl_2$ + Freeze-thaw

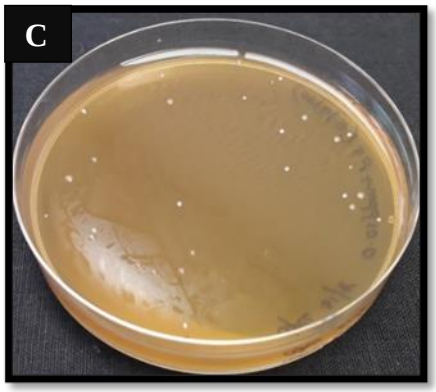
Plate 5. Colonies of *A. tumefaciens* EHA105 from cells transformed using different concentrations of silver nanoparticles along with calcium chloride and freeze-thaw



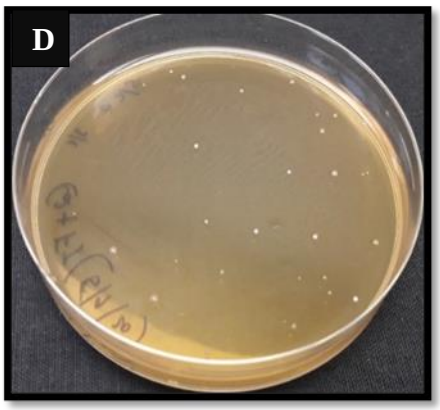
0.01 mgL^{-1} AgNPs + Freeze-thaw



0.5 mgL^{-1} AgNPs + Freeze-thaw



1 mgL^{-1} AgNPs + Freeze-thaw



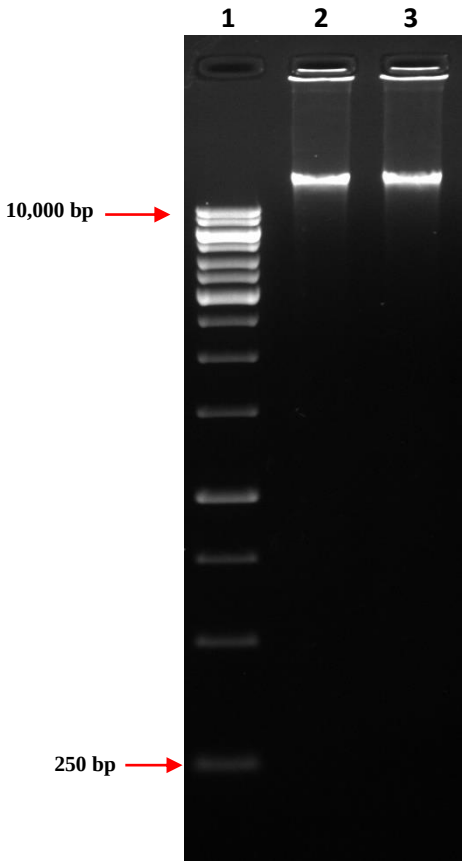
2 mgL^{-1} AgNPs + Freeze-thaw

Plate 6 Colonies of *A. tumefaciens* EHA105 from cells transformed using different concentrations of silver nanoparticles with freeze-thaw

4.5 Confirmation of transformation

Plasmid isolation was carried out from the cells transformed with 0.01 mgL^{-1} silver nanoparticles along with 20 mM calcium chloride and conventional technique. Good quality plasmid of predicted size (11.6 kb) was observed in agarose gel electrophoresis (Plate 7).

Transformed colonies obtained using different treatments were analysed by colony PCR using *npt*II gene specific primers. Single colonies of transformants from different treatments showed bands of predicted size (475 bp) confirming transformation (Plate 8).



Lane 1- 1kbp ladder

Lane 2- Plasmid isolated from transformed bacteria (20 mM $CaCl_2$ freeze-thaw technique)

Lane 3- Plasmid isolated from transformed bacteria (0.01 mgL^{-1} AgNPs + 20 mM $CaCl_2$)

Plate 7. Gel image of the plasmid isolated from transformed colonies

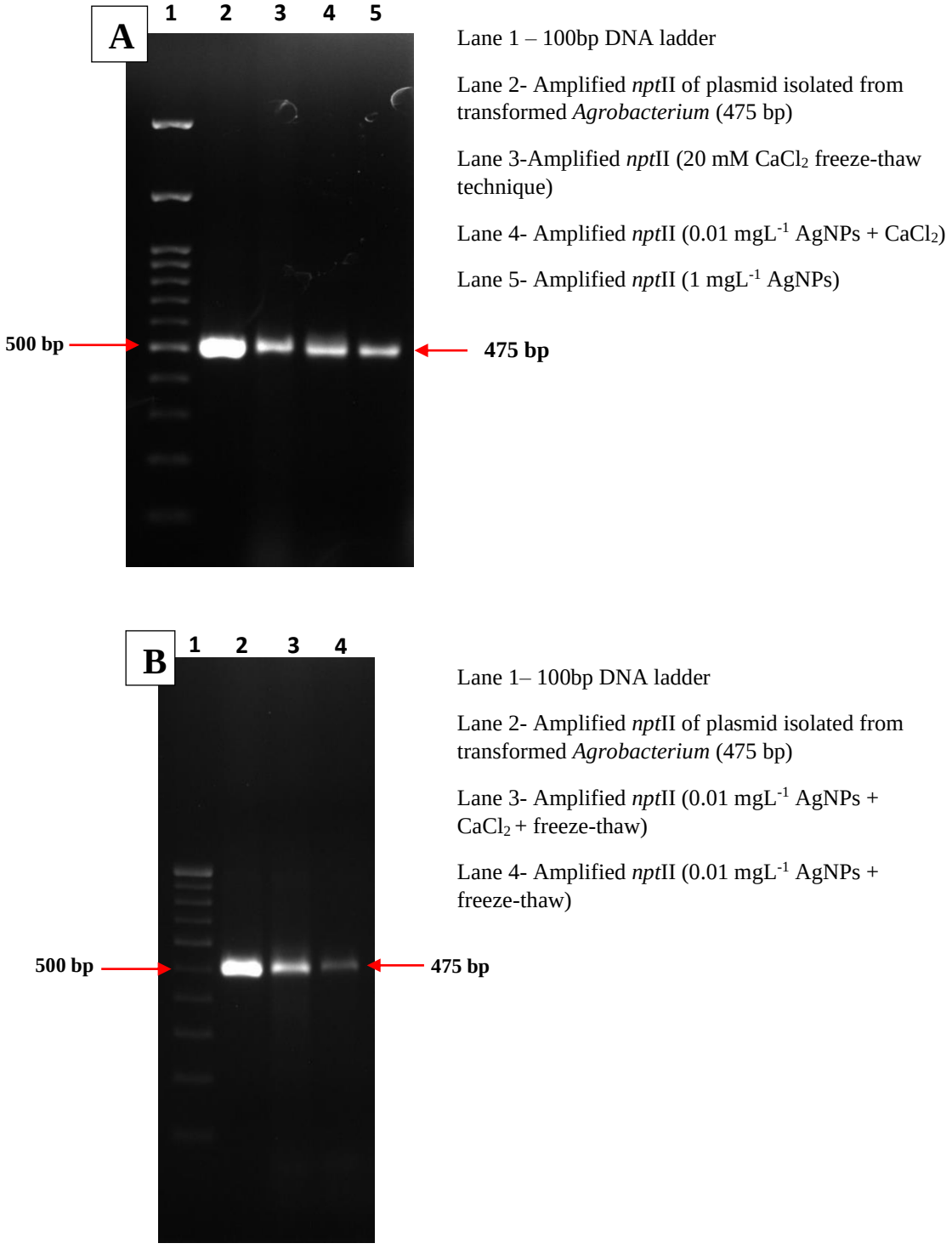


Plate 8. (A & B) Gel image of colony PCR using *npt*II primers in transformed *Agrobacterium tumefaciens* EHA105 by different treatments

Discussion

5. DISCUSSION

Bacterial transformation plays a pivotal role in genetic engineering technique. In recombinant DNA technology, an efficient technique for translocation of foreign genetic material across the biological membrane has an important role to play as majority of bacteria lack natural competency (Kotnik *et al*., 2015). Negative charges of both DNA and plasma membrane are the major hindrance in determining the efficiency of transformation in bacteria (Asif *et al*., 2017). Transport of exogenous DNA into the cytosol of Gram negative bacteria is a complex task as it involves movement across outer membrane, cell wall and plasma membrane (Chen and Dubnau, 2004).

Agrobacterium tumefaciens, a natural genetic engineer, is commonly used for plant genetic engineering. Induction of competency and transformation of *Agrobacterium* is a crucial step in transgenic plant development. Various methods reported for transformation of *Agrobacterium* include triparental mating, electroporation and freeze-thaw method (Wise *et al*., 2006). Triparental mating is a tedious process as it makes use of two *E. coli* strains for the transformation of *Agrobacterium*. Though electroporation gives high transformation efficiency the cost of equipment as well as the requirement of high cell density are two major limiting factors (Rebersek and Miklavcic, 2011). The most convenient method is freeze-thaw technique but low transformation efficiency is the major limitation. Therefore, attempts to improve the efficiency of introduction of DNA into *Agrobacterium tumefaciens* are in demand.

Nanotechnology has emerged as a most promising approach for both animal and plant genetic engineering. Nanotechnology-based carriers can deliver a variety of bioactive molecules into cells (Wang *et al*., 2019). Nanoparticles based carriers such as gold, silver and hydroxyapatite were reported to increase the bacterial transformation efficiency (Chatterjee and Sarkar, 2014; Deshmukh *et al*., 2019; Nagamani *et al*., 2019). Nanoparticle mediated gene transfer methods have the potential to directly transfer DNA into the cells, achieving stable integration and rapid expression of the transgene (Rai *et al*., 2015).

Silver nanoparticles disrupt the cell membrane, penetrate inside the cell and increase the porosity of the cell membrane (Dong e*t al*., 2019). It aids in the uptake of exogenous DNA by bacterial cells (Nagamani *et al*., 2019). Hence, in the present study an attempt was made to evaluate the efficiency of silver nanoparticles in improving the transformation efficiency of *Agrobacterium tumefaciens.*

There are several reports on efficient transformation using *Agrobacterium tumefaciens* strain EHA105 in different crop species. In the present study also transformation was attempted using *Agrobacterium tumefaciens* strain EHA105 as the host with pART27 as vector. Han *et al*. (2000) reported that *Agrobacterium* strain EHA105 was found to be superior over strains C58 and LBA4404 in transformation of cottonwood. According to Chetty *et al*. (2013), *Agrobacterium* strain EHA105 hasHig higher transformation efficiency and fewer transgene insertions in tomato. Bakhsh *et al.* (2014) reported higher transformation efficiency in tobacco using *Agrobacterium tumefaciens* strain EHA105 compared to C58C1 and AGL1. Wang and Pijut (2014) reported that *Agrobacterium* with pART27-PsAGRNAi construct showed higher transformation efficiency (21.7%) in black cherry than with pBI121-MDL4 or pBI121-PsTFL1 (5% each), indicating that the vector has a strong impact on transformation**.**

In the present study good quality and quantity of plasmid vector pART27 was isolated from *E. coli* by alkaline lysis method described by Bimboim and Doly (1979). Ehrt and Schnappinger (2003) have reported good quality plasmid isolation by this method from *E. coli*.

Growth curve of *A. tumefaciens* EHA105 was worked out by growing the bacteria in Luria Bertani medium supplemented with rifampicin (100 µg/mL) and by measuring absorbance at 600 nm at four hour intervals. The growth curve depicted the different phases of bacterial growth *viz*., lag phase, log phase and stationary phase. The lag phase was observed for initial 12 hours wherein it was preparing for cell division and hence no growth in cell volume was noticed. Subsequently, increase in cell number was noticed for 18 h indicating the log phase. Further, the OD value

remained constant indicating the stationary phase, as some of the cells are dying and others still growing and dividing. Similar pattern of growth curve was reported by Mendoza-de Gyves *et al*. (2010) in *Agrobacterium tumefaciens* strain GV2260.

The toxicity assay of silver nanoparticles on *Agrobacterium tumefaciens* strain EHA105 was carried out at exponential phase, as the doubling of bacterial cells occurs at a regular time interval. Among the different concentration of silver nanoparticles (Table 1), 0.01 mgL^{-1} exhibited minimum inhibition of growth (3.3%) and maximum inhibition of growth (91%) was exhibited by 20 mgL^{-1}. The half maximal effective concentration (EC_{50}) was statistically calculated as 8.707 mgL^{-1}. Increase in concentration of silver nanoparticles exhibited increase in toxicity. According to Amin *et al*. (2009), toxicity of silver nanoparticles on *E. coli* and *Staphylococcus capitis* was dose dependent. Nagamani *et al*. (2019) reported that silver nanoparticles of concentration 20 mgL^{-1} completely inhibited the growth of *E. coli* and concentration of 0.01 mgL^{-1} had no influence on the bacterial growth. Shrivastava *et al*. (2007) reported that the toxicity of silver nanoparticles was dose dependent and showed high inhibition on Gram negative bacteria compared to Gram positive ones. Toxicity is reported to decrease with increase in particle size. Silver nanoparticles of size 10 nm are more toxic than particles of 100 nm size (Cho *et al*., 2018).

Based on the result on the toxicity assay in the present study, silver nanoparticles of concentrations less than 8.70 mgL^{-1} were used for induction of competency in bacterial cells and transformation using pART27 vector. Nagamani *et al*. (2019) reported that concentration of silver nanoparticles below EC_{50} (4.75 mgL^{-1}) increased the transformation efficiency of *E. coli* when different vectors *viz*., pUC18, pCAMBIA and pBR322 were used for transformation.

Bacterial cell in early exponential phase are used for competent cell preparation and transformation. Chung *et al.* (1989) reported that transformation efficiency of *E. coli* cells decreased in a linear fashion as the cell density increased. According to Jyothiswaran *et al*. (2007) *Agrobacterium* cells in the early exponential phase (OD_{600} = 0.3) showed increased transformation efficiency and it decreased with

increase in absorbance from 0.3 to 0.6. Hence, in the present study also cells in the exponential phase were used for competency induction and transformation.

Competency induction and transformation efficiency of different treatments were assessed by counting the number of colonies on LB agar medium supplemented with rifampicin and spectinomycin as selectable marker. All the six treatments (T_1 to T_6) with silver nanoparticles alone showed threefold increase in transformation efficiency (2.74 to 2.91 log cfu/µg of DNA) compared to calcium chloride freeze-thaw technique (2.31 log cfu/µg of DNA) (Fig. 5). Nagamani *et al*. (2019) reported that *E. coli* cells treated with 100 nm silver nanoparticles at a concentration of 1 mgL^{-1} exhibited higher transformation (7.9×10^4 cfu/ng of DNA) compared to calcium chloride mediated method (2.3×10^3 cfu/ng of DNA). Increase in transformation efficiency might be due to the enhanced activity of silver nanoparticles on bacterial membrane resulting in larger pore size and larger surface area of the nanoparticles resulting in greater plasmid uptake. According to Weston *et al*. (1981), cations reduce the repulsive force between negatively charged DNA and outer membrane and facilitates better DNA membrane contact. Feng *et al*. (2000) reported that silver ions condense the DNA molecule resulting in reduced replication in *E. coli*. Sondi and Salopek-Sondi (2004) reported that *E. coli* cells treated with silver nanoparticles showed disruption in the surface of the cells characterised by pits in the cell walls.

Increase in concentration of silver nanoparticles resulted in decrease in transformation efficiency. Concentration of 1 mgL^{-1} gave a transformation efficiency of 2.91 log cfu/µg of DNA, whereas, at 6 mgL^{-1} concentration comparatively low transformation efficiency was recorded (2.74 log cfu/µg of DNA). This may be due to the fact that increased concentration of silver nanoparticles might hinder DNA replication as well bind to the thiol groups of proteins resulting in inactivation (Tang and Zheng, 2018). According to Shrivastava *et al.* (2007), silver nanoparticles bind to the negatively charged functional groups on bacterial cell wall and infiltrate into the cell. Navarro *et al*. (2008) reported that small size and large surface volume ratio of silver nanoparticles resulted in the release of silver ion into cell, resulting in increased toxicity on *Chlamydomonas reinhardtii.* Yan *et al*. (2018) confirmed by bioinformatic analysis that antibacterial mechanism of silver nanoparticles involves disruption of

cell membrane as well as generation of intracellular reactive oxygen species due to the release of silver ion.

Silver nanoparticles (0.01, 0.5, 1 and 2 mgL^{-1}) along with calcium chloride (20 mM) showed better transformation efficiency (3.25 to 3.34 log cfu/µg of DNA) compared to all other techniques. The transformation efficiency was ten-fold higher than the conventional technique (Fig.6). This may be due to enhanced uptake of plasmid DNA by the action of both silver nanoparticles and calcium chloride. Combined action of silver nanoparticles and calcium chloride might have increased the permeability of membrane. Wang *et al*. (2011) reported that competent cells of *Agrobacterium tumefaciens* strain EHA105 and LBA4404 prepared using calcium chloride in combination with dimethyl sulfoxide (DMSO) or polyethylene glycol 4000 (PEG4000) showed significantly higher transformation efficiency. The action of chloride ions of calcium chloride might reduce the toxicity effect of silver nanoparticles thus resulting in higher transformation efficiency. Chambers *et al*. (2014) reported that *E. coli* showed increased tolerance to AgNPs in the presence of high concentration of chloride ions due to dissolution of silver ion and formation of less toxic silver chloride.

The transformation efficiency of silver nanoparticles along with freeze-thaw was found to be significantly lower or comparable to conventional technique (Fig.7). Toxicity of silver nanoparticles along with stress caused by freeze-thaw might have resulted in low transformation efficiency.

Silver nanoparticles along with calcium chloride and freeze-thaw exhibited three-fold increase in transformation efficiency compared to conventional calcium chloride freeze-thaw technique (Fig.8). This might be due to combined action of silver nanoparticles and calcium chloride. The toxicity effect of silver nanoparticles might have been nullified by the action chloride ions. Garg *et al*. (2016) reported that oxidative dissolution of silver nanoparticles by the action of oxychloride ion formed less reactive silver chloride. However, transformation efficacy was lower than silver nanoparticles along with calcium chloride. This might be due to the stress caused by freeze-thaw.

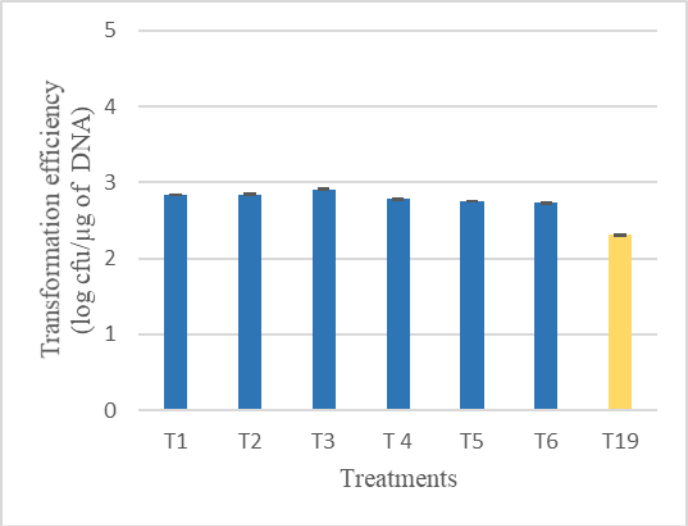


Fig.5 Comparison of transformation efficiency of silver nanoparticles alone and conventional calcium chloride freeze-thaw technique. T_1 - 0.01 mgL^{-1} AgNPs, T_2 - 0.5 mgL^{-1} AgNPs, T_3 - 1 mgL^{-1} AgNPs, T_4 - 2 mgL^{-1} AgNPs, T_5 - 4 mgL^{-1} AgNPs, T_6 - 6 mgL^{-1} AgNPs, T_{19} - 20 mM $CaCl_2$ with freeze-thaw technique

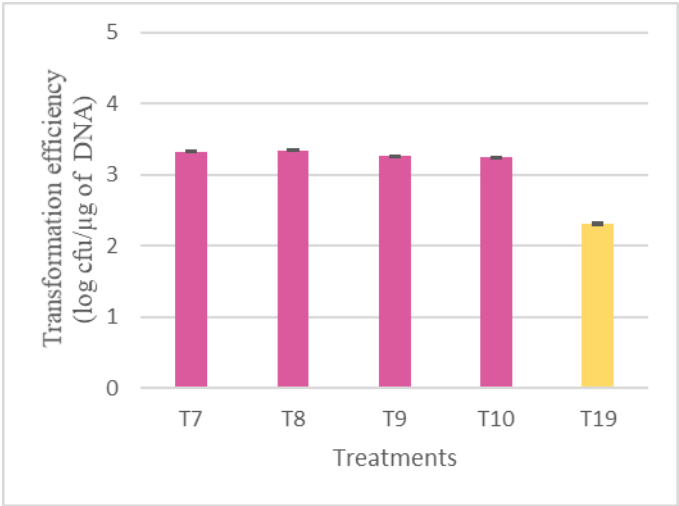


Fig.6 Comparison of transformation efficiency of silver nanoparticles along with calcium chloride and conventional calcium chloride freeze-thaw technique. T_7 - 0.01 mgL^{-1} AgNPs with 20 mM $CaCl_2$, T_8 - 0.5 mgL^{-1} AgNPs with 20 mM $CaCl_2$, T_9 - 1 mgL^{-1} AgNPs with 20 mM $CaCl_2$, T_{10} - 2 mgL^{-1} AgNPs with 20 mM $CaCl_2$, T_{19} - 20 mM $CaCl_2$ with freeze-thaw technique

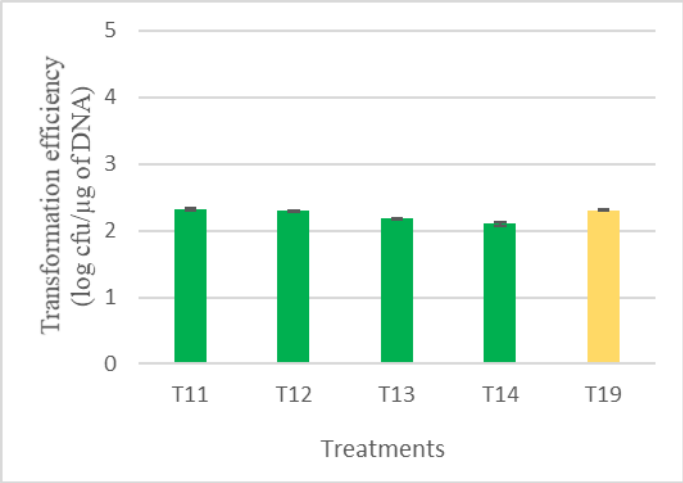


Fig.7 Comparison of transformation efficiency of silver nanoparticles along with freeze-thaw and conventional calcium chloride freeze-thaw technique. T_{11} - 0.01 mgL^{-1} AgNPs with freeze-thaw, T_{12} - 0.5 mgL^{-1} AgNPs with freeze-thaw, T_{13} - 1 mgL^{-1} AgNPs with freeze-thaw, T_{14} - 2 mgL^{-1} AgNPs with freeze-thaw, T_{19} - 20 mM $CaCl_2$ with freeze-thaw technique

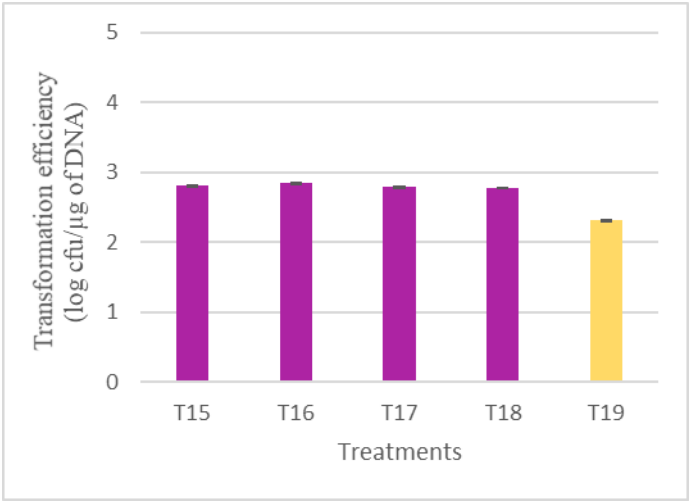


Fig.8 Comparison of transformation efficiency of silver nanoparticles along with calcium chloride and freeze-thaw and conventional calcium chloride freeze-thaw technique. T_{15} - 0.01 mgL^{-1} AgNPs with 20 mM $CaCl_2$ and freeze-thaw, T_{16} - 0.5 mgL^{-1} AgNPs with 20 mM $CaCl_2$ and freeze-thaw, T_{17} - 1mgL^{-1} AgNPs with 20 mM $CaCl_2$ and freeze-thaw, T_{18} - 2 mgL^{-1} AgNPs with 20 mM $CaCl_2$ and freeze-thaw, T_{19} - 20 mM $CaCl_2$ with freeze-thaw technique

According to Chatterjee and Sarkar (2014), transformation efficiency of glutathione functionalised gold nanoparticles mediated transformation of plasmid DNA (pUC19) into *E. coli* DH5α have higher (4.2 x 10^7 cfu/µg DNA) compared to conventional calcium chloride mediated method (2.3 x 10^5 cfu/µg DNA). Deshmukh *et al*. (2019) demonstrated that arginine-glucose functionalized hydroxyapatite nanoparticles (R-G-HAp NPs) can serve as an efficient plasmid delivery vehicle for both Gram positive and Gram negative bacterial transformation. R-G-HAp NPs mediated transformation in *E. coli* exhibited 10^2 and 10^3 fold higher transformation efficiency as compared to calcium chloride and electroporation, respectively.

Results of the present study indicated that silver nanoparticles of 100 nm size at a concentration of 0.5 mgL^{-1} or 0.01 mgL^{-1} along with 20 mM calcium chloride showed ten-fold increase in the transformation efficiency of *Agrobacterium tumefaciens* EHA105 with vector pART27 compared to conventional 20 mM calcium chloride freeze-thaw technique. Since, both the concentrations are on par in their effects, the lower concentration *viz*., 0.01 mgL^{-1} along with 20 mM calcium chloride was selected for further study.

Good quality of plasmid could be re-isolated from colonies transformed using 0.01 mgL^{-1} AgNPs along with 20 mM $CaCl_2$ by alkaline lysis method confirming transformation. Nagamani *et al*. (2019) have reported re-isolation of plasmid from *E. coli* cells transformed using 1 mgL^{-1} silver nanoparticles. Colony PCR using *npt*II primer also resulted in amplicon 475bp as reported by Jadhav (2019).

To conclude, silver nanoparticles at a concentration of 0.01 mgL^{-1} along with 20 mM calcium chloride is found to be an economically viable method to improve the transformation efficiency in *Agrobacterium tumefaciens* by ten-fold compared to conventional calcium chloride freeze-thaw technique.

Plant cells can be stably transformed by *Agrobacterium* and regenerated into fertile plants with inheritable features (Hwang *et al*., 2017). Co-cultivation of leaf discs with *Agrobacterium tumefaciens* transformed with pART27 using silver nanoparticles with calcium chloride and confirmation of transformation with *npt*II primer in regenerated plants needs to be taken up as a future line of work.

Summary

6. SUMMARY

The study entitled “Silver nanoparticles for *Agrobacterium* mediated genetic transformation” was carried out during 2018 - 2020 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to evaluate the efficacy of silver nanoparticles in improving transformation efficiency of *Agrobacterium tumefaciens.*

The growth curve of *A. tumefaciens* EHA105 was worked out by growing the bacteria in Luria Bertani medium supplemented with rifampicin (100 µg/mL) and measuring the absorbance at 600 nm at four hour interval. The growth curve depicted the different phases of bacterial growth *viz*., lag phase, log phase and stationary phase. The lag phase was observed for the initial 12 hours wherein it was preparing for cell division and hence no growth in cell volume was noticed. Subsequently, increase in cell number was noticed for 18 h indicating the log phase. Further, the OD value remained constant indicating the stationary phase as some of the cells are dying and others still growing and dividing.

The toxicity assay of silver nanoparticles on *Agrobacterium tumefaciens* strain EHA105 was carried out at the exponential phase. Different concentrations of silver nanoparticles (100 nm size) tried were 0.01, 1, 5, 10 and 20 mgL^{-1}. Among the different concentration of silver nanoparticles, 0.01 mgL^{-1} exhibited minimum inhibition of growth (3.3%) and maximum inhibition of growth (91%) was exhibited by 20 mgL^{-1}. The half maximal effective concentration (EC_{50}) was estimated as 8.707 mgL^{-1}.

As calcium chloride freeze-thaw technique is the conventional method for induction of competency and transformation, the efficiency of different treatments using silver nanoparticles were compared with calcium chloride freeze-thaw technique (positive control). The different treatments tried were replacement of calcium chloride and freeze-thaw with silver nanoparticles (ie., silver nanoparticles alone were used) or replacement of freeze-thaw (ie., silver nanoparticles with 20 mM calcium chloride) or replacement of calcium chloride (ie., silver nanoparticles with

freeze-thaw) in the conventional technique. Efficacy of addition of silver nanoparticles to conventional calcium chloride freeze-thaw technique for improvement of efficiency was also assessed.

Induction of competency and transformation of *Agrobacterium* was carried out in the early exponential phase using different concentrations of silver nanoparticles alone below EC_{50} value *viz*., 0.01, 0.5, 1, 2, 4 and 6 $mgL^{-1.}$ The best four treatments were tried for further treatments using silver nanoparticles with calcium chloride, silver nanoparticles with freeze-thaw, silver nanoparticles along with calcium chloride and freeze-thaw.

Transformation efficiency of different treatments were assessed by counting the number of colonies on LB agar medium supplemented with rifampicin and spectinomycin as selectable marker. Treatments with silver nanoparticles alone showed three-fold increase in transformation efficiency (2.74 to 2.91 log cfu/µg of DNA) compared to calcium chloride freeze-thaw technique (2.31 log cfu/µg of DNA). Increase in concentration of silver nanoparticles resulted in decrease in transformation efficiency. Concentration of 1 mgL^{-1} gave a transformation efficiency of 2.91 log cfu/µg of DNA, whereas, at a concentration of 6 mgL^{-1} comparatively low transformation efficiency (2.74 log cfu/µg of DNA) was recorded. The best four concentrations selected for combining with calcium chloride and freeze-thaw were 0.01, 0.5, 1 and 2 mgL^{-1}.

All treatments with silver nanoparticles along with calcium chloride showed significantly higher transformation efficiency compared to conventional technique. Among all the treatments tried, maximum transformation efficiency was exhibited by 0.5 mgL^{-1} silver nanoparticles along with 20 mM calcium chloride (3.34 log cfu/µg of DNA) which was on par with that obtained by treatment with 0.01 mgL^{-1} silver nanoparticles with 20 mM calcium chloride (3.33 log cfu/ µg of DNA).

The transformation efficiency of silver nanoparticles along with freeze-thaw was found to be significantly lower or comparable to conventional technique. The freeze-thaw method in combination with silver nanoparticles and calcium chloride showed significantly higher transformation efficiency compared to the conventional

calcium chloride freeze-thaw technique. However, it was lower compared to the treatment in which silver nanoparticles with calcium chloride was used. Transformation efficiency of treatments with silver nanoparticles along with calcium chloride and freeze-thaw technique were lower or comparable to treatments with low concentrations of silver nanoparticles alone. However, it was higher compared to higher concentrations of silver nanoparticles alone (4 mgL^{-1}, 6 mgL^{-1}).

Results of the present study indicated that silver nanoparticles of 100 nm size at a concentration of 0.5 mgL^{-1} or 0.01 mgL^{-1} along with 20 mM calcium chloride showed ten-fold increase in the transformation efficiency of *Agrobacterium tumefaciens* EHA105 harbouring the vector pART27 compared to conventional 20 mM calcium chloride freeze-thaw technique. Since, both the concentrations are on par in their efficiency, the lower concentration *viz*., 0.01 mgL^{-1} along with 20 mM calcium chloride was selected for further study.

Good quality of plasmid could be re-isolated from colonies transformed using 0.01 mgL^{-1} AgNPs with 20 mM $CaCl_2$ by alkaline lysis method confirming transformation. To conclude, use of silver nanoparticles at a concentration of 0.01 mgL^{-1} along with 20 mM calcium chloride is an economically viable method to improve the transformation efficiency of *Agrobacterium tumefaciens* by ten-fold compared to conventional calcium chloride freeze-thaw technique.

Evaluation of efficacy of transformed *Agrobacterium tumefaciens* EHA105 with pART27 using silver nanoparticles with calcium chloride in plant transformation and confirmation of transformation with *npt*II primer in regenerated plants needs to be taken up as a future line of work.

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Appendices

APPENDIX I

Chemical composition of Luria Bertani (LB) media

Ingredients	g/L
Casien hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	10.00
Final pH (at 25 ^{o} C)	7.5 ± 2

In case of LB agar media, 15 g/L of agar was added and dissolved before sterilization.

APPENDIX II

Preparation of stock solutions of antibiotics

SI. No.	Antibiotic	Solvent
1	Rifampicin	Alcohol
2	Spectinomycin	Water

Stock solution of rifampicin (50 mg/mL) was prepared by dissolving in alcohol and adding 2 to 3 drops of 10 N sodium hydroxide solution. Spectinomycin (50 mg/mL) was prepared by dissolving in water. Both the stock solutions were stored at -20 ^{o}C as aliquots.

APPENDIX III

Buffers for isolation of plasmid DNA from host cells

1. Glucose EDTA Tris (GET) buffer (10 mL)

Glucose : 90.1 mg

EDTA (0.5 M) : 200 µL

Tris- Cl (0.5 M) : 500 µL

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

2. Lysis buffer (10 mL)

NaOH (1 N) : 2 mL

SDS (10%) : 0.1 g

Freshly made up using sterile autoclaved water (Not autoclaved)

3. Neutralizing solution (10 mL)

Potassium acetate (5 M) : 6 mL

Glacial acetic acid : 1.15 mL

Water : 2.85 mL

Not autoclaved, stored at room temperature.

APPENDIX IV

Preparation of agarose gel electrophoresis buffer

TBE (Tris borate EDTA) buffer

Tris base : 10.8 g

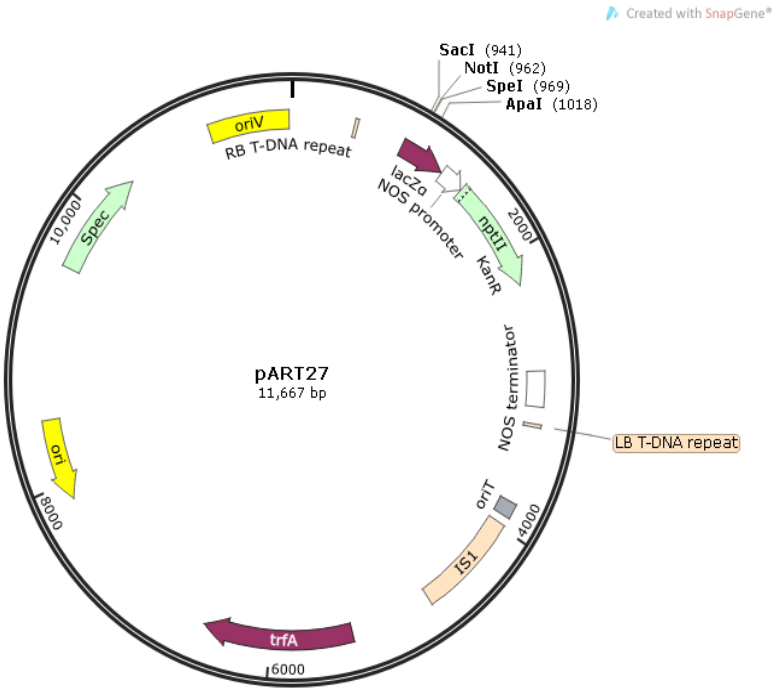
Boric acid : 5.5 g

EDTA (0.5 M) : 4 mL

Volume made up to 1 L with double distilled water.

APPENDIX V

Physical map of pART27



SILVER NANOPARTICLES FOR *AGROBACTERIUM* MEDIATED TRANSFORMATION

by

AMALA BENNY

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ABSTRACT

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VELLAYANI, THIRUVANANTHAPURAM- 695 522

KERALA, INDIA

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ABSTRACT

The study entitled "Silver nanoparticles for *Agrobacterium* mediated transformation" was carried out during 2018 - 2020 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to evaluate the efficacy of silver nanoparticles in improving the transformation efficiency of *Agrobacterium tumefaciens.*

Growth kinetics of *Agrobacterium tumefaciens* strain EHA105 was determined by plotting Absorbance (OD_{600}) *vs* Time. The exponential phase started 12 h after inoculation. Toxicity assay of silver nanoparticles (100 nm) on *Agrobacterium* cells was carried out in the exponential phase by exposing to different concentrations of silver nanoparticles *viz*., 0.01, 1, 5, 10 and 20 mgL^{-1}. Maximum toxicity (91%) was recorded at a concentration of 20 mg L^{-1} and minimum (3.3%) at a concentration of 0.01 mgL^{-1}. The half maximal effective concentration (EC_{50}) was estimated as 8.707 mgL^{-1} by carrying out Probit analysis using the SPSS software.

Efficacy of different concentrations of silver nanoparticles (below EC_{50} value) for induction of competency and transformation of *Agrobacterium tumefaciens* with pART27 (11.6 kb) plasmid vector was assessed and compared with conventional calcium chloride (20 mM) freeze-thaw technique. The different techniques tried were silver nanoparticles alone, silver nanoparticles with calcium chloride, silver nanoparticles with freeze-thaw and silver nanoparticles with calcium chloride along with freeze-thaw. For the treatments involving silver nanoparticles alone, six different concentrations (0.01, 0.5, 1, 2, 4, 6 mgL^{-1}) were tried. The four concentrations (0.01, 0.5, 1, 2 mgL^{-1}) that recorded better transformation efficiency were selected for inclusion in combinations with calcium chloride and freeze-thaw technique. All the treatments were carried out with cultures at an early exponential phase and the transformants were selected on Luria Bertani agar supplemented with rifampicin (100 µg/mL) and spectinomycin (100 µg/mL). Transformation efficiency of different techniques was assessed.

Colonies started appearing in the plates after 48 h in conventional technique and treatments involving silver nanoparticles with calcium chloride, whereas, in all other treatments involving silver nanoparticles, colonies were observed after 60 h of plating. Size of the colonies that emerged from cells involving treatments with silver nanoparticles were smaller compared to that of conventional technique. However, all treatments involving silver nanoparticles showed significantly higher or comparable transformation efficiency with conventional technique (2.31 log cfu/µg of DNA) except two treatments (1 mgL^{-1} or 2 mgL^{-1} of silver nanoparticles with freeze-thaw). Maximum transformation efficiency (3.34 log cfu/µg of DNA) was recorded in the treatment with combination of 0.5 mgL^{-1} silver nanoparticles along with calcium chloride (20 mM) which was on par with 0.01 mgL^{-1} silver nanoparticles along with calcium chloride (3.33 log cfu/ µg of DNA).

Transformation was confirmed by the re-isolation of plasmid from the transformed colonies. Colony PCR of the transformed colonies using *npt*II gene specific primer exhibited an amplicon of predicted size (475 bp) confirming transformation.

Results of the present study indicated that silver nanoparticles can improve transformation efficiency in *Agrobacterium tumefaciens*. Silver nanoparticles of size 100 nm at a concentration of 0.01 mgL^{-1} with calcium chloride (20 mM) showed ten-fold increase in the transformation efficiency (3.33 log cfu/µg of DNA) of *Agrobacterium tumefaciens* compared to conventional calcium chloride freeze-thaw technique. From the study it was inferred that induction of competency using silver nanoparticles is economically feasible.