Studies on Nanoparticles-Assisted Genetic Transformation of *Stevia rebaudiana* Bertoni

A

Dissertation Report

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DECLARATION

I hereby declare that the work being presented in the dissertation report entitled **"Studies on Nanoparticles-Assisted Genetic Transformation of *Stevia rebaudiana* Bertoni"** submitted by me for the award of the degree of **Master of Technology** in Department of Biotechnology, TIET University, Patiala is true and original record of my own independent and original research work carried out under the joint supervision of Dr. Anil Kumar and Dr. Shekhar Agnihotri. Further, I declare that no part of this dissertation has been submitted to any other University/Institute for the award of any degree in India or abroad.

Vasudha

Dated: 22-08-2018

Vasudha Verma

CERTIFICATE

This is to certify that the dissertation work entitled **"Studies on Nanoparticles-Assisted Genetic Transformation of *Stevia rebaudiana* (Bertoni)"** submitted by Vasudha Verma (Roll. No. 601604010) in partial fulfillment for the award of degree of Master of Technology in Biotechnology from Thapar Institute of Engineering and Technology, Patiala, Punjab, is the record of the candidate's own independent and original research work carried out under our supervision and guidance. The matter embodied in this dissertation has not been submitted in part to any other University/Institute for the award of any degree or diploma in India or Abroad.

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Abbrevations

%	Percent
°C	Degree Celsius
2,4-D	2,4 Dichlorophenoxy acetic acid
BA	6-Benzyladenine
cm	Centimeter
et al.	et alia alii (Latin) = and other people
Fig	Figure
g	Gram
GUS	□-Benzyladenine
hrs	Hours
IAA	Indole acetic acid
l	Liter
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Mill molar
MS	Murashige and Skooge (1962) medium
MS-1	Murashige and Skooge (1962) medium + 10 μM silver nitrate
N	Normal
NAA	1-Naphtalene acetic acid
nm	Nano meter
nos	Nopaline synthase

NPs	Nano particles
Npt II	neomycin phosphor transferase
O.D.	Optical density
PGR's	Plant growth regulator(s)
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by volume
X-gluc	5-bromo-4-chloro-3-indolyl-D-glucronic acid
µg	Microgram
µl	Microliter
µM	Micro molar

Abstract

Stevia rebaudiana Bertoni is a medicinally important plant, which is well known for its non-caloric sweetening properties. Because of its natural properties it is in huge demand but the rate of seed germination is very low and seed propagation does not lead to the production of homogenous populations. There are many limitations with the traditional breeding technique. Thus, biotechnological tools such as genetic engineering and genetic transformation are seen as important alternatives. The present report is focused on the use of silver nanoparticles for the improvement of genetic transformation efficiency of *Stevia rebaudiana* Bertoni. Effect of different combinations and concentration of 2,4-D, NAA and BA on shoot organogenesis and somatic embryogenesis was investigated. In MS-1 medium supplemented with 5 μM BA (92%) of explants resulted in shoot organogenesis. MS-1 Medium supplemented with 1 μM 2,4-D (95%) and 1 μM NAA (96%) showed maximum explant differentiating into embryo like structures.

The present study is aimed at the development of an efficient method genetic transformation in *Stevia rebaudiana* Bertoni through *Agrobacterium tumefaciens* and particles. In *Agrobacterium tumefacien* mediated genetic transformation explants pre-cultured (2 days) before bacterial infection ($O.D_{600}$ - 0.6) showed maximum transient GUS expression (80%). The genetic transformation through chitosan stabilized silver nanoparticles coated with pBI121 plasmid was attempted while the effect of pristine silver nanoparticles in the presence of chitosan as a stabilizer was also studied. The explants injured silver NPs and transformed with *Agrobacterium tumefacien* showed (70%) of transient GUS expression while there was decline in transformation frequency in explants injured with chitosan stabilized silver NPs and transformed with *Agrobacterium tumefacien* showed (30%). GUS activity and molecular studies confirm stable transformation in regenerated shoots. To optimize the use of nanoparticles in genetic transformation further studies need to be conducted.

Introduction

Stevia rebaudiana Bertoni commonly known as candy leaf (Carakostas *et al.* 2008) is a medicinal plant, which belongs to 'Asteraceae' family. *S.rebaudiana* is a small perennial herbaceous, semi-bushy endemic shrub native to Amambay region of Paraguay. It grows in a subtropical region with temperature ranging from -6 °C to 43°C (Yadav *et al.* 2011). It reaches a height of 65-80 cm, with 2.5 cm sessile oppositely arranged leaves (Goyal *et al*. 2010). The flowers are small in size (15mm-17mm), white in color having both male and female organs (Goettemoeller *et al.* 1999, Marsolasis *et al.* 1998). Flowering in *Stevia* occurs from January to March and from September to December (Chalapathi *et al.* 1997).

The seeds produced by *Stevia* are achene type with feathery pappus and are about 3 mm in length (Brandle *et al*.1998, Goettemoeller and Ching 1999). The endosperm is very small, the seedlings are slow to develop because of the small size of seeds. The plant responds well to soil, which is rich in water, and has high organic matter (Singh & Rao 2005; Ramesh *et al*. 2006). *Stevia* has been cultivated in many states of India: Rajasthan, Maharashtra, Kerala and Orissa, currently the major market of *Stevia* is in Japan. There are few countries like China, Taiwan, Malaysia, Thailand, Korea, which are cultivating *Stevia* commercially (Thiyagarajan & Venkatachalam 2012).

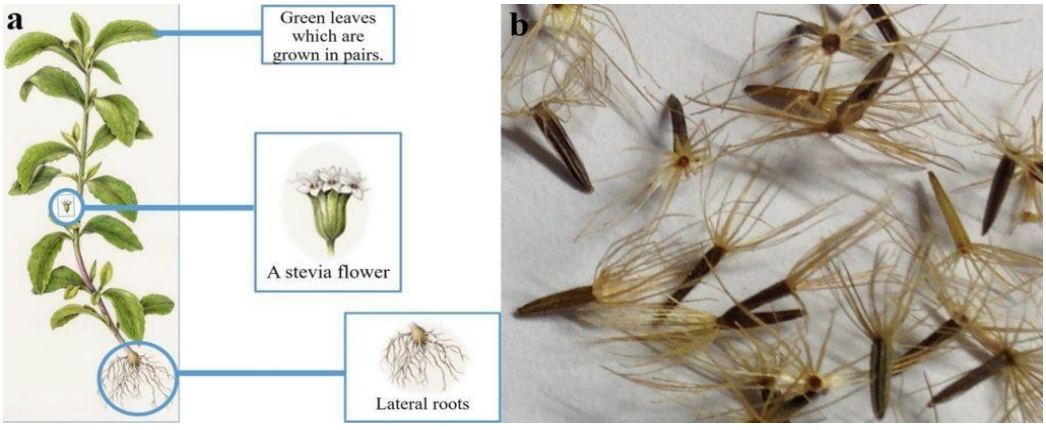

Fig:-1 Morphology of *Stevia rebaudiana* (source: http://mecklenburghsquaregarden.org.uk/*Stevia-rebaudiana*-a-sugar-substitute)

Leaves of *Stevia* produce low calorie glycosides (stevioside and rebaudioside), which are 300-400 times sweeter then sugar (Megeji *et al*. 2005; Rajasekaran *et al*. 2007). Presence of stevioside and rebaudioside contributes to its sweetness (Gupta *et*

al. 2013). Since ancient times *Stevia* has been used in medicines. It is a major source of high-potency sweetener for food and pharmaceuticals preparations. The genus *Stevia* comprises of about 130 species out of which *Stevia rebaudiana* Bertoni is the sweetest (Soejarto *et al*. 1982; Kinghorn *et al*. 1984).

Properties of plant

Stevia is a medicinally important plant with varied bioactive compounds (Lemus-Mondaca et al. 2012). The leaves are used for the treatment of chronic diseases as well as non-chronic diseases (Gupta *et al.* 2013). This plant is used for natural management of diabetes and also helps to control weight gain (Jeppesen *et al.* 2000). It is primarily used as a sweetener and is also used to normalize the blood pressure, the extract of the plant also have antibacterial, antifungal and antiviral properties (Goyal *et al.* 2010) (table 1).

Table 1: The biological activity of leaf extract reported in literature

Disease	**Plant part**	**Action**	**Reference**
Cancer	Leaf extract	Stevioside, steviol and isosteviol present in plant extract cause the inhibition of tumor initiation	Akihisa *et al.* 2004
Diabetes	Leaf extract	Steviol present in leaf extract cause suppression of plasma glucose level	Megeji *et al.* 2005
Inflammatory bowel disease	Leaf extract	Steviol and stevioside inhibit intestinal smooth muscle contraction	Shiozaki *et al.* 2006
Blood pressure	Leaf extract	Systolic and diastolic blood pressure can be normalized by stevioside present in the hot water extract of *Stevia* leaf	Gupta *et al.* 2013

The food industries use this plant as a sweetening and flavoring agent in candies and soft drinks (Sorjarto *et al.* 1982), due to these properties the demand of commercial cultivation is increasing but traditional cultivation techniques are not enough to meet the increasing demand. *Stevia* is a cross-pollinated crop and seeds are mostly

nonviable, they lose their viability after a very short time period and because of cross pollination they don't produce a homogenous plant population (Tamura *et al.*1984). The cultivation by stem cutting is time consuming and produce less number of plantlets from selected plants and also results in pathogen accumulation during repeated cycles of vegetative propagation (Mishra *et al.* 2010) thus leads to inconsistent production and supply of glycosides in market. Genetic transformations and regeneration can lead up to varieties with improved traits, to acquire desired traits plants are genetically modified for which an efficient plant regeneration protocol from desired explant is required.

Two common pathways of *in-vitro* propagation are shoot organogenesis and somatic embryogenesis or non-zygotic embryogenesis.

Shoot organogenesis is the process in which the undifferentiated mass of cells develops into an organized plant structure. Shoot organogenesis can be direct or indirect, direct shoot organogenesis infers to direct regeneration from explant whereas indirect organogenesis occurs from callus. Direct and indirect organogenesis relies on the inherent plasticity of plant tissue. The plant growth regulators are used to manipulate the morphogenetic response of cultures, thus the organ formation is effected by the concentration of PGR's. auxin to cytokinin ratio determines roots or shoots formation.

Somatic embryogenesis is the process in which groups of somatic cells lead to the formation of embryo that has resemblance to zygotic embryos. Somatic embryos either form directly from tissue without callus formation or indirectly from intermediate callus. Auxins play an important role in somatic embryogenesis initiation. Most common auxins used for somatic embryogenesis are 2,4-D, NAA (Dodeman *et al.* 1997). Other auxins like IAA, IBA, dicamba and picloram are also used for somatic embryogenesis (Arnold *et al.* 2002). *In-vitro* propagation being an efficient method is used for rapid and homogenous production of *Stevia* plants at commercial level and for specific genetically modification of plants, for which optimization of regeneration protocol is prerequisite.

Genetic transformation is performed to get higher yielding varieties with improved traits, like disease resistance, biotic and abiotic stress tolerance, and efficient yield of

desirable phytomolecules. Different methods of genetic engineering include transformation through *Agrobacterium*, microprojectile bombardment, electroporation, microinjection, nanoparticles etc. (Demirer *et al.* 2017). *Agrobacterium tumefacien* mediated transformation is an effective process for transformation because of its natural ability to introduce genetic material into the nuclear genome.

Nanotechnology is yet other emerging technique, which is gaining importance for genetic modification in plants. Gold, starch, mesoporous silica, calcium phosphate, ZnS nanoparticles have been used to penetrate the cell and deliver the gene of interest into the living systems as transgenic vehicle (Siddiqui *et al.* 2015). Nanoparticles can serve as 'magic bullets', containing herbicides, pestisides, or genes**.** It is believed that engineered nanoparticles can efficiently be introduced into plants cells, and can transport DNA into plant cells via sonication, gene gun, etc. (González-Melendi *et al*. 2007). Particularly, nanoparticles assisted genetic transformation is of considerable importance in plant biotechnology as the manipulation at nano scale, offers new possibilities to target specific gene manipulations and expression in plant. The scientists are exploring efficient vectors for efficient transformation (Abdin *et al.* 2017).

The present study is aimed on the development of an efficient protocol for the genetic transformation of *Stevia rebaudiana* using *Agrobacterium tumefaciens* and silver nanoparticles.

Review of literature

Stevia rebaudiana is a medicinal plant, the extract of *Stevia* is used by people of South Africa as a traditional medicine (Kinghorn *et al.* 2002). It is used as a digestive aid, a skin care product and in treatment of various other wounds (Ravindran 2017). Goyal *et al*. (2010) and Mahmood *et al.* (2015) have reported the anti-diabetic, anti-bacterial, anti-cancerous, anti-hypertensive etc. activities of the plant.

The rate of seed germination in *Stevia* is very low and seedlings are very slow to establish (Singh *et al.* 2005). Moreover there is a huge variability in plant population (Sivaram *et al.* 2003). The improvement of the plant for sweetness is carried out through conventional breeding (Yadav *et al.* 2010). Varieties improved with glycoside content are RSIT 94-1306, RSIT 94-751, synthetic cultivar AC black bird with improved glycoside content RSIT-95-166-13 with high rebaudioside C content have been developed through conventional breeding (Brandle *et al*. 1998, Marsolais *et al*. 1998). There are few limitation of breeding technique like limited possibility of trait specific improvement, self-incompatibility, labor intensiveness and time consumption (Yadav *et al.* 2010). Therefore, biotechnological approaches like genetic engineering, genetic transformation are used as alternatives (Pande and Gupta 2013).

Genetic engineering has the ability to undertake trait specific improvement in wide variety of plant species, Genetic engineering is a common approach for the production of plants with improved traits (Rashid *et al*. 2016) and for successful and efficient transformation of plant an efficient regeneration protocol is prerequisite (Yadav *et al*. 2010). Many studies are available on the regeneration of *Stevia* (Ferreira and Handro 1988; Banerjee and Sarkar 2008; Preethi *et al*. 2011). Tamura *et al*. (1984) has given the first report on shoot regeneration in *Stevia* from stem tip they have reported the clonal propagation from stem tips as explants when cultured on MS-medium supplemented with 10 mg/l kinetin. Many studies on direct and indirect regeneration from various explants has been reported. Ferreira and Handro (1988) have reported the regeneration from suspension culture using leaves as explant when grown on MS-medium supplemented with 2mg/l BA in light conditions and 2 mg/l BA + 2 mg/l NAA in dark conditions. Sivaram and Mukundan (2003) have reported the adventitious shoot formation from leaf explants of *Stevia*

on MS-medium supplemented with 8.87 μM BA and 5.71 μM IAA. Mitra and Pal (2007) have reported the higher shoot proliferation from nodal explant when cultured on MS-medium supplemented with 1 mg/l IAA 10 mg/l Kinetin and 30 mg/l adenine sulphate. Banerjee and Sarkar (2008) have reported the maximum shoot proliferation from leaf as expalnt when cultivated on MS-medium with cynobacterium (5ml/l and 10 ml/l). Sreedhar *et al*. (2008) have reported the direct shoot induction on both side of midrib from adaxial surface of leaf after 5 weeks on MS-medium supplemented with 8.88 μM NAA, 4.65-6.98 μM kinetin. Other studies on regeneration are listed in (table-2).

Table:- 2 Some important reports on direct regeneration in *Stevia rebaudiana*

S.no.	Explant	Medium	Response	Reference
1	Leaf, shoot discs and internode	MS-medium+3 mg/l BA, 0.5 mg/l NAA	Multiple shoot induction	Neelima and Sarita (2009)
2	Shoot tip and node	MS-medium+4.44 μM BA & 0.80 μM NAA	Shoot buds from shoot tips	Giridhar *et al*. (2010)
3	Flower	MS-medium+2 mg/l BA	Shoot organogenesis	Ahmed *et al*. (2011)
4	Leaf	MS-medium+2.0 mg/l, Kn 0.5 mg/l and NAA 0.1 mg/l.	Direct shoot organogenesis	Preethi *et al*. (2011)
5	Node	MS-medium+1 mg/l BA and 0.5 mg/l IBA	Shoot bud initiation	Laribi *et al*. (2012)

In these studies that have been mentioned in table 2, it has been observed that direct shoot organogenesis can be obtained in media supplemented with BA and NAA. The combination of BA with 2,4-D or NAA has been used for the induction of callus. BA with kinetin can also result in direct shoot organogenesis (Sreedhar *et al*. 2008). The use of spermine and adenine sulphate is reported beneficial for shoot organogenesis in *S.rebaudiana* (Mitra and Pal 2007; Gurucharan and Chinnagounder 2013). Photoperiod also affected the shoot organogenesis (Ferreira and Handro 1998, Huang *et al*. 2000). It has been reported that dark conditions in presence of auxins and cytokinin in suspension cultures promoted shoot regeneration whereas under light conditions with a photoperiod cycle of 14-16 hour lead to maximum regeneration in comparison to the photoperiod of less than 11 hour (Okporie and Ekpe 2008; Zeevaart 1971).

Indirect regeneration occurs when explant produce undifferentiated mass of cells, which then differentiate into the plant tissue like leaf, stem or root. Indirect regeneration includes either callus formation or somatic embryogenesis.

Somatic embryogenesis is another way to obtain plants as vegetative cells develop into whole plant. Only few reports are available on somatic embryogenesis in *Stevia*. It is an important pathway of cell differentiation in plants (Zimmerman 1993). Somatic embryogenesis can occur from any living tissue (Filho and Hattori 1997; Banerjee and Sarkar (2008); Arellano *et al*. 2015; Nazneen *et al*. 2014) most of these reported protocols have applied 2,4-D as an important part of medium combination. Studies on somatic embryogenesis in *Stevia* were initiated by Filho *et al*. (1993).

Table:- 3 Various reports on somatic embryogenesis in *Stevia rebaudiana*

S.no.	Explant	Medium	Response	Reference
1	Leaf	MS medium + 2,4-D (25 μM) + BA (1 μM)	Somatic embryogenesis, no regeneration	Filho *et al.* (1993)
2	Floret	MS medium + 2,4- D (9.05 μM)	Somatic embryogenesis and regeneration	Filho and Hattori (1997)

3	Leaf and node	MS medium + 2,4- D (4.87 μM)	Somatic embryogenesis	Banerjee and Sarkar (2008)
4	Leaf and node	MS medium + 2,4- D (2 mg/l) + BA (0.2 mg/l) + TDZ (0.2 mg/l)	Embryogenic callus	Banerjee and Sarkar (2009)
5	Leaf	MS-medium+ MS medium+2,4- D (0.5-2.0 mg/l NAA)+BA (1.0 mg/l+500 mg/l)	Somatic embryogenesis and regeneration	Arellano *et al.* (2015)
6	Leaf	MS-medium+3.6 μM NAA and 2.2 μM BA	Friable callus	Swanson *et al*. (1992)
7	Shoot buds	MS-medium+10.7 μM NAA and 8.8 μM BA, MS-medium+4.4 μM BA	Callus, multiple	Smitha *et al*. (2005)
8	Leaf, node and internode	MS-medium+2,4-D (4.87-19.48 μM).	Callus	Uddin *et al*. (2006)
9	Leaves	MS-medium+0.75 mg/l NAA and 1 mg/l 2,4- D	100% callusing	Gupta *et al*. (2010)

Nazneen *et al.* (2014) studied stages of somatic embryogenesis through Scanning Electron Microscopy (SEM). Naranjo *et al*. (2016) reported that genotype also affects the embryogenesis the maximum numbers of somatic embryos were developed by genotype SRQ-93 on medium supplemented with 2,4-D (18.09 μM) and 2iP (7.38 μM). Das *et al*. (2006) has shown that kinetin in combination with 2,4-D and NAA had better callus initiation. BA and NAA in half strength MS-medium is best for maintaining callus. Sadeak *et al*. (2009) and Tiwari (2010) also reported the essentiality of 2,4-D for the induction of callus from any of the explant.

Khan *et al.* (2014) have reported the genetic transformation of *Stevia* through *Agrobacterium tumefaciens* (EHA-105 harboring pCAMBIA 1304), and have achieved the development of transgenic plant. Many studies have been reported on the transformation of *Stevia* using *Agrobacterium rhizogene* (Giri *et al.* 2001; Yogananth and Basu 2009). Studies listed in table:-4 comprises of the transformation of other species that belongs to Asteraceae family.

Table:-4 *Agrobacterium tumefacien* mediated transformation of other plants of Asteraceae family

S.no.	**Plant**	**Key findings**	**Reference**
1	*Cichorium intybus*	Transformed plants (resistant to chlorine sulfon) having *Arabidopsis* acetolactate synthase gene were obtained	Vermeulen *et al.* (1992)
2	*Lactuca sativa*	Transformation efficiency Selection methods of transgenic plants	Liu-Jingmei *et al.* (2001)
3	*Lactuca sativa*	Resistance to abiotic stresses, like low temperate and drought by overexpression of *ABF3, P5CS* genes	Pileggi *et al.* (2001); Pileggi *et al.* (2002); Vanjildorj *et al.* (2005);
4	*Gerbera hybrida*	Plant was transformed with gene having resistance to tomato spotted wilt virus by LBA 4404 *A.tumefaciens*	Korbin *et al.* (2002)

5	*Lactuca sativa*	They established an efficient *Agrobacterium* mediated genetic transformation protocol using LBA4404 *A.tumefaciens* containing pBIPTA plasmid with an aphidicidal gene, *pta*	Ahmed *et al.* (2007)
6	*Cichorium intybus*	Plants were transformed with *AtNHX1* gene to obtain salinity resistant lines	Zhao *et al.* (2009)
7	*Bidens pilosa*	Transformation conditions were optimized and the halcon synthase gene was transferred	Wang *et al.* (2012)
8	*Cichorium intybus*	Efficient regeneration protocol was developed and *Agrobacterium tumefaciens* strain C58C1 (pGV2260) (pTJK136) was used to obtain an efficient regeneration protocol	Maroufi *et al.* (2012)
9	*Dahlia pinnata*	Efficient regeneration protocol for *Agrobacterium*-mediated transformation using EHA101 harbouring pIG121-Hm strain was developed	Otani *et al.* (2013)
10	*Artemisia annua*	Transformation protocol was optimized using *Agrobacterium tumefaciens* for the enhancement of artemisinin	Elfahmi *et al.* (2014)

According to the existing literature, there are no reports on the use of nanoparticles for the genetic transformation of *Stevia* by nanoparticles. Many other studies of plant transformation by Nanoparticles are reported. Protoplast of *Nicotiana tabacum* was transformed by mesoporous silica nanoparticles harboring pER8-GFP plasmid DNA (Torney *et al.* 2007). pEGAD plasmid DNA was delivered into the calli cells

of *Dioscrea zigiberensis* by fluorescence starch nanoparticles (Liu *et al.* 2008). pCAMBIA 1301 and pCAMBIA 2301 plasmid DNA were delivered into the *Nicotiana tabacum, Oryza sativa* and *Leucaena leucocephala* by carbon supported gold nanoparticles (Vijayakumar *et al.* 2010). pCAMBIA 1301 plasmid encapsulated in CaP NPs were used for hypocotyls transformation of *B. juncea* and *Pusa Jaikisan* (Naqvi *et al.* 2011). Leaf discs of *Nicotiana tobacum* were transformed by ZnS nanoparticles harboring pBI121 plasmid DNA (Fu *et al.* 2012).

Objectives

1. Optimization of an efficient protocol for shoot organogenesis and/or somatic embryogenesis
2. Development of *Agrobacterium tumefaciens* mediated genetic transformation protocol for *Stevia rebaudiana*
3. Exploring the use of silver nanoparticles for efficient genetic transformation of *Stevia rebaudiana*

Material and methods

Plant material and explant preparations:

Cultures of *Stevia rebaudiana* that were maintained in plant tissue culture laboratory of TIFAC-CORE, T.I.E.T, Patiala, were used. The cultures were maintained on MS medium containing 3% sucrose (w/v) and 0.7% agar (w/v), the cultures were multiplied through regular sub-culturing period of 21 days. The leaves of tissue cultured grown *Stevia* are observed to be small, thus for the expansion of leaves the shoots were cultured on the MS-1 Medium (Kaur *et al.* 2017) containing 1 μM BA.

Chemicals and glassware:

All the fine chemicals were purchased from Hi-Media laboratories, Mumbai, India. Plant growth regulators (PGRs), antibiotics, CTAB, EDTA, agarose (molecular grade) were purchased from Sigma Chemicals. Chitosan (deacetylation degree 85%, purity 99%, p.H. 5-6, molecular weight 1,00,000-3,00,000) was purchased from Hi-media, Silver nitrate and sodium borohydrate were purchased from sigma aldrich,. All glassware's such as measuring cylinder, conical flasks were purchased from Borosil Glass Works Ltd. (Mumbai, India). Culture bottles 300ml capacity were purchased from Kasablanka Corporations, (Mumbai, India). Plasticware like sterile disposable filter sterilization unit were purchased from Merck Millipore (Merck Specialties Pvt Ltd Mumbai, India). Pertidishes, microtips, microfuge tubes, measuring cylinder, beaker etc. were purchased from Tarson Products Pvt Ltd (Kolkata, India).

Preparation of culture medium and stock solutions:

MS-medium (Murashige and Skoog 1962) with 3% sucrose and gelled with 0.75% (w/v) agar (basal MS medium) was used for the experiments. Various plant growth regulators (PGRs) like BA, NAA, 2,4-D were added to the medium in various concentrations and combinations. The stock solutions of all macronutrients, micronutrients and vitamins were prepared individually, which were further used to prepare the medium (Annexure:-I). The 2.5mM stock solutions of plant growth regulators (PGRs) were prepared by dissolving these in respective solvents (1N KOH/NaOH and 1N HCL) and finally volume was raised by distilled water

(Annexure:-II). The stock solutions were stored in -4°C refrigerator. MS medium was prepared from these stock solutions and used for the tissue culture studies. The pH of medium was adjusted to 5.8 with 1N HCl or 1N KOH using pH-meter (Cyberscan 510, Eutech Instruments, Singapore). After the preparation of medium 50 ml each is transferred into 300 ml glass culture bottles (Kasablanka, Mumbai) containing 0.75% w/v agar. After that medium was sterilized in an autoclave (121°C, 15psi, 15min, Equitorn, Mumbai, India). For antibiotic medium stock solutions of antibiotics like kanamycin, rifampicin, cefotaxime with required concentrations were prepared and filter sterilized using sterile filter of 0.22 μM pore size and were stored in -20 °C deep freezer (vest frost, India).

Culture growth conditions

The cultures were incubated at 25±1 °C under white fluorescent light (CFL) (Philips India Ltd, Mumbai) with 42 μmol m^{-2} s^{-1} light intensity with 16hr light and 8hr dark cycle.

Preparation of explants and establishment of cultures

The *in-vitro* cultures of *Stevia rebaudiana* were established using young stems from *S.rebaudiana* maintained in PTC laboratory of TIFAC-CORE T.I.E.T, on MS-1 medium supplemented with 1 μM BA and was further used for micro- propagation, regeneration and genetic transformation experiments. Leaf disks were used for regeneration and transformation experiments.

Effect of PGRs on regeneration of plant

The expanded leaf disks from microshoots of *S.rebaudiana* maintained on MS-1 medium supplemented with 1 μM BA were taken as explant for the optimization of regeneration protocol. Leaves were cut transversely along the midrib and were inoculated on modified MS medium supplemented with various concentration and combinations of PGRs (0 to 25 μM of BA & NAA, 2,4-D & NAA and BA & 2,4-D respectively) with their adaxial surface facing the medium.

Sensitivity of leaf explant towards kanamycin

The effect of kanamycin (50 mg/l) on shoot organogenesis from leaves was tested. The stock of kanamycin (50 mg/ml) in different concentrations (0 to 100 mg/ml) was added to the medium supplemented with 5 μM BA after autoclaving.

Agrobacterium tumefacien* mediated genetic transformation of *Stevia rebaudiana

The experiments of transformation were carried out using *Agrobacterium tumefaciens* strain EHA105 harboring pBI121 binary vector (Fig:-2). The map of pBI121 binary vector carrying a reporter gene *uidA* (β-glucuronidase) with CaMV 35S promoter and NOS terminator and *nptII* gene (neomycin phosphotransferase II) used as a selection marker gene, with NOS promoter and terminator within t-DNA region

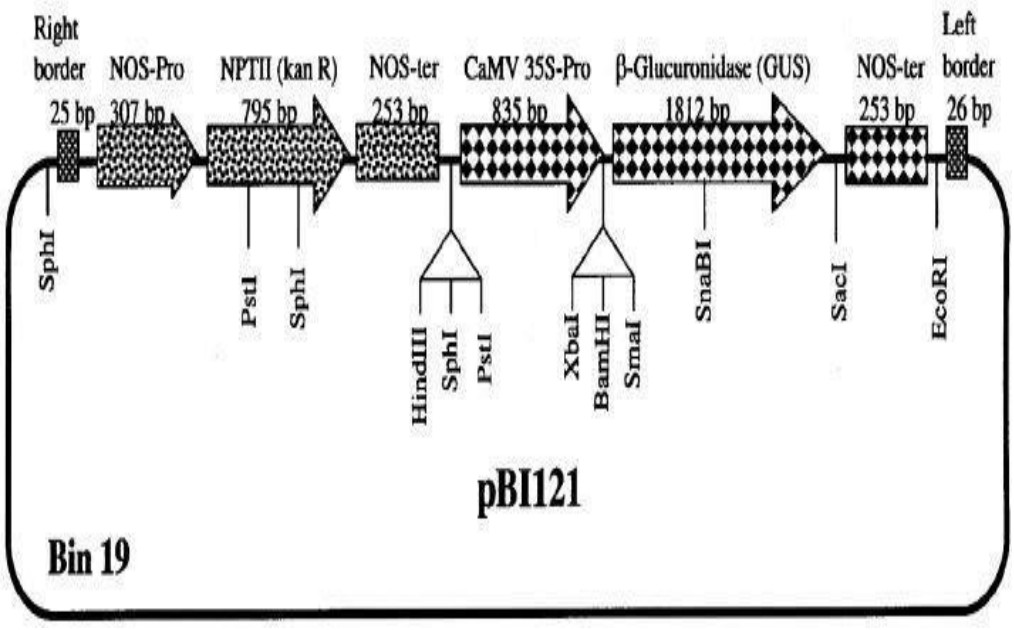

Fig 2:- Diagrammatical representation of pBI121 plasmid vector (The T-DNA region is indicating the localization of *nptll* gene which is encoding neomycin phosphotransferase (*nptll*) and the *uidA* gene, encoding Glucuronidase (GUS), NOS terminator, NOS promoter, CaMV35S promoter (cauliflower mosaic virus 35S promoter).

Source of explant for transformation experiments

The leaves from the actively growing micro-shoots of *Stevia rebaudiana* maintained on MS-1 medium supplemented with 1 μM BA were taken as explant for genetic transformation experiments.

Transformation with *Agrobacterium tumefaciens*

The *Agrobacterium tumefacien* strain EHA105 harboring pBI121 binary vector was inoculated in 25 ml LB medium supplemented with kanamycin (25 µg/ml) and rifampicin (15 µg/ml) and was incubated at 28°C on shaker (130 rpm) for 16-18 hrs to attain the desirable OD_{600}. Explants were injured with sterile surgical blade in sterilized petriplate and put in bacterial suspension for 15 mins then after the leaves were dried on autoclaved paper. These injured leaves were then co-cultivated on MS-medium supplemented with 5 µM BA (best regeneration was achieved on this combination). After co-cultivation the explants were washed with sterilized distilled water and dried on autoclaved paper. The explants were then cultured on MS-medium supplemented with 5 µM BA, 50 mg/l kanamycin and 300 mg/l cefotaxime. The transient GUS assay of leaves was done after 2 days of culture.

Factors affecting *Agrobacterium tumefacien* mediated genetic transformation

For optimization of genetic transformation protocol different factors like infection time, bacterial OD_{600}, pre-culture, co-cultivation time and photoperiod were studied

Table:- 5 factors for the optimization of *Agrobacterium*-mediated transformation

S.no.	Factors	Variable
1	Effect of infection time	5, 10, 15, 20, 30 mins
2	Effect of bacterial OD_{600}	0.4, 0.6, 0.8,1
3	Effect of pre-culture days	0, 1, 2, 3, 4, 5 days
4	Effect of co-cultivation	12, 24, 36, 48, 72 hrs
5	Effect of photoperiod	16 hrs light & 8 hrs dark, 24 hrs dark and 24 hrs light

Transformation with silver nanoparticles

Synthesis of $AgNO_3$ nanoparticles

Silver nanoparticles were synthesized as per the established protocol (Agnihotri *et al.* 2014) with modifications. In brief, 45 ml of freshly prepared aqueous solutions containing 3.8 mg $NaBH_4$ and 50 mg TSC were mixed and heated to 60°C for 30 mins in the dark with continuous stirring at 400 rpm to ensure the formation of homogenous solution. After 30 mins, 5 ml of 10 mM $AgNO_3$ solution was added drop wise to the mixture where the color of solution changed instantly from colorless to golden yellow confirming the formation of silver nanoparticles. The nanoparticles suspension was then mixed for additional 5 mins and was allowed to cool at room temperature. The nanoparticles were then used for further characterization and stored at 4 °C till further use.

Synthesis of ch-$AgNO_3$ nanoparticles

Chitosan stabilized silver nanoparticles were prepared as per the earlier protocol (Jiang *et al.* 2012) with substantial modifications. As described, 0.2% (w/v) chitosan in 1% acetic acid solution was introduced where due to the low solubility of chitosan the mixture was kept overnight in stirring conditions. Next day the solution was filtered and 2 ml of 0.01 M $AgNO_3$ solution was added to 47 ml of chitosan solution, stirred for 30 mins after that 10 ml of 0.1 M $NaBH_4$ solution was added drop wise to the mixture and again stirred for 90 mins. The nanoparticles were then collected and used for further characterization and stored at 4°C till further use.

Plasmid DNA isolation

Single colony of transformed *E.coli* having pBI121 plasmid was picked and inoculated in LB medium for 16-18 hrs at 37°C. 1.5 ml of culture was transferred to eppendorf and centrifuged at 5000 rpm for 5 mins. Supernatant was discarded, 200 µl of solution I (GTE buffer) was added to the pellet and was retro-pipetted. 200 µl of freshly prepared solution II (4.5 ml H_2O, 200 µl SDS, 100 µl NaOH) was added to it, incubated on ice till it became translucent (approximately 5 mins) then 300 µl of solution III (potassium acetate) was added to it, mixed and incubated on ice for 5 mins centrifugation at 12000 rpm for 10 mins was done. 1 µl of RNAse was added to

the supernatant and incubated for 1 hr at 37°C. Equal volume of phenol chloroform isoamyl alcohol (25:24:1) was added to it and centrifuged at 12000 rpm for 10 mins. The aqueous phase was taken and equal volume of isopropanol was added to it and incubated at -20°C for 45 mins. Centrifugation at 12000 rpm for 15 mins was done. The supernatant was discarded and pellet was washed with 70% ethanol, the pellet was dissolved in 30 µl of TE buffer, quantification and quality check of plasmid DNA was done and stored at -20°C.

Coating of chitosan stabilized silver nanoparticles with DNA

Ch-$AgNO_3$ (0.11 µg/ml) nanoparticles and pBI121 plasmid DNA (1 µg/µl) at different volumetric ratios were incubated for 1 hour as shown in table:-6. Agarose gel (1%) electrophoresis was performed to validate the complex formation between DNA and Ch-Ag NPs.

Table:- 6 DNA nanoparticles complex formation

S.no.	**DNA (**1µg/µl**)**	**Nanoparticles (**0.11 µg/ml)	**TE buffer**
1	1µl	5 µl	14
2	2.5µl	5 µl	12.5
3	5µl	5 µl	10
4	7.5µl	5 µl	7.5
5	10µl	5 µl	5
6	15µl	5 µl	-

Protection of DNA from ultrasound damage by silver nanoparticles

To identify whether the coated DNA can be protected from ultrasonic damage, DNA-Nanoparticles complex were treated with ultrasound of power 120/480 W, 35 kHz for 5, 10, 20 mins. Plasmid DNA was used to measure the extent of DNA damage and agarose gel (1%) electrophoresis was done for detection of plasmid DNA damage.

Nanoparticles mediated genetic transformation

The leaf explants were pre-cultured for 2 days. The explants were cut into disks in sterilized petri plate, then they were immerged in 5 ml ultrasonic buffer (15 mM NaCl, 1.5 mM tri sodium citrate, 5% DMSO)(FU Yu-qin *et al.* 2012) and 200 µl DNA+ Ch-Ag NPs complex and was subsequently ultrasonicated for 5, 10 and 15 mins. The leaves were dried on autoclaved tissue. These injured leaves were then co-cultivated for 24, 48 and 72 hrs on MS-medium containing 5 µM BA. After co-cultivation half of the explants were washed with sterilized distilled water and dried on autoclaved tissue. The explants were then transferred on MS-medium containing 5 µM BA 50 mg/l kanamycin. GUS assay of leaves were done after 2 days for transient expression.

Nanoparticles assisted *Agrobacterium tumefaciens* mediated genetic transformation

The two days pre-cultured explants were immerged in $AgNO_3$ nanoparticles and chitosan stabilized silver nanoparticles separately and ultrasonicated for 15 mins. One set of leaves was washed and another set was kept unwashed, and infected with *Agrobacterium tumefacien* for 15 mins, the leaves were dried on autoclaved tissue. These infected leaves were then co-cultivated for 48 hrs on MS-medium containing 5 µM BA (as best regeneration was achieved on this combination). After co-cultivation the explants were washed with sterilized distilled water and dried on autoclaved tissue. The explants were then shifted on MS-medium supplemented with 5 µM BA 50 mg/l kanamycin and 300 mg/l cefotaxime. GUS assay of leaves were done after 2 days for transient expression.

Histochemical Gus assay

Beta glucuronidase gene is used as marker for analysis of gene expression in transformed plants. GUS staining refers to the reporter gene assay based on beta-glucuronidase, the enzyme when incubated with colorless substrates can transform them into blue colored product. (Fig-3) (Jefferson *et al.* 1987). Transient GUS expression was done after 2 days of incubation.

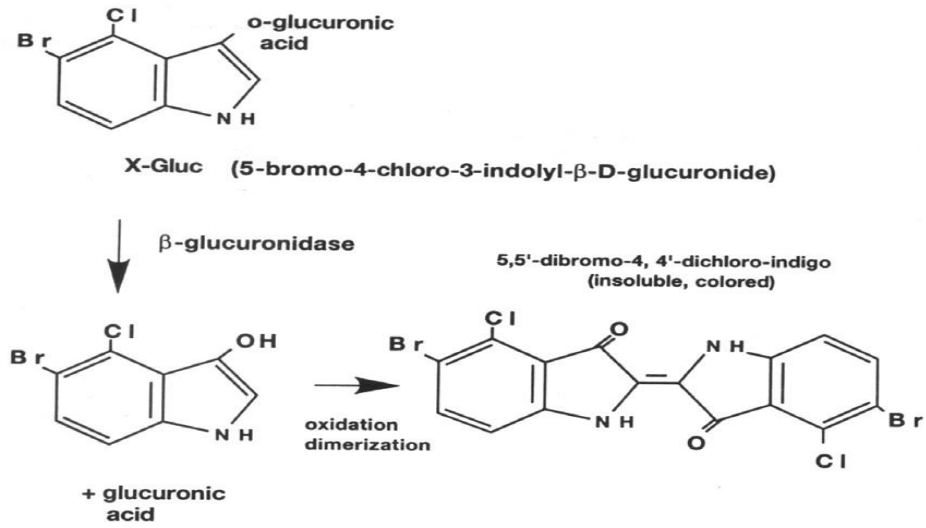

Fig:- 3 Mechanism of GUS assay–X-Gluc is used as the non fluorescent substrate which is converted to a blue colored product beta-glucuronidase

Procedure for GUS-Assay:

Transformed leaves were incubated in eppendorf containing GUS-histochemical staining solution (Annexure III) overnight at 37°C. The staining solution was removed after 24 hrs and leaves were washed and dipped in ethanol, to remove chlorophyll for better visualization of stained tissue. After the complete removal of chlorophyll, the tissue was observed for the presence of blue color.

Results

Multiplication of cultures:- The cultures of *Stevia rebaudiana* maintained on Murashige and Skoog (1962) medium are observed to be having small leaf size, thus for the expansion of the leaves the shoots were cultured on the MS-1 Medium containing 1 μM BA. The microshoots with improved leave size were further used for experimentation.

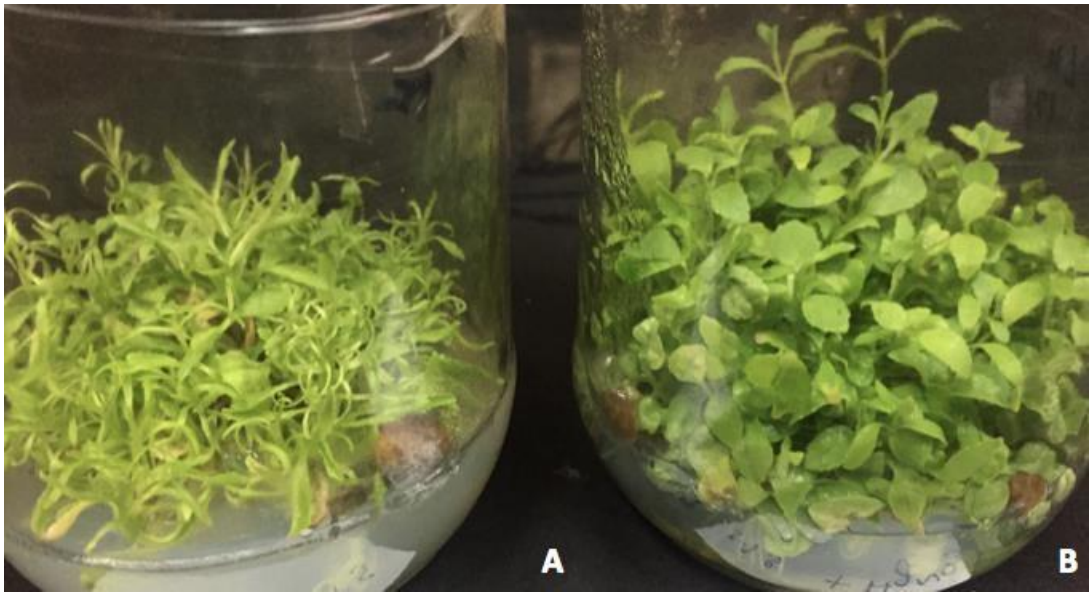

Fig 4:- Cultures of *Stevia rebaudiana* growing on A) Basal medium; B) MS-1 Media supplemented with 1 μM BA

Effect of PGRs on regeneration of plant:- Effect of BA and NAA in different concentration (0 to 25 μM) on regeneration was studied (table:-7). Shoot organogenesis were observed in the medium supplemented with BA where as combination of NAA with BA lead to the decrease in the shoot organogenesis. Nodular callus was observed in the medium supplemented with NAA. Maximum shoot organogenesis was observed in medium containing 5 μM BA.

Table 7:- Effect of BA and NAA in MS-1 medium on shoot organogenesis

S.No.	NAA	BA	Response	%Explant showing shoot organogenesis
1	0 µM	0 µM	No response	0 o
2	1 µM	0 µM	Nodular callus	92±4.89 h
3	5 µM	0 µM	Nodular callus	72±4.89 n
4	25 µM	0 µM	Nodular callus	92±4.89 i
5	0 µM	1 µM	Shoot organogenesis	48±11.97 l
6	1 µM	1 µM	Shoot organogenesis	86±3.99 m
7	5 µM	1 µM	Compact callus	100 f
8	25 µM	1 µM	Compact callus	100 e
9	0 µM	2.5 µM	Compact callus	100 d
10	1 µM	2.5 µM	Nodular callus	100 c
11	5 µM	2.5 µM	Compact callus	100 b
12	25 µM	2.5 µM	Compact callus	100 a
13	**0 µM**	**5 µM**	**Shoot organogenesis**	**92±7.98 g**
14	1 µM	5 µM	Shoot organogenesis	90±7.98 j
15	5 µM	5 µM	Compact callus	88±7.98k
16	25 µM	5 µM	Compact callus	88±4.8 l

Data recorded after 9 weeks of culture and analyzed by ANOVA. Means were compared using LSD. Values are the mean of five replicated (5 explant in each replicate).

Effect of PGRs on somatic embryogenesis:- Leaves of *stevia* were used to study the effect of 2,4-D, BA and NAA in different concentration (0 to 25 µM) for the induction of somatic embryogenesis. The leaf curling was observed in the 1st week and the callus induction was observed in the 4th week of initial culture. Nodular callus, globular embryos were observed in the 6th week of culture. Maximum

globular embryos were observed in the medium containing 1 µM 2,4-D and 1 µM NAA.

Table 8: - Effect of 2,4-D and NAA on somatic embryogenesis from leaf explant taken from microshoots.

S.No.	2,4-D	NAA	Response	%Explant showing nodular callus
1	0 µM	0 µM	No response	0 o
2	1 µM	0 µM	Nodular callus	95±3.97 b
3	2.5 µM	0 µM	Nodular callus	80±6.28 g
4	5 µM	0 µM	Nodular callus	72±7.95 l
5	**0 µM**	**1 µM**	**Nodular callus**	**96±9.73 a**
6	1 µM	1 µM	Nodular callus	72±11.92 j
7	2.5 µM	1 µM	Nodular callus	76±7.43 k
8	5 µM	1 µM	Compact callus	72±4.86 n
9	0 µM	5 µM	Nodular callus	72±4.86 m
10	1 µM	5 µM	Nodular callus	76±7.43 j
11	2.5 µM	5 µM	Compact callus	80±6.28 f
12	5 µM	5 µM	Compact callus	88±4.86 c
13	0 µM	25 µM	Compact callus	84±7.43 d
14	1 µM	25 µM	Nodular callus	84±3.97 c
15	2.5 µM	25 µM	Nodular callus	76±7.43 i
16	5 µM	25 µM	Nodular callus	76±7.43 h

Data recorded after 9 weeks of culture and analyzed by ANOVA. Means were compared using LSD. Values are the mean of five replicated (5 explant in each replicate).

Table 9: - Effect of BA and 2,4-D on somatic embryogenesis from leaf explant taken from microshoots.

S.No.	BA	2,4-D	Response	%Explant showing nodular callus
1	0 µM	0 µM	No response	0 [p]
2	1 µM	0 µM	Compact callus	56±14.67 [l]
3	2.5 µM	0 µM	Compact callus	84±7.47 [g]
4	5 µM	0 µM	Compact callus	92±4.89 [c]
5	**0 µM**	**1 µM**	**Nodular callus**	**95±7.98 [a]**
6	1 µM	1 µM	Friable callus	88±7.98 [d]
7	2.5 µM	1 µM	Compact callus	92±7.99 [b]
8	5 µM	1 µM	Friable callus	84±7.47 [f]
9	0 µM	5 µM	Nodular callus	76±7.47 [h]
10	1 µM	5 µM	Compact callus	84±9.78 [e]
11	2.5 µM	5 µM	Compact callus	68±4.89 [k]
12	5 µM	5 µM	Compact callus	76±3.99 [i]
13	0 µM	25 µM	Friable callus	48±10.18 [n]
14	1 µM	25 µM	Compact callus	48±4.89 [m]
15	2.5 µM	25 µM	Compact callus	10.95±4.89 [o]
16	5 µM	25 µM	Compact callus	72±13.54 [j]

Data recorded after 9 weeks of culture and analyzed by ANOVA. Means were compared using LSD. Values are the mean of five replicated (5 explant in each replicate).

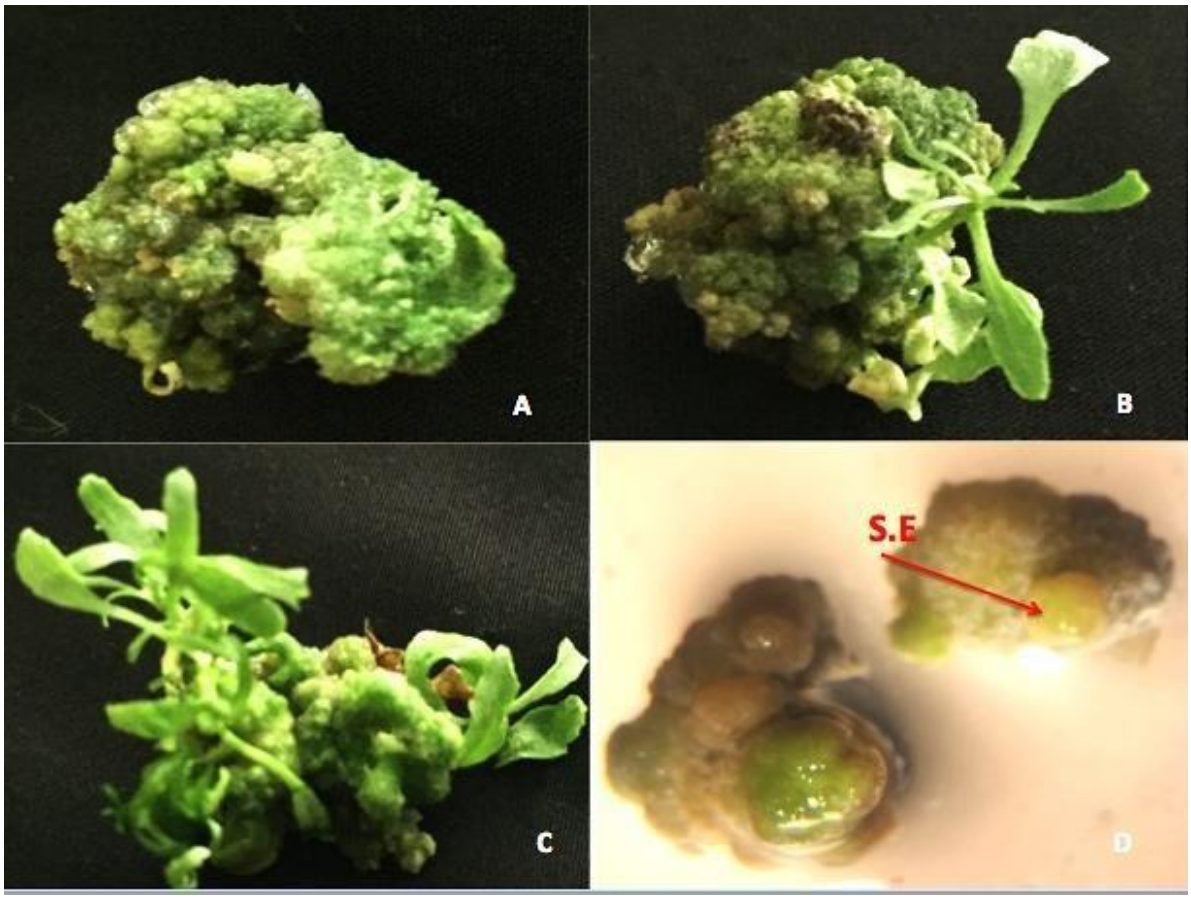

Fig 5: - Shoot organogenesis and somatic embryogenesis A) Callus; B) Shoot initiation; C) Regenerated shoot; D) somatic embryo like structures

Antibiotic sensitivity: - The kanamycin sensitivity of the *Stevia rebaudiana* leaves for transformed tissue selection was checked by culturing the leaves on MS media supplemented with different concentration of kanamycin (0, 15, 25, 50, 75, 100 mg/l). Decrease in survival rate was observed on media supplemented with kanamycin. All explants died on media supplemented with 50 mg/l, this concentration was further used in the experiments for the selection.

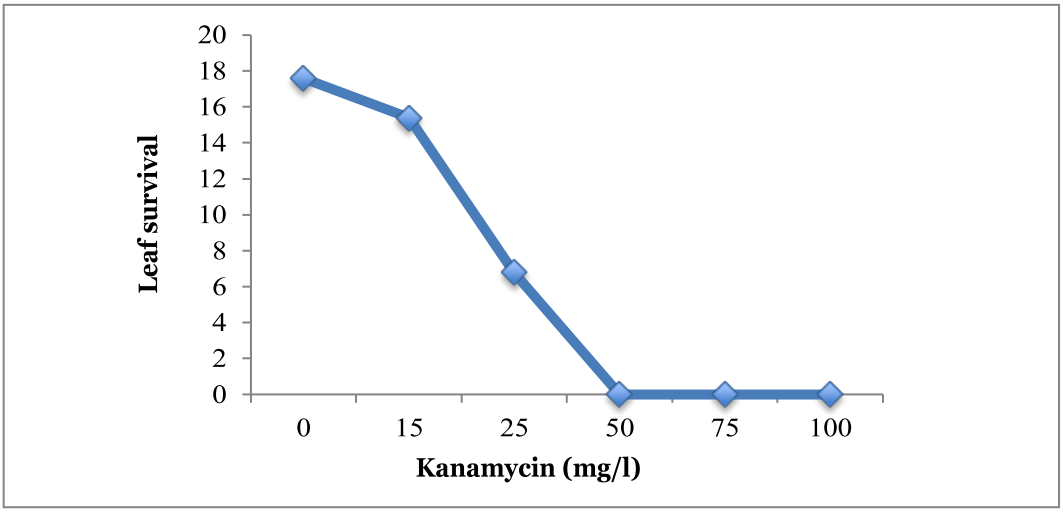

Fig 6:- Effect of kanamycin on the survival of leaf explant of *S.rebaudiana*. Data recorded after 5 weeks of culture values are the mean of five replicated (20 explant in each replicate).

Effect of different transformation factors:- Factors like optical density, pre-culture days, infection time, co-cultivation and photoperiod that are influencing genetic transformation of *S. rebaudiana* were studied.

Effect of bacterial OD_{600}

Table 10:- Effect of bacterial OD_{600} on genetic transformation

S.No.	Variable	%GUS expression
1	0.4	40
2	0.6	70
3	0.8	75
4	1	50

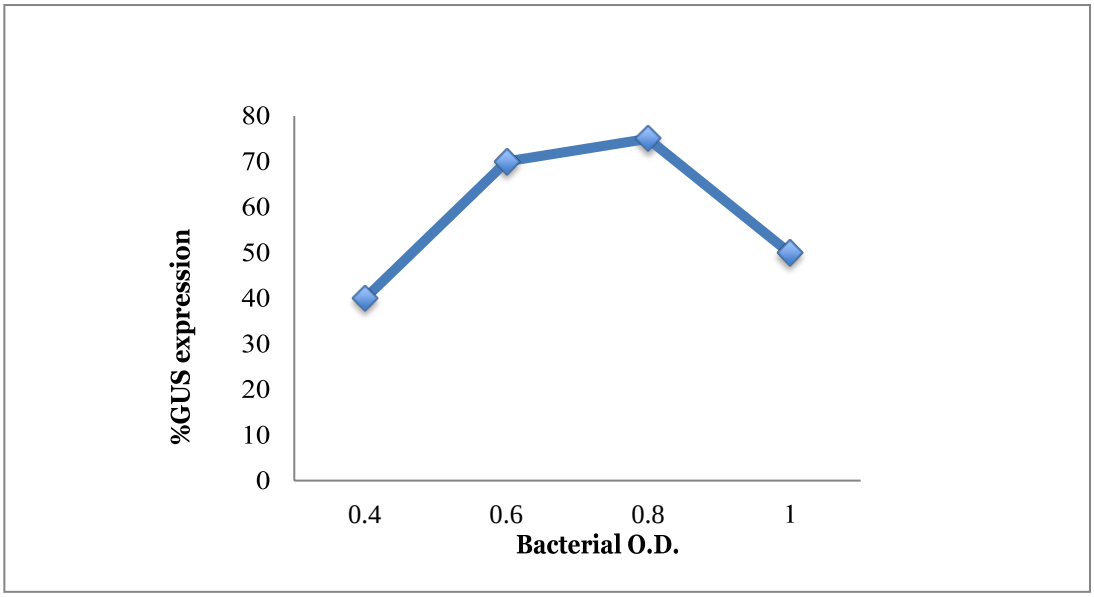

Fig:- 7 Effect of bacterial O.D. on genetic transformation of *S. rebaudiana.* Data recorded after 2 days of transferring the tissue on selection media

OD_{600} of *Agrobacterium tumefacien* suspension used for the infection of leaf explants also influence the GUS expression. The explants infected with bacteria suspension with
0.6 OD_{600} showed maximum transient GUS activity. At higher OD_{600} bacterial contamination was observed with lead to death of explant.

Effect of pre-culture days

Table 11: - Effect of pre-culture days on genetic transformation

S.No.	Variable	%GUS expression
1	0 days	50
2	1 days	75
3	2 days	80
4	3 days	70
5	4 days	55
6	5 days	40

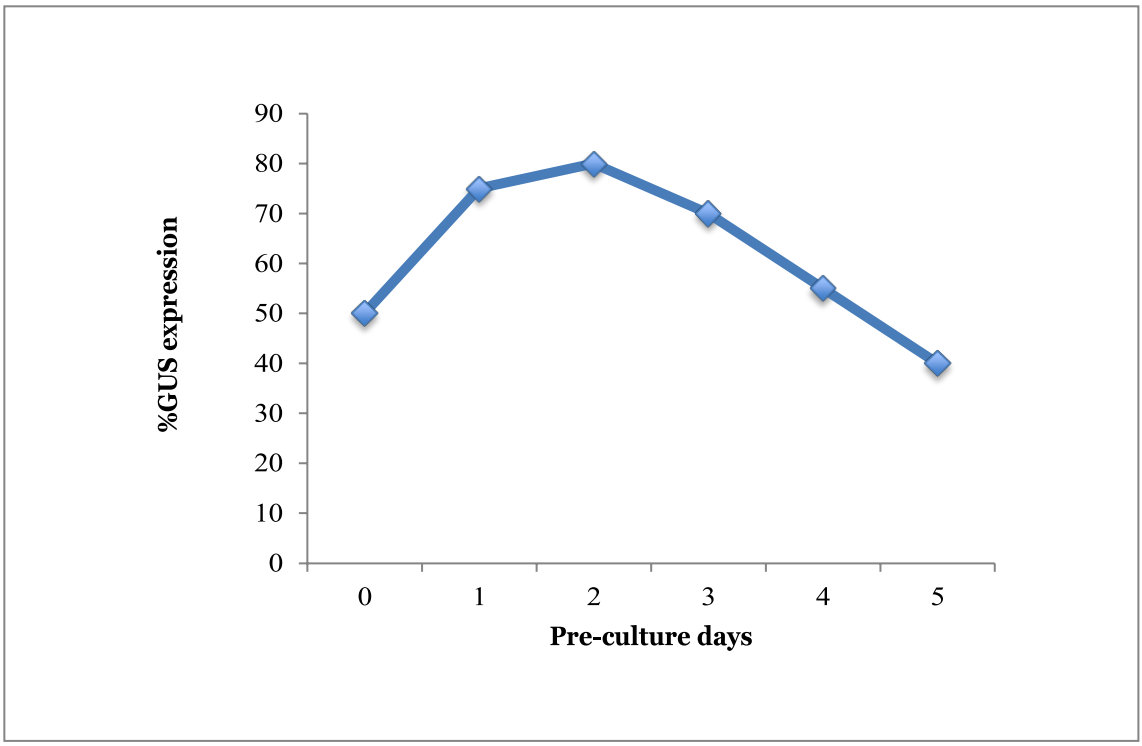

Fig:- 8 Effect of pre-culture days on genetic transformation of *S. rebaudiana*. Data recorded after 2 days of transferring the tissue on selection media

Leaves of *S.rebaudiana* were pre-cultured on M.S-medium containing 5µM BA for 0,1,2,3,4,5 days respectively and incubated under 16 hr light cycle. The leaves pre-culture for 2 days showed maximum transient GUS activity i.e. 80%.

Effect of infection time

Table 12: - Effect of infection time on genetic transformation

S.No.	Variable	% GUS expression
1	5 mins	40
2	10 mins	60
3	15 mins	75
4	20 mins	80
5	30 mins	70

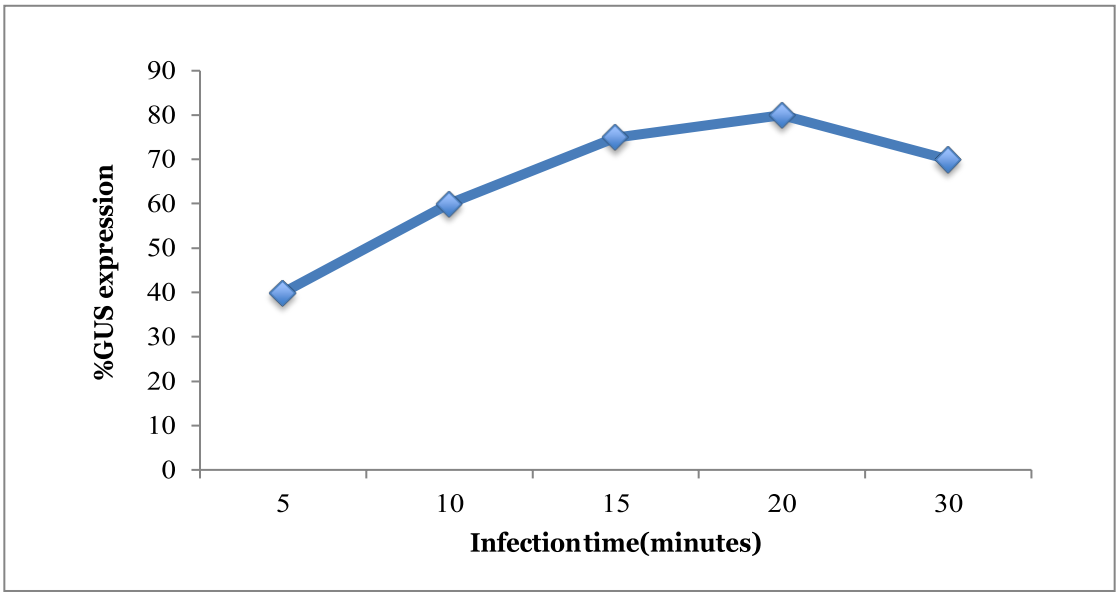

Fig 9: - Effect of infection time on genetic transformation of *S. rebaudiana*. Data recorded after 2 days of transferring the tissue on selection media

The duration of time interval at which the leaves are exposed to *A.tumefaciens* influences the transformation frequency of explants. Leaves were incubated with *A.tumefaciens* for 5, 10, 15, 20, 30 mins respectively. Significantly increased frequency was shown by explants that were incubated for 15 mins with *A.tumefaciens*. Exposure to *A.tumefaciens* for more than 15 mins resulted in decline in transformation frequency and bacterial contamination. However the 30 mins infection was associated with problems such as elimination of the *A.tumefaciens* subsequent to co-cultivation and loss of viability of the explants resulting from the overgrowth of bacteria.

Effect of co-cultivation

Table 13:- Effect of co-cultivation time on genetic transformation

S.No.	Variable	%GUS expression
1	12 hrs	20
2	24 hrs	35
3	36 hrs	65
4	48 hrs	80
5	72 hrs	Bacterial contamination

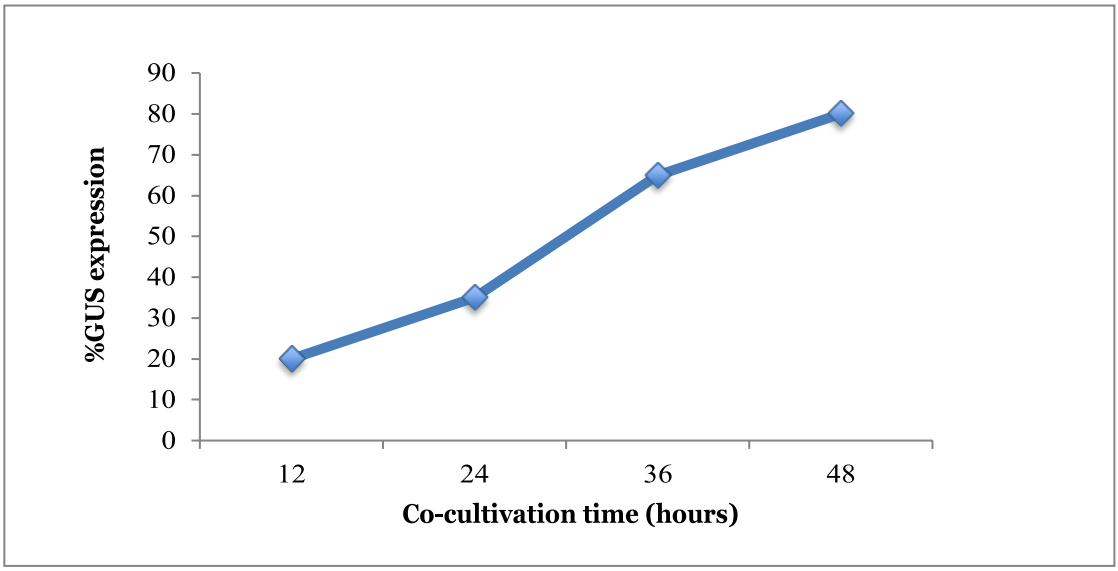

Fig:-10 Effect of co-cultivation time on genetic transformation of *S. rebaudiana*. Data recorded after 2 days of transferring the tissue on selection media

The Co-cultivation period after the *A.tumefaciens* infection plays a major role in the delivery of T-DNA and it also influence the transient GUS expression. Explant co-cultivated for 48 hrs showed maximum transient GUS activity Co-cultivation period of less than 48 hrs leads in decrease transient GUS activity. Co-cultivation period of more than 48 hrs caused excessive growth of bacteria leading to death of explants.

Effect of photoperiod

Table 14:- Effect of photoperiod on genetic transformation

S.No.	Variable	% GUS expression
1	16 hrs light & 8 hrs dark	30
2	24 hrs dark	45
3	24 hrs light	70

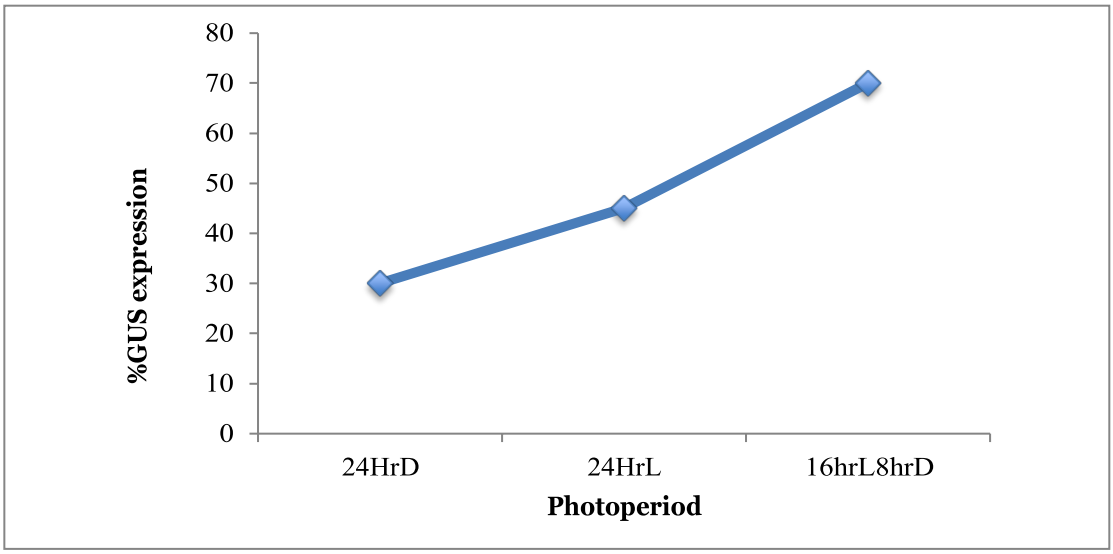

Fig:-11 Effect of photoperiod on genetic transformation of *S. rebaudiana*. Data recorded after 2 days of transferring the tissue on selection media

Photoperiod during co-cultivation influences the transient GUS activity. Photoperiod of 16 hrs light and 8 hrs dark showed maximum transient GUS activity.

After optimization of various parameters of *Agrobacterium tumefaciens* mediated genetic transformation of *S.rebaudiana*, the optimized transformation protocol is pre-culturing of leaf explants of *S.rebaudiana* on modified MS medium for two days, then in sterilized petridish infection of leaf explants for 15mins *A .tumefaciens* strain EHA105 (harboring pBI121) suspension culture grown overnight having density

0.6 at OD_{600} After infection, blotting of infected explants on sterile filter paper. Then co-cultivated for 48 hrs on modified MS medium supplemented with 5 µM BA having pH 5.8 under photoperiod of 16 hrs light and 8 hrs dark. Washing of leaf explant after co-cultivation with autoclaved water containing 300 µg/ml cefotaxime,

blotting of leaf explants on sterile filter paper and transfer to culture bottles containing modified MS medium supplemented with 5 μM BA, 50 mg/l kanamycin and 300 mg/l cefotaxime. Histochemical GUS assay was carried out after two days of incubation on modified MS medium supplemented with 5 μM BA, 50 mg/l kanamycin and 300 mg/l cefotaxim, for scoring transient expression. The tissue showing transient GUS expression (blue color) was scored.

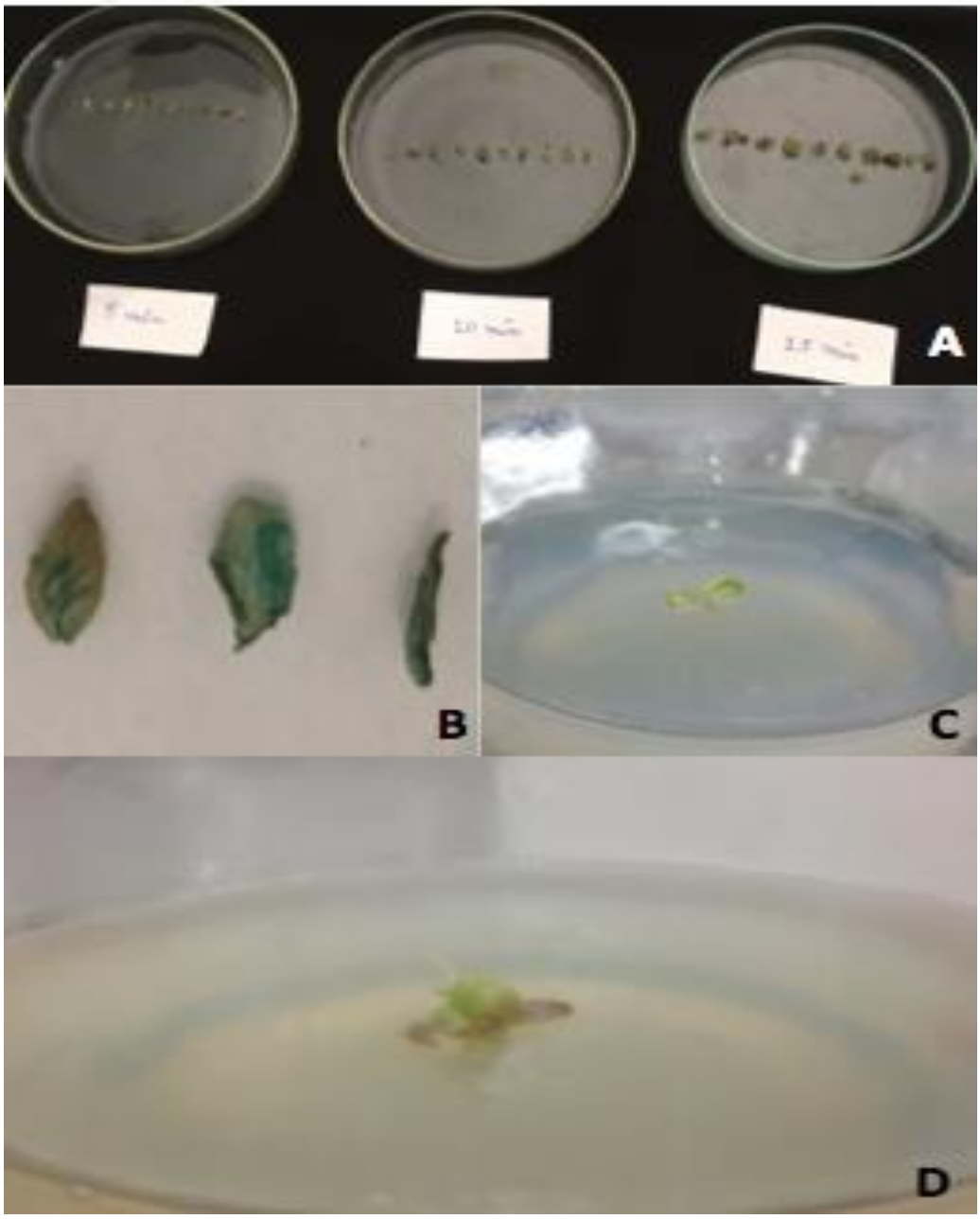

Fig 12: - (A&B) Leaf explant showing transient GUS expression; (C&D) Transformed shoots growing on selection medium containing kanamycin

Synthesis of silver nanoparticles:- The silver nanoparticles of 0.65 µM concentration were successfully synthesized by chemical reduction method using $NaBH_4$ and TSC as reducing agents. An instant color change in silver nitrate solution from colorless to golden yellow is attributed to the formation of silver nanoparticles. Synthesized $AgNO_3$ NPs were then Characterized through UV-Vis spectroscopy and dynamic light scattering (DLS).

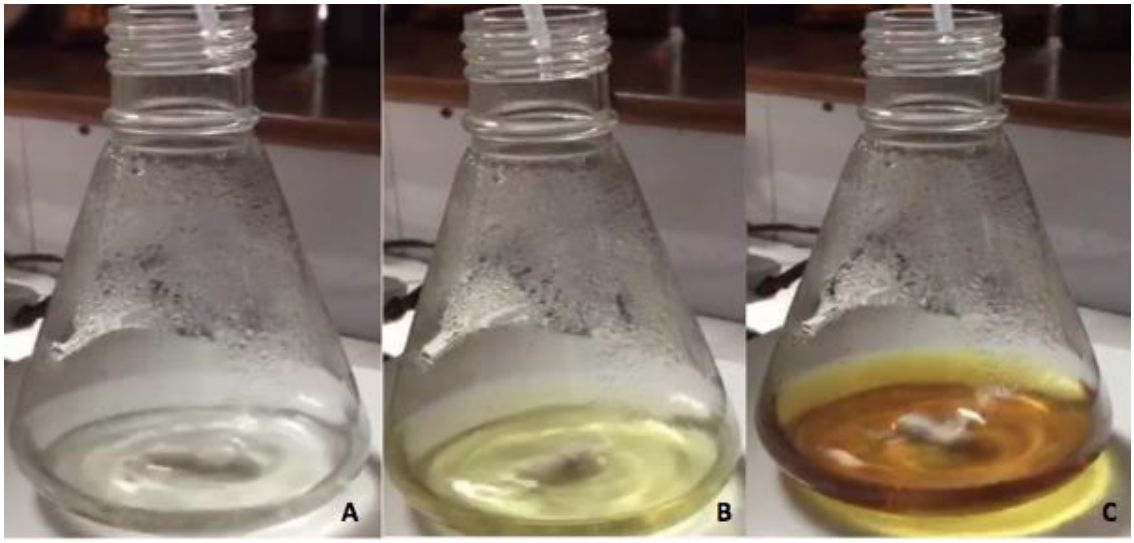

Fig13:- Synthesis of silver nanoparticles A) sodium borohydride and TSC B) addition of $AgNO_3$ C) silver nanoparticles

UV-Vis Spectra

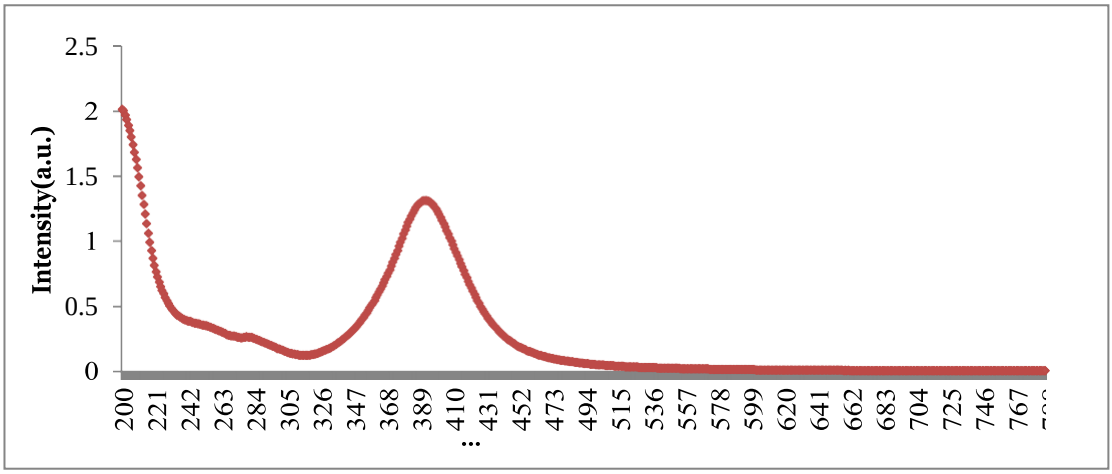

Fig:- 14 UV-Vis absorption peak of synthesized silver nanoparticles

UV-Vis absorption peak was observed around 400 nm, which confirmed the formation of AgNPs in the suspension.

Dynamic light scattering

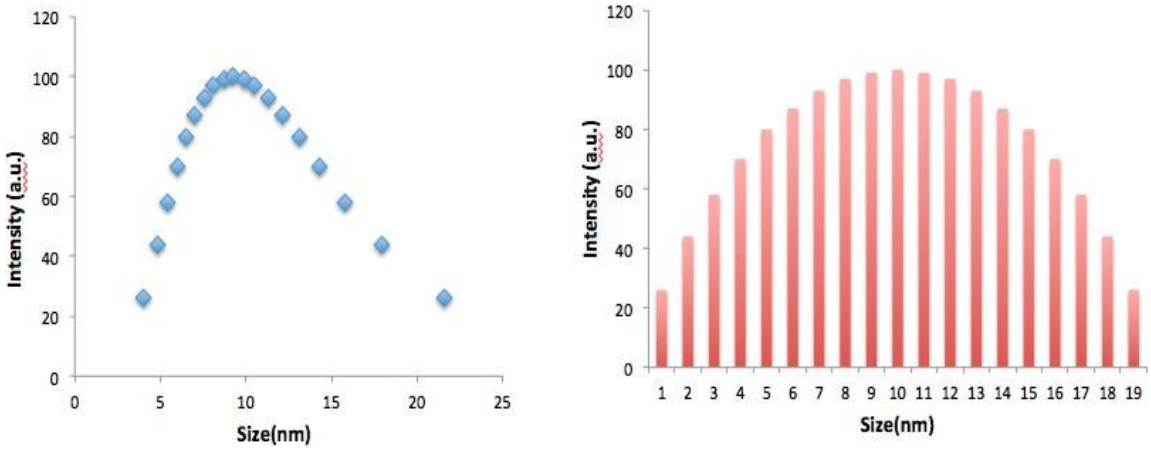

Fig 15:- DLS of synthesized silver nanoparticles

The particle size distribution (PSD) of synthesized silver nanoparticles contains particles of different sizes, with average size around 10 nm.

Zeta potential analysis:- Zeta potential measurements of synthesized silver nanoparticles was -39 mV, which is an indicative of highly stabilized AgNPs.

Chitosan stabilized silver nanoparticles:- Ch-Ag nanoparticles of 0.42 nM concentration were successfully synthesized from $AgNO_3$ by chemical reduction with $NaBH_4$. An instant color change from colorless to yellow is attributed to the formation of Ch-Ag NPs, synthesized NPs were also characterized through UV-Vis spectroscopy and dynamic light scattering (DLS).

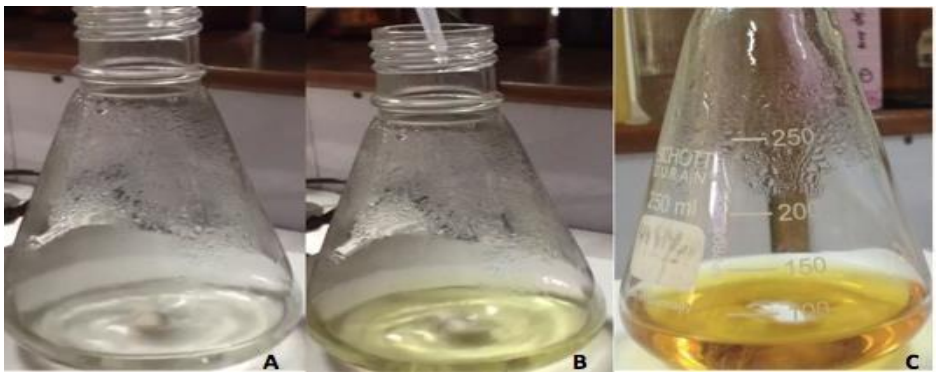

Fig 16 :- Synthesis of chitosan stabilized silver nanoparticles A) chitosan + $AgNO_3$ B) addition of $NaBH_4$ C) Ch-Ag NPs

UV-Vis Spectra

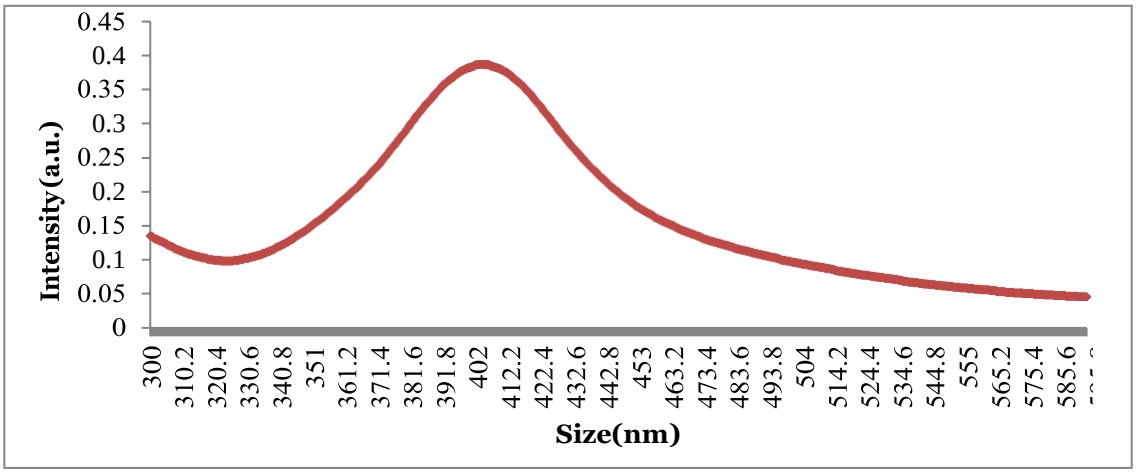

Fig:- 17 UV-Vis absorption peak of chitosan stabilized silver nanoparticles

UV-Vis absorption peak was observed around 406 nm, which confirmed the formation of Ch-Ag NPs in the suspension. A slight red shift also evidenced the formation of chitosan layer over AgNPs, which resulted in the increase in hydrodynamic size of AgNPs.

Dynamic light scattering

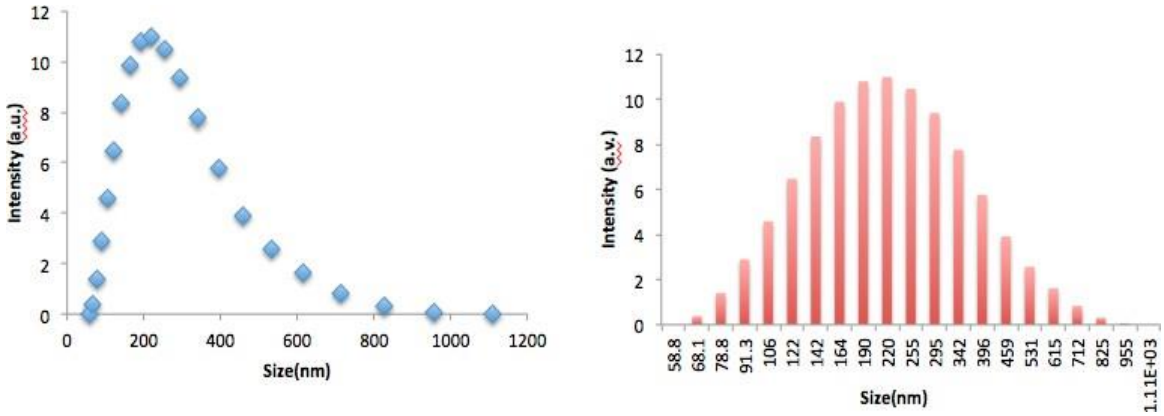

Fig 18:- DLS of chitosan stabilized silver nanoparticles

The particle size distribution (PSD) of synthesized silver nanoparticles contains particles of different sizes, with average size around 220 nm.

Zeta potential analysis:- Zeta potential measurements of synthesized silver nanoparticles was 42.4 mV, which is an indicative of highly stabilized Ch-Ag NPs.

Coating of Ch-Ag NPs with plasmid DNA

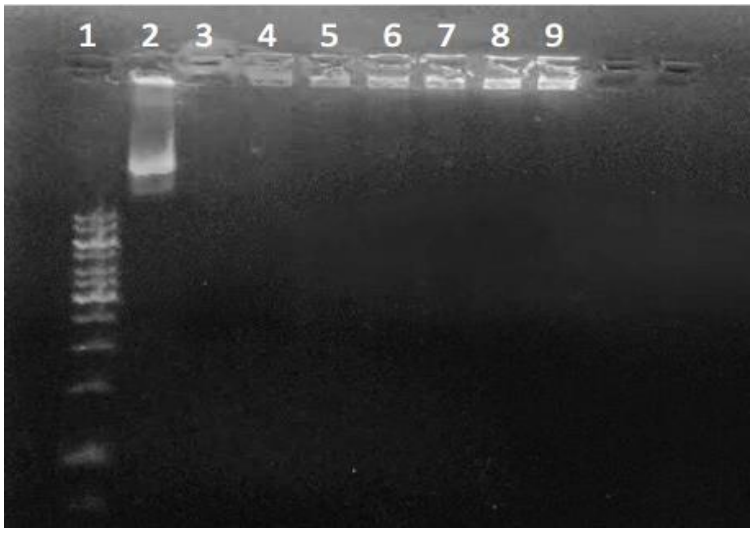

Fig 19:- Well 1 ladder, 2 plasmid DNA, 3 Ch-Ag NPs, 4-9 plasmid DNA+ Ch-Ag NPs complex

pBI121 plasmid was used for the binding to Ch-Ag NPs. The complex of plasmid DNA and Ch-Ag NPs complexes were detected by agarose gel electrophoresis. The Ch-Ag NPs + plasmid complexes were trapped in the wells of gel (lane 4-9) due to the large size as compared to uncombined plasmid DNA alone (lane 2) in lane, thus this confirms the binding of plasmid DNA to Ch-Ag NPs.

Protection of DNA from ultrasound damage by silver nanoparticles

To identify weather the Ch-Ag NPs + plasmid DNA complex can protect the plasmid DNA from ultrasound damage, the complex were ultrasonicated for 5, 10, 20 mins respectively with ultrasound of 120/480 W, 35 kHz using plasmid DNA and nanoparticles as control

Effect of 5 mins sonication on pBI121 plasmid and Ch-Ag NPs + plasmid DNA complex

Fig 20:- Well 1 ladder, 2 plasmid DNA, 3 Ch-Ag NPs, 4 sonicated plasmid DNA, 5 sonicated Ch-Ag NPs, 6-11 plasmid DNA+ Ch-Ag NPs complex

Five mins sonication with 120/480 W, 35 kHz ultrasound has no effect on plasmid DNA and plasmid DNA+ Ch-Ag NPs complex.

Effect of 10 mins sonication on pBI121 plasmid and Ch-Ag NPs + plasmid DNA complex

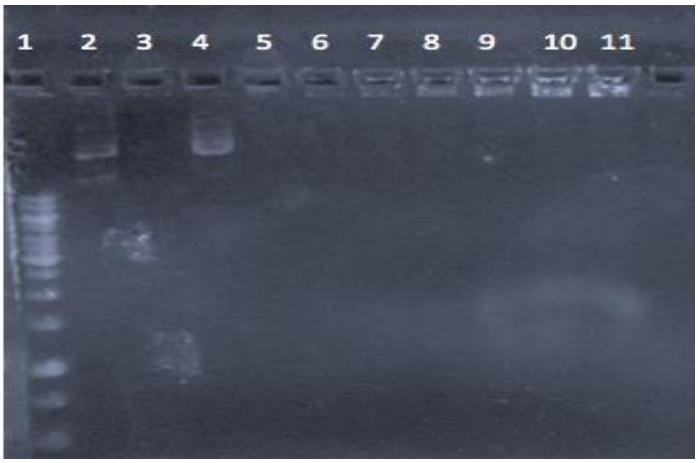

Fig 21:- Well 1 ladder, 2 plasmid DNA, 3 Ch-Ag NPs, 4 sonicated plasmid DNA, 5 sonicated Ch-Ag NPs, 6-11 plasmid DNA+ Ch-Ag NPs complex

Ten mins sonication with 120/480 W, 35 kHz ultrasound has no effect on plasmid DNA and plasmid DNA+ Ch-Ag NPs complex.

Effect of 20 mins sonication on pBI121 plasmid and Ch-Ag NPs + plasmid DNA complex

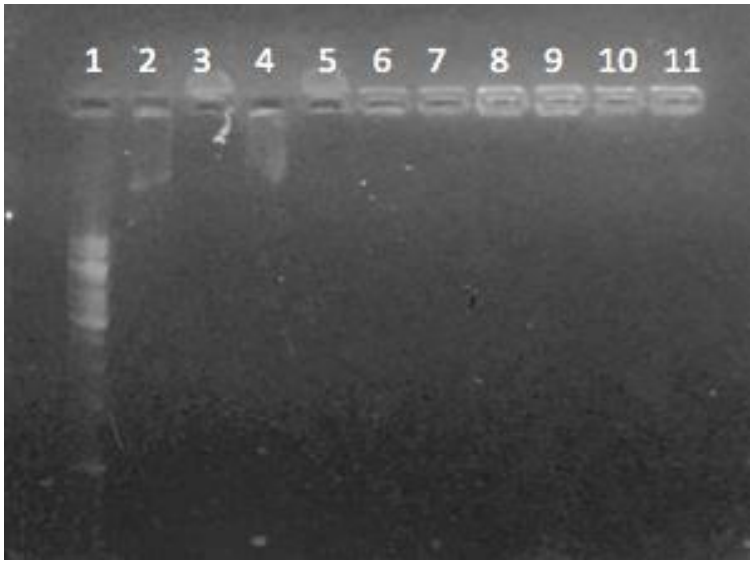

Fig:- 22 Well 1 ladder, 2 plasmid DNA, 3 Ch-Ag NPs, 4 sonicated plasmid DNA, 5 sonicated Ch-Ag NPs, 6-11 plasmid DNA+ Ch-Ag NPs complex

Twenty mins sonication with 120/480 W, 35 kHz ultrasound showed that the plasmid DNA was severely sheared by ultrasound whereas there was no effect on plasmid DNA+ Ch-Ag NPs complex that indicates that Ch-Ag NPs can protect the plasmid DNA from ultrasonic damage.

Nanoparticles mediated transformation

Ch-Ag NPs + DNA complex was tried to introduce into the *Stevia* leaf disks with the help of ultrasonication. Transformation of *Stevia* was performed with Ch-Ag NPs + DNA complex by following optimized transformation protocol. The survival of explant was observed for 21 days. Further stable Gus expression and molecular studies will be performed with regenerated shoots.

Nanoparticles assisted *Agrobacterium tumefaciens* mediated genetic transformation

Table 15:- Effect of chitosan stabilized silver nanoparticles and silver nanoparticles on *Agrobacterium* mediated transformation

S.No.	Variable	%GUS expression
1	*Agrobacterium tumefaciens*	80
2	*Agrobacterium tumefaciens* + $AgNO_3$ nanoparticles (unwashed)	60
3	*Agrobacterium tumefaciens* + $AgNO_3$ nanoparticles (washed)	50
4	*Agrobacterium tumefaciens* + Ch-$AgNO_3$ nanoparticles (unwashed)	30
5	*Agrobacterium tumefaciens* + Ch-$AgNO_3$ nanoparticles (washed)	20

The leaves injured with silver and chitosan stabilized silver nanoparticles respectively were transformed according to the optimized protocol, the explants injured with blade and transformed with *Agrobacterium tumefaciens* alone showed a significantly increased frequency of transformation (80%) whereas the explants injured with silver NPs and kept unwashed showed (60%) transformation efficiency, followed by explants infected with silver NPs and were washed. There was decline in transformation frequency in explants infected with chitosan stabilized silver NPs, the unwashed explants infected with chitosan stabilized silver NPs showed (30%) of transformation efficiency where as washed explants infected with chitosan stabilized silver NPs showed (20%) of transformation efficiency.

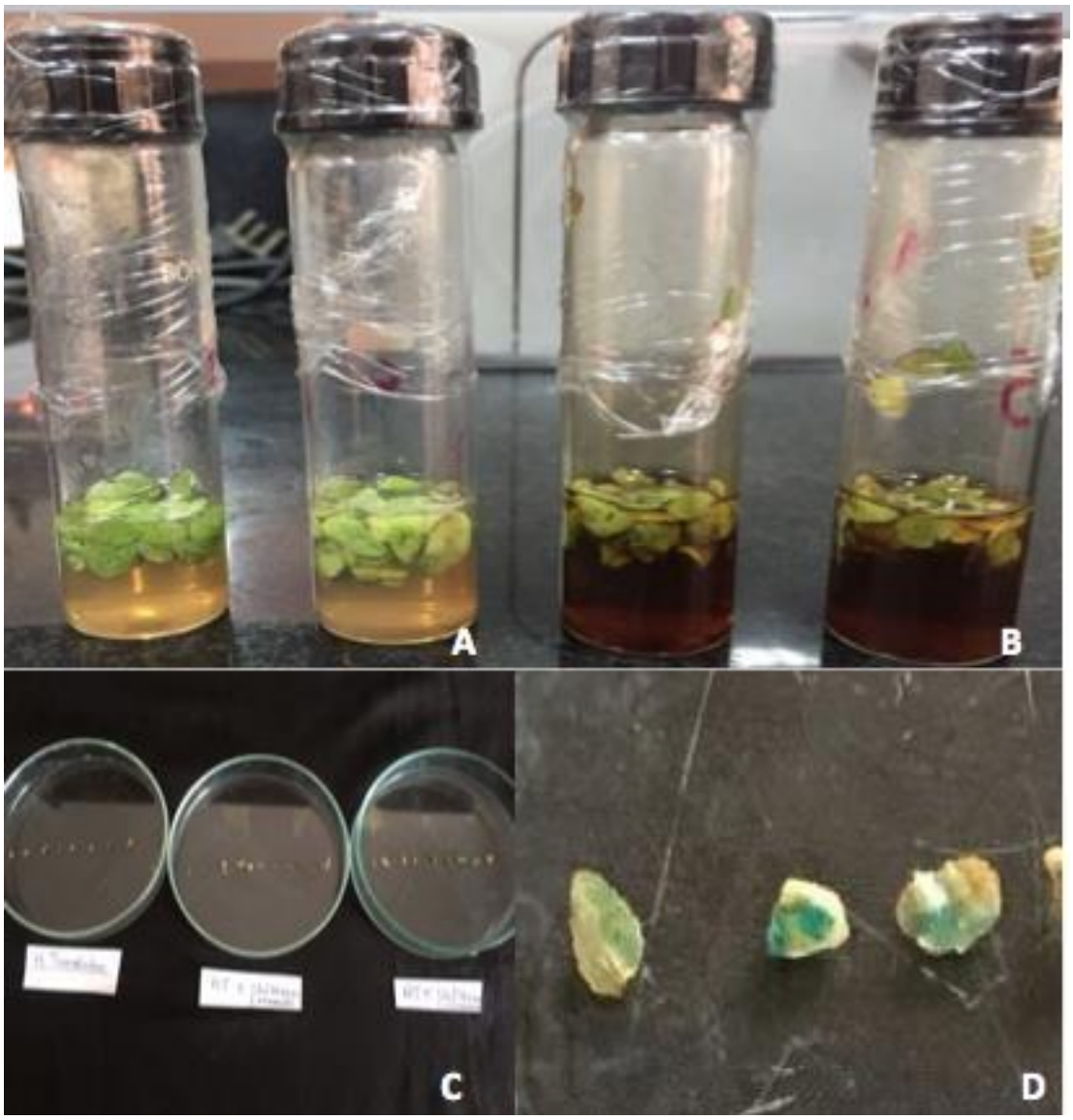

Fig 23:- A) *Stevia* explants submerged in chitosan stablized silver NPs; B) *Stevia* explants submerged in silver NPs; C&D) Leaf explant showing transient GUS expression

Discussion

Genetic transformation is a technique, which is performed to get higher yielding varieties with improved traits and to get efficient yield of desirable phytomolecules. *Agrobacterium tumefaciens* mediated transformation is traditionally a method of choice while nanotechnology is yet other emerging technique, which is gaining importance for genetic modification in plants. An efficient regeneration protocol is a prerequisite for the genetic modification of plants. Many studies are there on the regeneration of *Stevia* (Ferreira and Handro 1988; Banerjee and Sarkar 2008; Preethi *et al*. 2011), Firstly it was reported by Tamura *et al*. 1984. Somatic embryogenesis is another important pathway adopted by cells to redifferentiate into whole plant (Dodeman *et al.*1997). The present study is focused on development of nanoparticles assisted genetic transformation protocol of *Stevia rebaudiana* Bertoni.

Cultures of *Stevia rebaudiana* grown on MS-1 medium for shoot bud induction and formation of somatic embryos. Effect of plant growth regulators (PGRs) was evaluated using different concentration and combinations of 2, 4-D, BA, NAA. Cytokinins play an important role in cell division and in shoot bud development. In present study, 5 μM BA showed maximum shoot organogenesis (92%).

Auxins play an important role in somatic embryogenesis (Liu *et al.* 1993).. It has been reported that 2, 4-D is an important auxin for the induction of somatic embryos but its high concentration inhibits the process during later stages of somatic embryogenesis (Cooke *et al.* 1993; Liu *et al.* 1993). 1 μM 2,4-D (95%) and 1 μM NAA (96%) showed maximum explant differentiating into embryo like structures.

After establishment of successful regeneration protocol, factors affecting *Agrobacterium tumefaciens* mediated transformation were studied for the optimization of transformation protocol of *Stevia rebaudiana*. The transformation through nanoparticles was also attempted and nanoparticles assisted *Agrobacterium tumefaciens* mediated transformation was achieved. For the selection of transformed tissue antibiotic sensitivity needs to be checked. Kanamycin is used as selection marker, as pBI121 exhibits *nptII* gene resistance. Kanamycin concentration of 50 mg/l was found to be the minimum concentration at which the explants died. The effect of various factors (optical density, pre-culture days, infection time, co-cultivation time and photoperiod) that influence genetic transformation of

S.rebaudiana were studied.

The density of cells effects the transformation efficiency (Mathysse 1986). $O.D._{600}$ 0.6 showed maximum transient GUS expression of (70%), as bacterial infection efficiency is maximum in log phase (Mondal 1999). At higher $O.D._{600}$ the bacterial cells effect the survival of explants due to the overgrowth of bacteria. The increase in the co cultivation time resulted in necrosis of explant.

Pre culture is reported as one of the important factor for transformation efficiency (Barik *et al.* 2007). In this report the leaves pre-cultured for 2 days on MS medium supplemented with 5μM BA showed maximum transient GUS expression (80%). The higher efficiency in pre-cultured leaves is due to the presence of cytokinin resulting in the increased cell division (Lawrence and Koundal 2000).

The explants infected for 15 mins gave maximum GUS expression (75%), the increase in infection time resulted in problems such as elimination of *Agrobacterium tumefaciens* and loss of viability of explant resulting from the overgrowth of bacteria. It is also reported that increase in infection time leads to the death of explant (Dilshad *et al.* 2016).

The transformation efficiency is also affected by the co-cultivation time, the transformation efficiency increased with the increase in co-cultivation time, the maximum (80%) transient GUS expression was shown by explants that were co-cultivated for 48 hrs after this the efficiency decreased and lead to bacterial contamination and death of explant due to overgrowth of bacteria.

Variation in light conditions during co-cultivation affects the transient GUS activity (Zuker *et al.* 1999). The photoperiod of 16-h light and 8 h dark gave the maximum transient GUS expression. After studying these various factors, the protocol for transformation in *S.rebaudiana* was optimized.

Due to higher transformation efficiency of nanoparticles as compared to *Agrobacterium tumefaciens* mediated transformation (Naqvi *et al.* 2012), the transformation through nanoparticles was also attempted. CaP nanoparticles were used for the transformation of *Brassica juncea* for increase in transformation efficiency (Naqvi *et al.* 2012).

$AgNO_3$ nanoparticles with a size of 10 nm and ch-$AgNO_3$ nanoparticles with a size of 220 nm were synthesized and used as gene carriers. The characterization and size analysis of NPs was done by UV visible spectroscopy and dynamic light scattering.

Chitosan stabilized silver nanoparticles were used as a gene carrier for the transformation of *S.rebaudiana.* In the present study ultra sonication was used as a mode of injury and pBI121 plasmid DNA was coated on ch-AgNPs. The results indicate that ch-Ag NPs can effectively bind with pBI121 DNA. The release kinetics of pBI121 and NPs depicted that ch-Ag NPs can prevent the DNA from ultrasonic damage. The pBI121 plasmid DNA ch-Ag NPs complex was introduced into the leaves of *S.rebaudiana* with the help of ultra sonication. The effect of silver nanoparticles and chitosan stabilized silver nanoparticles was also studied through nanoparticles assisted *Agrobacterium tumefaciens* mediated genetic transformation. The maximum transformation efficiency was achieved by silver nanoparticles (10 nm) followed by chitosan stabilized silver nanoparticles (220 nm). The present study reports that smaller the size of nanoparticles more is the penetration rate. More damage corresponds to additional *Agrobacterium tumefaciens* binding sites, enhancing the rate of transformation.

Conclusions

The present study paves a new path for the development of an efficient delivery vehicle for the genetic transformation in *Stevia rebaudiana*.

- The cultures of *S.rebaudiana* were multiplied on MS-1 medium for the expanded leaf size.
- Shoot organogenesis was achieved on MS-1 medium supplemented with 5 μM BA.
- $AgNO_3$ nanoparticles were successfully synthesized with the size of 10 nm, charge – 39 mV and ch-$AgNO_3$ nanoparticles with size of 220 nm and charge of 42.4 mV.
- pBI121 plasmid DNA was coated on positively charged chitosan stabilized silver nanoparticles and release kinetics was studied through agarose gel electrophoresis.
- Silver nanoparticles and chitosan stabilized silver nanoparticles were successfully used for the *Agrobacterium tumefaciens* mediated genetic transformation.

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Annexure I

Composition of Murashige and Skoog medium (MS, 1962)

S.No.	Component	Amount (mg/l)
1	NH_4NO_3	1650
2	KNO_3	1900
3	$CaCl_2.2H_2O$	440
4	$MgSO_4.7H_2O$	370
5	KH_2PO_4	170
6	$Na_2MoO_4.2H_2O$	0.25
7	H_3BO_4	6.20
8	$MnSO_4.H_2O$	16.9
9	$ZnSO_4.7H_2O$	8.6
10	KI	0.83
11	$CuSO_4.5H_2O$	0.025
12	$CoCl_2.6H_2O$	0.025
13	Glycine	2
14	Nicotinic Acid	0.5
15	Pyridoxine HCl	0.5
16	Thymine HCl	0.1
17	FeEDTA.$2H_20$ (sodium salt)	30
18	Myo-inositol	100
19	Sugar	3000

Annexure II

Growth regulator's stock solution preparation

S.no	Growth Regulator	Amount (mg)	Solvent	Volume make up (100 ml)
1.	2.5 µM BA	56.315	HCl	Distilled water
2.	2.5 µM NAA	46	KOH	Distilled water
3	2.5 µM 2,4-D	55.26	-	Distilled water

Annexure III

Composition of GUS histochemical solution

Stock solution Final	**Concentration**	**Reagent Mix (µl /ml)**
NaPO4 buffer, pH 7.0	1 M	100
EDTA, pH 8.0	0.25 M	40
K-ferricyanide pH 7.0	0.005 M	100
K- ferrocyanide pH 7.0	0.005 M	100
X-gluc	0.02 M	50
Triton X-100 (optional)	10%	10
Distilled water		600
Final volume		1 ml