7. Genetic Transformation

7.1. Introduction and Review of Literature

Various species of bacteria are capable of transferring genes to higher plant species (Broothaerts et al. 2005). Among them, most widely studied ones are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. Plant tissue transformed by *A. tumefaciens* or *A. rhizogenes* can be readily distinguished by their oncogenic phenotype – tumours or hairy roots respectively (Spano et al. 1982). Various plant species differ greatly in their susceptibility to infection by *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* and other bacterial species which are capable of gene transfer to plants (Broothaerts et al. 2005).

*Agrobacterium tumefaciens* is a soil bacterium that can genetically transform plant cells with a segment of DNA (transfer DNA, abbreviated as T-DNA) from a tumor-inducing plasmid (Ti plasmid) with the resultant production of a crown gall, which is a plant tumor. The detailed mechanisms of the transfer of DNA from *A. tumefaciens* have been reviewed by many authors (Klee et al. 1987; Zambryski, 1992; Zupan and Zambryski 1992). Wounding of the plant allows the entry of bacteria and provides phenolic compounds that activate the DNA-transfer machinery of *A. tumefaciens*. The bacteria multiply in the wound sap and attach to the walls of plants cells. T-DNA is transferred to plant cells and integrated into the plant genome. Genes encoded by the T-DNA are expressed in the plant cells even before integration (Janssen and Gardner, 1989). Tumors develop as a result of cell divisions that are triggered by the continuous production of auxin and cytokinin by enzymes encoded in the T-DNA. In addition, the tumor cells produce and excrete opines that are synthesized by enzymes that are also encoded in the T-DNA, and these opines are consumed specifically by the infecting bacteria.
In 1907, Smith and Townsend demonstrated that the Gram-negative soil bacterium *Agrobacterium tumefaciens*, can cause crown gall tumors in plants as a result of bacterial infection, usually at the wound sites (Herrera-Estrella et al. 2004). Later in the 1980s, scientists in Europe and USA successfully used the T-DNA region to insert foreign genes into tobacco and showed that foreign genes became integrated and functionally expressed in the plant cells. Since then, T-DNA region has been largely modified so that the disarmed Ti plasmids, which contain a T-DNA lacking genes involved in tumor formation (disarmed plasmid), can be purchased and used for genetic modification of plants. The disarmed plasmid will allow the *Agrobacterium* to insert foreign DNA into plant cells without causing crown gall tumors.

A number of sophisticated plant-transformation vectors, designed on the basis of this naturally occurring gene-transfer mechanism, have been developed and such vectors are widely employed in plant molecular biology and in the genetic engineering of plants (Fraley et al. 1986). The use of such vectors for gene delivery to higher plants has several advantages in addition to its convenience and efficiency; it allows the transfer of large segments of DNA with minimal rearrangement and the integration of small numbers of copies of genes into plant chromosomes (Klee et al. 1987; Zambryski et al. 1988; Hamilton et al. 1996). Foreign genes delivered by this method are usually transmitted to progeny plants in a Mendelian manner (Budar et al. 1986; Feldmann and Marks 1987).

The bacterium is presumably attracted to a wounded plant in response to signal molecules released by the plant cells to which it then becomes attached (Zambryski 1992). Wounded tobacco (*Nicotiana* sp.) cells exude phenolic compounds, such as 4-acetyl-2,6-dimethoxyphenol (acetosyringone) and 4- (2-hydroxy-acetyl)-2,6-dimethoxyphenol (α-hydroxy acetosyringone), which activate *vir* genes on Ti plasmids (Stachel et al. 1985). Bolton et al. (1986)
found that seven phenolic compounds induced the expression of vir genes. These signal molecules appear to be very important in the recognition by *A. tumefaciens* of suitable hosts. Some plants, in particular grasses, appear not to produce these compounds, or even if they do, the levels are insufficient to serve as signals (Smith and Hood 1995).

Plant genetic transformation is a core research tool in modern plant biology and agricultural biotechnology. *Agrobacterium tumefaciens* remains the preferred method to genetically transform plants (Gheysen et al. 1998). In spite of progress in *Agrobacterium*-based technology, efficient transformation methods are still restricted to a few genotypes of a limited number of plant species. This is reflected in the fact that crop improvement through transgenesis involves usually a few elite and/or local varieties. Moreover, for most commercially important plants, currently available transformation procedures are too inefficient to be used in high-throughput applications. Improving transformation frequencies remains, therefore, one of the major challenges in plant transgene technology (Gheysen et al. 1998; Hansen and Wright 1999). Many environmental factors as well as plant and bacterial genes that affect DNA transfer from *Agrobacterium* to plants have been identified (Tzfira and Citovsky 2002), and this forms the basis for improving transformation procedures.

Early experiments with *Agrobacterium* indicated that wounding of the host plant is required for development of tumors (Lippincott and Lippincott 1975). Binns and Thomashow (1998), pointed out the distinct correlation in the literature between the wound-induced division of cells and the competence of such cells to be transformed by *A. tumefaciens*. They proposed that processes related to the synthesis of DNA and cell division are required for incorporation of foreign DNA into a host genome (Binns and Thomashow 1998). Evidence consistent with this hypothesis has been reported in the case of both

One of the most commonly used techniques in the transformation of dicotyledonous plants is the addition of phenolic compounds, such as acetosyringone, to co-cultures or bacterial cultures. Their addition is indispensable if the plant does not produce a sufficient level of signal molecules. Hiei et al. (1994) demonstrated that acetosyringone at 100 μM is a key to successful transformation of rice. The level of transient expression of β-glucuronidase (GUS), a marker enzyme, after co-cultivation was extremely low when acetosyringone was omitted. Chan et al. (1993) included the medium from a suspension culture of potato cells, which is a rich source of phenolic compounds, in co-cultures. Transfer of T-DNA is initiated at an early stage of co-cultivation in the presence of acetosyringone, and pre-treatment of bacteria with acetosyringone is not essential. However, pre-treatment might slightly increase the efficiency of gene transfer (Aldemita and Hodges 1996).

Other factors during co-cultivation, such as an acidic pH (Alt-Morbe et al. 1989; Turk et al. 1991), culture temperature below 28 °C (Alt-Moerbe et al. 1988), and high osmotic pressure (Usami et al. 1988) have also been reported to be important for the expression of vir genes. Veluthambi et al. (1989) found that, opines stimulated the induction of vir genes. In addition, Shimoda et al. (1990) and Cangelosi et al. (1990) reported that a group of aldoses, such as D-glucose and some non-catabolizable sugars such as 2-deoxy-D-glucose and 6-deoxy-D-glucose, markedly enhanced the expression of vir genes. The effects of sugars were clear when levels of phenolic inducers were limited and Hiei et al. (1994) confirmed the importance of these factors. Transient expression of GUS in calli derived from scutella after co-cultivation with Agrobacterium was strongest when co-cultures were incubated between 22 °C and 28 °C, and the
pH of the co-cultivation medium was between 4.8 and 6.2. The addition of D-glucose, D-galactose or L-arabinose, and of nopaline did not have any significant synergistic effect in the presence of 100 μM acetosyringone.

High regeneration capacity of the target cells is however, not always sufficient for successful transformation. Additional genotypic factors also influence the transformation ability. The most important factors are sensitivity to tissue damage for transformation and susceptibility to Agrobacterium when using Agrobacterium-mediated transformation and for both methods, sensitivity to selective agents (Kumlehn et al. 2006). However, the use of hypervirulent Agrobacterium strains and vectors, which contains extra copies of vir-genes has now partly overcome (Kumlehn et al. 2006). Susceptibility to Agrobacterium does, however, still vary among many genotypes and many elite cultivars show low or no transformation response (Murray et al. 2004; Hensel et al. 2008).

The first reported genetically engineered sunflower involved de novo regeneration of hypocotyl-induced callus, combined with Agrobacterium-mediated gene transfer using private inbred lines (Everett et al. 1987). Transforming cotyledons from germinating seedlings of the highly morphogenic responding HA300B did not lead to the regeneration of stable transformants (Laparra et al. 1995). The use of embryonic axes from mature (Schrammeijer et al. 1990; Bidney et al. 1992; Malone-Schoneberg et al. 1994; Grayburn and Vick 1995; Burrus et al. 1996; Alibert et al. 1999; Rao and Rohini 1999) or immature embryos (Lucas et al. 2000; Muller et al. 2001) as starting material proved to be generally applicable for the production of transgenic sunflower.

The molecular mechanism by which dicotyledonous plant cells are transformed to crown gall cells by Agrobacterium tumefaciens may involve the
transfer of DNA from the bacterium to the plant cell. Chilton et al. (1977) have demonstrated the presence of DNA in a cloned line of tobacco crown gall tissue culture cells that is homologous to approximately 3.5 mega daltons of \textit{A. tumefaciens} plasmid DNA. It was believed that part of the plasmid DNA may be transcribed in the crown gall cell because mRNA isolated from crown gall tissue cultures will hybridize with plasmid DNA. Also, hybridization is limited to that portion of the plasmid that is believed to be transferred to the plant cell. Although there is no direct evidence for functional bacterial gene products in crown gall tissues, their presence has been inferred from the work cited by Petit (1970).

The soil-borne gram-negative bacterium \textit{Agrobacterium rhizogenes} is the causative agent of hairy root disease in dicotyledonous plants. This disease results from the transfer and integration of transferred-DNA (T-DNA) of root-inducing (Ri) plasmid to the plant genome (Schmülling et al. 1989) and thereby inducing hairy root at the site of infection in natural condition (Brillanceau et al. 1989). The transformed roots can be excised and grown \textit{in vitro} as hairy root cultures. Hairy roots have several properties that have promoted their use for plant biotechnological applications. Their fast growth, genetic and biosynthetic stability, low doubling time, ease of maintenance and have the ability to synthesize a range of chemical compounds that makes them a suitable system for \textit{in vitro} production of secondary metabolites (Giri and Narasu 2000). Depending on synthesis and degradation of opine, \textit{A. rhizogenes} have been classified into three strains \textit{viz.}, agropine, mannopine and cucumopine. Agropine strains of \textit{Agrobacterium rhizogenes} is the most thoroughly studied strains, contain a T-DNA, divided into two regions, T\textsubscript{L} and T\textsubscript{R} (Huffman et al. 1984) physically separated on the plasmid by a non-transferred DNA sequence. The transfer of T-DNA is mediated by virulence genes, which form the \textit{vir} region of the Ri-plasmid and the bacterial \textit{chv} genes (Giri and Narasu 2000). Transformed roots induced as a result of integration of
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The TL and TR region of root inducing (Ri) plasmid into plant genome and their subsequent expression (White and Nester 1980). The TL region of Ri plasmid contains 18 open reading frames (ORF) (Slightom et al. 1986). ORFs 10, 11, 12 and 15 represent the root locus, i.e rol A, rol B, rol C and rol D, respectively. Root loci (rol) have been found to be essential for hairy root induction (White et al. 1985; Jouanin et al. 1987). Among these rol B plays central and most important role while rol A, rol C and rol D promote the root formation synergistically (Aoki and Syono 1999). The TR-DNA of the agropine-type Ri plasmid contains genes that are homologous to the iaa M and iaa H genes of the Ti T-DNA (Huffman et al. 1984). The ags gene responsible for opine biosynthesis in the transformed tissue are also located in TR region of Ri plasmid (Binns and Tomashow 1988).

Gene transfer into intact cells and tissues by particle or microprojectile bombardment is becoming an important tool in plant molecular biology (Klein et al. 1990). Particle bombardment into plant cells was first described by Sanford et al. (1987). Tungsten microprojectiles of approximately 4 μm in size were accelerated into onion epidermal cells and directly visualized using an inverted microscope. Transient gene expression in onion and later in maize (Klein et al. 1988) proved in the concept that tungsten microprojectiles could carry functional DNA into intact plant cells. The β-glucuronidase (GUS/uidA/gusA) gene (Jafferson et al. 1987) or an anthocyanin gene from maize (Ludwig et al. 1990) has furthered the development of improved procedures with biolistic technology because transformed cells can be directly visualized.

The efficiency of gene transfer by particle bombardment is highly dependent on various physical parameters that influence the particles momentum and distribution upon the target tissue. The size and shape of the particle will also influence its potential for cell proliferation. The number of particles per unit area that impact the target tissue is controlled to a large
degree by method of acceleration. Another important parameter controlling the efficiency of the process is the method used to adsorb DNA to the particles. Biological factors such as the size of the target cells, their turgor pressure and stage of development are also of importance in particle bombardment.

To-date no reports are available in the pertinent literature pertaining to genetic transformation of *Gymnema sylvestre*.

7.2. Materials and Methods

7.2.1. Transformation with *Agrobacterium tumefaciens*:

7.2.1.1. Plant material

Seeds of *G. sylvestre* were washed under running tap water for 5 min, treated for 10 min with a 3% (v/v) aqueous solution of Tween-80 and rinsed three to four times with sterile-distilled water. The seeds were then surface sterilized with 0.1 % (w/v) aqueous solution of mercuric chloride for 3 min followed by five rinses in sterile-distilled water. Surface sterilized seeds were germinated on MS basal medium (Murashige and Skoog 1962) solidified with 0.8% agar-agar, at 25 ± 1 °C and 60% relative humidity under continuous light provided by cool-white fluorescent tubes at an intensity of 35 μmol photons m⁻² s⁻¹. The leaves (0.5-0.8 cm discs) from 15-20 day old seedlings and nodal segment (less than 1 cm segments) were used in all studies. All cultures were maintained at 25 ± 1 °C under a 16/18h (day/night) photoperiod with light provided by white fluorescent tubes.

7.2.1.2. Bacterial strain

*Agrobacterium tumefaciens* strain LBA4404 carrying the binary plasmid pK1W1105 (Janssen and Gardner, 1993) was used. The T-DNA of pK1W1105 possesses neomycin phosphotransferase (*nptII*) gene conferring resistance against kanamycin as selection marker gene and β-glucuronidase (*gusA*) gene as reporter gene under 35S CaMV promoter. The bacterial culture was grown
overnight in the dark (16-18h, 28 °C, 200rpm) in liquid YEP (Sambrook et al.,1989) medium supplemented with 50 mg l⁻¹ kanamycin monosulphate and 10 mg l⁻¹ rifampicin.

7.2.1.3. Determination of phytotoxic levels of selective and bactericidal antibiotics

To determine the effect of kanamycin on the induction of callus/shoot and to screen an appropriate concentration of kanamycin for selection of transformants, the leaf/nodal segments were transferred to the callus induction medium (CIM) MS medium supplemented with 0.5 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg l⁻¹ 6-benzyl adenine (BA), 2 mg l⁻¹ naphthaleneacetic acid (NAA) or shoot induction medium (SIM) MS medium with 1 mg l⁻¹ BA, 0.2 mg l⁻¹ Kinetin (Kn), 0.05 mg l⁻¹ NAA. Both CIM and SIM were supplemented with different concentrations of kanamycin (filter sterilized solution) (0, 25, 50, 75 and 100 mg l⁻¹). Similarly, in a separate study the leaf/nodal segments were transferred to CIM/SIM supplemented with cefotaxime at different concentrations (0, 250, 500 and 750 mg l⁻¹). Antibiotic solutions were filter-sterilized prior to addition to the autoclaved media. Ten explants were used per treatment with three replicates each, number of explants showing callus/shoot bud induction was recorded for a time period of 40 days.

7.2.1.4. Transformation

A single bacterial cell colony was inoculated into liquid YEP medium (20 ml) containing 50 mg l⁻¹ kanamycin and grown in the dark (16-18 h, 28 °C, 200 rpm). Bacterial cells corresponding to an optical density (OD) of approximately 0.6-0.8 at 600 nm were pelleted by centrifugation (5,000 rpm, 10 min), followed by washing twice with half-strength MS basal medium and the final cell density was adjusted. Pre-cultured explants were submerged in the bacterial suspension for different durations (0.5, 1, 5, 10 min) with gentle shaking, blot dried on sterile paper and finally transferred to co-cultivation
medium without kanamycin and kept in dark for different time periods ranging from 1-4 days. Following different durations of co-cultivation, the explants were washed twice with sterile distilled water, then with 250 mg l\(^{-1}\) cefotaxime solution and blot-dried. The explants were then transferred onto 0.8% agar-solidified CIM/SIM containing bacteriostatic antibiotic cefotaxime (250 mg l\(^{-1}\)) and kanamycin (75 mg l\(^{-1}\)). Following a 2-week culture period, explants were sub-cultured onto fresh medium supplemented with cefotaxime (250 mg l\(^{-1}\)) as well as the kanamycin (75 mg l\(^{-1}\)). The kanamycin-resistant explants were sub-cultured at every 15-day intervals.

**7.2.1.5. Evaluation of factors influencing transformation**

**7.2.1.5.1. Pre-culture, bacterial cell density, Infection, Co-cultivation**

To determine the optimum conditions for *Agrobacterium* infection, the effect of different durations of pre-culture was investigated. Explants were pre-cultured on MS medium solidified with 0.8% (w/v) agar for 0, 1, 2, 3 and 4 days before infection. In order to evaluate the effect of bacterial cell density on transformation, a range of five concentrations (OD\(_{600}\) 0.2-1) of *Agrobacterium* suspension were investigated. To test the importance of infection period explants were infected with *Agrobacterium* suspension for various periods (0.5-10 min).

To determine the length of co-cultivation period, the explants were infected with *Agrobacterium* for 1 min, then co-cultivated on MS agar-solidified medium (0.8% w/v) for 1, 2, 3 and 4 days. Each treatment was replicated three times with at least 10 explants and repeated twice. Transient GUS expression was determined in a sample of explants from each treatment after transformation by histochemical assay and *Agrobacterium* infection frequency (number of GUS-positive explants/number of explants examined) was determined.
Immediately after co-cultivation, explants were washed first in excess of sterile distilled water, then washed twice (5 min, per wash) in liquid MS medium with 250 mg l\(^{-1}\) cefotaxime, blotted dry on sterile filter paper, and then abaxial side up, placed on CIM/SIM, subcultures were done for every 10 days.

7.2.2. Direct DNA transfer into intact plant cells by microprojectile bombardment

In an attempt to study the possibility of direct DNA transfer to *Gymnema sylvestre*, the callus samples were used for particle bombardment. Transformation experiments were conducted with a Biolistics PDS 1000/He system using tungsten M-25, 1.7 μm in diameter (Bio-Rad, Richmond, CA, USA).

7.2.2.1. Preparation of callus for microprojectile bombardment

Approximately 10-15 calli tissues each about 0.5 cm diameter, were sliced into smaller pieces (2-3 mm) and placed in the middle of a petriplate containing osmotic medium (MS basal medium, 0.5 M D-mannitol, 30g l\(^{-1}\) sucrose, 2.5 mg l\(^{-1}\) 2, 4 D and 3g l\(^{-1}\) Phytagel) for 4 hours prior to bombardment. The calli were left on the same medium overnight following the bombardment before being transferred to subculture medium.

7.2.2.2. Preparation of microprojectiles for bombardment

60 mg of tungsten powder (BioRad Tungsten M-17; with each particle diameter 1.7 μm, Bio-Rad, Richmond, CA, USA) was suspended in 1 ml of absolute ethanol, subjected for centrifugation for 2 min in a microfuge at 3000 rpm. Pelleted tungsten particles were suspended in 1ml distilled to obtain a microcarrier suspension (0.75 mg in 50 μl) subjected for centrifugation again, repeated for 3 times. Finally thus obtained tungsten particles were resuspended the in 1.0 ml of 50% glycerol, stored at −20 °C for further usage.
7.2.2.3. Coating of plasmid DNA to microprojectiles and Bombardment

The microcarrier suspension prepared in 50% glycerol (60 mg/ml) vortexed for at least 5 min on a platform to disrupt agglomerated particles and also to maximize uniform sampling in suspension. 5μl of microcarrier suspension was removed and transferred into a 1.5 ml microfuge tube. While vortexing vigorously, 5μl DNA (pKIWI105; 1μg/ml), 50μl CaCl₂ (2.5M), 20μl spermidine (free base, 0.1 M) were added in serial order and continued vortexing for another 3 min. The micro carriers were allowed to settle for 3 min by spinning in microfuge (at approximately 5000 rpm), supernatant was discarded and 150 μl of 70% ethanol was added without disturbing the pellet. The pellet was saved and carefully washed for two times with 150 μl of absolute ethanol each time. After discarding liquid (saving the pellet) the pellet was resuspended into 55-60 μl of absolute ethanol. 6 μl aliquots of microcarrier suspension were transferred to the center of a macro carrier.

The particles were finely dispersed with ultrasonic cleaner (Sonicor Instrument Corporation, Copiague, NY, USA) before bombardment. Before shooting, all the discs and stopping screens were sterilized by placing absolute ethanol in a petri plate for 5-1 and air-dried under sterile conditions in the tissue culture hood. Similarly the gun parts like holder for the rupture discs, the stage, the five metal ring holders for the flying discs and holder assembly for the flying discs, and the platform for the leaf samples were also surface sterilized by wiping with absolute ethanol and also the interior of the vacuum chamber and door were wiped with absolute alcohol. Bombardment was done at different bombardment pressures (1100 or 1500 p.s.i.) and distances from the launching plate (6 or 9 cm) were evaluated for transformation efficiency. Per bombardment approximately 1 pg of plasmid, one flying disc (the large orange discs), and one stopping screen (the wire mesh screens) were used (as suggested in the manufacturer’s kit manual, Bio-Rad, Richmond, CA, USA).
7.2.3. Histochemical staining for GUS activity

Histochemical staining for GUS activity was examined following the procedure described by Jafferson et al. (1987) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as a substrate. Transient GUS expression was determined in samples of explants from each experiment after co-cultivation with Agrobacterium. Similarly GUS histochemical staining was performed with calli transformed directly using biolistics. The calli that were bombarded with gus gene coated to tungsten particles were incubated in dark on the mannitol media. The next day early morning the calli were transferred onto selection media supplemented with different concentrations of kanamycin, after three days of incubation on selection media the calli were subjected to GUS histochemical staining to confirm the gus gene expression.

Plant tissues (Agrobacterium co-cultivated explants and directly transformed calli were individually incubated with 1 mM X-Gluc in 50 mM phosphate buffer (pH 7.5) containing 10 mM EDTA, 0.1% Triton x-100 for 18h at 37 °C, washed once with sterile distilled water and finally dipped in 70% ethanol overnight to extract any chlorophyll that may be present in the tissue. Explants were examined under a dissecting microscope and scored for blue coloration and GUS expression was documented by photography. The number of explants showing blue coloration divided by the total number of inoculated explants was taken as GUS expression frequency. Thin transverse hand sections of the leaf and node randomly selected after co-cultivation with Agrobacterium was taken for transient GUS expression, similarly callus and shoot sections from randomly selected putative transformed (kanamycin-resistant) shoots from each explant were taken for calculating stable expression.
7.2.4. PCR analysis of transformants

The PCR analysis was carried out on genomic DNA extracted from 300-500 mg fresh weight of tissue of control and putative transformed samples following the cetyltrimethylammoniumbromide (CTAB) method (Doyle and Doyle 1987). Amplification was performed in a 20 μl reaction volume consisting of 10x buffer, 50 mM KCl, 1.5 mM MgCl₂, 100mM dNTPs, 0.5U Taq DNA polymerase, 250 nM primers and 20ng template DNA. The PCR amplification profile consisted of an initial denaturation at 94 °C for 4 min followed by 30 cycles at 94 °C for 1min, at 45 °C for 1min 30 s and at 72 °C for 2 min and the was concluded by a final cycle of 94 °C for 7min. The primer pairs 5'-TGTAGAACCCCAA- CCCGTGAAAT-3' and 5'-GTCCCGCTAGTGCTTGTCCAGTT-3' were used for amplifying the gusA gene. Non-transformant tissue DNA was included as control. PCR products were observed under UV light after electrophoresis on a 1% agarose gel with 0.1% ethidium bromide and the molecular marker of 1-kb ladder was used.

7.2.5. Transformation with Agrobacterium rhizogenes

7.2.5.1. Bacterial Strain

Agrobacterium rhizogenes strain A4 was used for hairy root induction. Bacterial culture was grown overnight on YEM broth at 28°C on a rotary shaker at 100 rpm in the dark. After 16 h the cells were collected by centrifugation at 5000 rpm for 10 min (C-24 BL, REMI, India), washed with MS liquid medium and resuspended in MS liquid medium.

7.2.5.2. Plant material and hairy root induction

Leaf, petiole and nodal segments from 20 days old seedling were used as explants for hairy root induction. Explants were cut into small pieces (0.5-1 cm) and infected with bacterial culture with OD₆₀₀ ranging from 0.2-1 for different time periods (5, 10, 15 and 20 min.) in separate treatments. Explants were blotted dried on sterile filter paper and co-cultivated in dark for 1-3 days
on hormone-free full and half strength MS media containing 3% sucrose in separate trials. After co-cultivation explants were rinsed with sterile water, then washed in 500 mg{l} cefotaxime and transferred onto hormone-free full and half-strength MS media to test the induction of hairy roots. Explants were subcultured at regular intervals (10-15 days) till the induction of hairy roots.

7.3. Results and Discussion

7.3.1. Transformation with Agrobacterium tumefaciens:

*Agrobacterium tumefaciens* strain LBA4404 carrying the binary plasmid pKIWI105 (Janssen and Gardner, 1993) was used. The T-DNA of pKIWI105 possesses neomycin phosphotransferase (*nptII*) gene conferring resistance against kanamycin as selection marker gene and β-glucuronidase (*gusA*) gene as reporter gene under 35S CaMV promoter (Figure-1).

![Figure-7.1: Structure of the T-DNA region of plasmid pKIWI1105](image-url)

7.3.1.1. Phytotoxic levels of selective and bactericidal antibiotics

The sensitivity of the leaf and nodal segments to kanamycin was studied to determine the optimum level for callus and shoot induction. The effect of increasing the concentrations of kanamycin (0, 25, 50, 75, 100 mg{l}¹) was assessed. Kanamycin was apparently ineffective at 25 mg{l}¹ for both leaf and nodal segments. About 70-80% of the explants were bleached by day 30 in culture medium containing 50 mg{l}¹ kanamycin, this was more prominent in
case of leaf segments. The phytotoxic effect of kanamycin was noticeable at higher than 75 mg l\(^{-1}\) in the medium as explants started to bleach between 10 days and 15 days following inoculation, resulting in a total loss of chlorophyll pigmentation (bleaching) by day 30. All explants became necrotic, and there was an absolute arrest of any kind of response from both types of explants at this concentration. Hence, in subsequent experiments, both CIM and SIM were supplemented with 75 mg l\(^{-1}\) kanamycin for the selection of putative transformed explants (Figure-7.2). In most of the studies kanamycin at concentrations between 50-100 mg l\(^{-1}\) was found to safe for the plant cells (Niu et al. 2000; Barik et al. 2005; Sandhya and Kamlesh 2007; Pandey et al. 2010; Ntui et al. 2010; Guo et al. 2010).

![Figure-7.2: Effect of kanamycin concentration on growth of explants](image)

The antibiotic cefotaxime was used at various concentrations (0, 250, 500 and 750 mg l\(^{-1}\)) to eliminate the background \textit{Agrobacterium}. Cefotaxime had a detectable effect on callus/shoot induction also. In the present study, 750 mg l\(^{-1}\)
cefotaxime was found to be effective on bacterial elimination, but showed detrimental effect on the plant growth, while 250 mg l⁻¹ cefotaxime in the medium showed prominent effect on bacterial elimination without adversely affecting morphogenesis (Fig. 3). Therefore 250 mg l⁻¹ cefotaxime was considered to be optimum concentration for further experiments. The response of explants to bactericidal antibiotics has been observed in many species *Pinus taeda* (Tang et al. 2004), *Glycine max* (Meurer et al. 1998), *Salvia miltiorrhiza* (Yan and Wang 2007), *Colocynth is citrullus* (Ntui et al. 2010), *Eupatorium adenophorum* (Guo et al. 2010) (Figure-7.3).

![Figure-7.3: Effect of cefotaxime concentration on tissue growth](image)

7.3.1.2. Explant pre-culture

Explant pre-culture has been reported to be a useful parameter in *Agrobacterium*-mediated transformation. During the present study it was observed that, prior to co-cultivation with *Agrobacterium*, explants were
cultured for a period varying from 1 day to 4 days. Shorter duration of pre-culture less than 2 days resulted more transformants than longer duration.

The significance of explant pre-culture has been reported to be a useful parameter in *Agrobacterium*-mediated transformation in several plant species including *Vigna mungo* (Karthikeyan et al. 1996), *Cajanus cajan* (Lawrence and Koundal 2000), *Sesbania drummondii* (Priya and Shivendra 2009), *Eupatorium adenophorum* (Guo et al. 2010).

### 7.3.1.3. Bacterial strain and growth phase

To determine the right stage of *Agrobacterium* growth for efficient transformation, different stages of bacterial growth phase was studied. At the late-log phase, corresponding to $\text{OD}_{600} = 0.4-0.6$, the maximum transformation rate was obtained as indicated by measuring transient GUS expression. Any changes in the OD value above or below its optimum value were not conducive for transformation, because of bacterial overgrowth. Higher OD results in extensive tissue damage, while lower OD value results in poor transformation. Range of duration tested for explant infection with *Agrobacterium* culture, the maximum transformation was obtained with explants treated with diluted *Agrobacterium* culture (1:1) for less than 5 min. To test the importance of the infection period, explants were infected with an *Agrobacterium* suspension ($\text{OD}_{600} = 0.6$) for varying periods (0.5 -10 min). The frequency of transient GUS expressing explants was high when explants were infected for 1 min. Infection period shorter than 5 min was found to be optimum. A longer infection period (above 5 min) decreased the frequency due to cell necrosis (Figure-7.4). A decline in transformation efficiency with increase in bacterial cell density has been reported in safflower (Orlikowska et al. 1995) and black gram (Saini and Jaiwal 2007), which may be attributed to variation with regard to plant species, explanted tissue, duration of co-cultivation and mode of regeneration.
7.3.1.4. Co-cultivation

The effect of different co-cultivation periods on transformation was compared by conducting a transient expression experiment after co-cultivation. Explants were co-cultivated for an increasing length of time (1-4 days). 2 days of co-cultivation period was found to be optimum for maximum transformation. Even though transient GUS expression was detectable in explants co-cultivated for 1 day, but percentage of GUS positive cells was less compared to explants co-cultivated for increasing length of time and it was highest after two days (Figure-7.5). Extending the co-cultivation period above two days, did not increase GUS activity, but caused necrosis and death of cells due to excessive growth of Agrobacterium. Therefore, a co-cultivation period for 2 days could be considered as optimum for efficient transformation.
Optimal co-cultivation duration is critical for plant transformation, and different co-cultivation times appear to be specific to different species, varying from 1 to 7 days (Vergauwe et al. 1998; Suzuki et al. 2001). A problem with excessive growth of *Agrobacterium* with an increase in co-cultivation period has been reported in other plants as well (Cervera et al. 1998; Saima and Bushra 2004; Wang and Zu 2007; Srivastava et al. 2009; Shilpa et al. 2010). As in the present study, a co-cultivation period of 2 days was adopted in other plant species such as *Antirrhinum majus* (Holford et al. 1992), *Glycine max* (Li et al. 2004), *Vigna radiata* (Saima and Bushra 2004), *Nicotiana tabacum* (Uranbey et al. 2005), *Carthamus tinctorius* (Shilpa et al. 2010).

### 7.3.1.5. Transformation and Tissue proliferation.

In the present study, leaf and nodal segments excised from 15-20 days old seedlings were used to study *Agrobacterium* mediated transformation. Leaves were cut into 0.5-0.8 cm disc while nodes were cut into less than 1 cm segment
and pre-cultured for two days on two different media, leaf segments on callus induction media (CIM) containing MS basal supplemented with 0.5 mg l⁻¹ 2, 4-D, 2 mg l⁻¹ BAP and 2 mg l⁻¹ NAA and nodal segments on shoot induction media (SIM) containing MS basal supplemented with BAP 1mg l⁻¹, Kn 0.2 mg l⁻¹ and NAA 0.05 mg l⁻¹. Two days pre-culture found to enhance the transient GUS expression frequency (71%) as compared with 0, 1, 3 or 4 days pre-culture (40%, 66%, 55%, 50%, respectively). The explants infected with Agrobacterium culture for less than 5 min and the transient GUS expression frequency for 1 min was 78%, found to be better than 5 min (74%) (Figure-7.6). Following 2 days of pre-culture, 1 min infection time, 2 days of co-cultivation, explants were washed in 500 mg l⁻¹ cefotaxime to remove bacterial overgrowth here after explants were cultured on respective CIM and SIM media. Explants were monitored regularly for any residual bacterial growth and such cultures were rewashed. After 1 week explants were transferred to respective CIM and SIM media supplemented with kanamycin 75 mg l⁻¹ and 250 mg l⁻¹ cefotaxime. Callus induction started from leaf segments after 20 days on CIM supplemented with 75 mg l⁻¹ kanamycin and 250 mg l⁻¹ cefotaxime, similarly nodal explants showed shoot bud outgrowth after 25 days on SIM supplemented with 75 mg l⁻¹ kanamycin and 250 mg l⁻¹ cefotaxime. Explants were subcultured every 15 days of time period. Selection for minimum of 4-5 cycles was required for obtaining stable transformants.
Callus induction started from leaf segments after 20 days on CIM supplemented with 75 mg l\(^{-1}\) kanamycin and 250 mg l\(^{-1}\) cefotaxime, similarly nodal explants showed axillary bud outgrowth after 25 days on SIM supplemented with 75 mg l\(^{-1}\) kanamycin and 250 mg l\(^{-1}\) cefotaxime. Explants were subcultured every 15 days of time period. Selection for minimum of 4-5 cycles was required for obtaining stable transformants.

7.3.2. Direct DNA transfer into intact plant tissue by microprojectile bombardment

7.3.2.1. Transformation

The results of direct gene transfer by biolistic transformation revealed that, of the different pressures and (1100 or 1500 psi) and distances from the launching plate (6 or 9 cm) tested for transformation efficiency, 1100 psi...
pressure and 9 cm target cell/tissue distance from launching plate have resulted in better transformation efficiency compared to other trials. produced highest GUS positive calli (Figure-7.9-T2). The callus was observed to be significantly differed from other two stages. More intensive blue coloration was also seen at 9 cm target distance compared to other two treatments (Figure-7.9).

Figure-7.7: Gene gun used for direct gene transfer into Gymnema calli with gus gene in plasmid pKlW1105
7.3.1.6. Histochemical staining for GUS activity

Expression of the GUS-intron gene is a reliable indicator of plant transformation, since the gene only can express efficiently in plant cells but not in Agrobacterium (Van-canneyt et al. 1990; Narasimhulu et al. 1996). GUS positive blue coloration was visibly detected in all transformed tissue stained with X-Gluc reagent, where as no blue coloration was observed in non-transformed control tissue (Figure-7.8).

(a) Shoot regeneration on kanamycin selection medium; (b) Leaf callus induction on kanamycin selection medium; (c) GUS-positive of transformed shoot; (d) Leaf callus showing transient GUS expression; (e) Callus showing transient GUS expression; (f) Callus showing stable GUS expression;

Figure-7.8: Transformation of Gymnema sylvestre
C - Control; T1 - Callus at target distance 6cm; T2 - Callus at target distance 9cm; T3 - Callus at target distance 12cm.

Figure-7.9: Effect of target cell distance on transformation *Gymnema* calli by Biolistics method
7.3.1.7. PCR analysis

PCR analysis was carried out to identify the presence of gusA gene in kanamycin-resistant plants. The expected approximately 500 bp (465bp) sequence was amplified using gusA gene–specific primers in the kanamycin and GUS-expressing putatively transformed callus and regenerated plants. All the kanamycin-resistant and GUS-expressing putatively transformed callus and regenerated plants tested by PCR analysis showed 500 bp band in the gel corresponding to gusA gene amplification, indicating that the regenerated plants were most likely transformed. No specific amplification products were detected in the non-transformant control sample (Figure-7.10).

PCR Analysis

![PCR Analysis Image]

T1 - GUS positive transformed calli; T2 - GUS positive transformed shoot; C1, C2 - Respective controls

Figure-7.10: PCR analysis of GUS-putative positive transformants

7.3.1.7.1. GUS Primers designed for GUS gene amplification:
7.3.1.7.1. GUS Primers designed for GUS gene amplification:

Forward - TGTAGAAACCCCAACCCGTGAAAT
Reverse - GTCCCGCTAGTGCCTTGTCAGTT

35s promoter+ gus gene

5'CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGACATGCAGATCGGCAGGTCCCCA
GATTAGCCTTTTCAATTCCAAAGAAAGATGCCTAACCACAGATGGATTAGAGAGCT
TACGCAGCAGGTCTCATTCAAGACGATCTACCCAGAATATACTCCAGGAAATCA
AATACCTTTCCCCACTGAAATCAAAGCCATGGAGTCAAAGATTCAAATAGAG
GACCTAACAGAAGCTCGCACTAAAGACTCGCGAACAGTCTACTATGCCCGCTATAC
GACTCAATGCAAAGAAAGAAATCTTCTGACACATGGTGAGCGACGACACACTTTG
TCTACTCAAAATATCAAAGATACGATCTGACTGAGACCAGACCGAAATGAGAC
TTTCACAAAGGGGTAAATATCCGGGAAACCTCCTCGGATTCCATTGCCCAGCTATC
TGTCACTTATTTGTAAGATAGTGGAAAAGGAAGGTTGGTGCCTCTACAAATGCCATC
ATTGCAGATAAACAGAAGGCAATCGTTGAAAGATGCTCTGCAGACAGTGGTCCCA
AAGATGGACCCCCCAACACAGAGCATCGTGAAAGAGAAAGAGACGTCCTATATCGA
CGTCTTTAAAGCAAGTGGATTGATGATACTATCACCACGTAGTAGGAGTAGACG
AACAATCCCCACTTCTCTCGACAGCACCTCTCCTATATAGGGAAGTGTCATCTTTT
TGGAGAGAAACCGGGGACTCTAGAAGAAATCTCGAGCCATTGCCCAGCGTTTACT
CTAGTACGATTCTGTAATTGAGCCTGTTTCTGCTATGAAATGCTTCTTACTTCA
TACCAGAAAGTGGCCAGGGCAGCCAGCTTCGTTCTCAGTGTCAGTGCTACCTCGA
ATTAGAGAAGCTCGGCAGGAACTTGCGATGACGTTGATGTGAAAGAGGCAC
GTACAAGAAAGGGCAATTTGCTGTTGCAAGGATGTTGATTGAAAGGCAC
GATGCAATATTCCAATTTGAGGGGAAACTGCTGTTGTTGTTGAAAGGACGCT
ATACGCAGATATTCTGTAATTGAGGCAACAGCTGTTGTAATCAGCCGAAGCTTTCT
TACCCGAAAGGTTGCCCAGGTACGCATTCTTCGTTGAAATGCAGGGCTAGCTC
ATTAGGGAAAGTGGTTGTCAAATATCGGAAGATGTAATCCGGCAGCGC
ATACGCACATTTCGAAAGCCGATGCACAGCTGTTATTGATTGCGCCGAAAGATGTACG
TCATACCGTATTTGGTAAACAGAAACTGCAACTGCAAGACTCCTCCCGCGGGAATG
GTGATTACCGACAGAACAGGCAAGAAAGCAGTCTTTACTTTATGTATATTTTTA
ACTATGCCGGAATCCATCGCAGCGTAGCTATGCTTACTACACACGCAGGACACCTGGGT
Chapter-7 Genetic Transformation

The data on Agrobacterium tumefaciens mediated transformation of Gymnema sylvestre presented here is the first attempt to transform this plant using leaf and nodal explants. In our efforts we could able to obtain

GGACGATATCACCAGTGCTGACGATGTCGCTCGAGACGACTGTAACCACCTGCTGTCTGTT
GACTGGCAACTGATGTGATGCCCAATATGGTCTGCTAGCGTTAAGCTGCTGAGTGTGTTGGAATTTCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAA
CAAGAAAGGGATTTCCATTCTCATACCCGACATGAGAAGTAGTACGATGTCGCTGATG

35s F-TGTAGAAACCCCAACCCGTGAAAT
GUS F-GGGCAGGCCAGCGTATCGTG
GUS R-GTCCCGCTAGTGCCTTGTCCAGTT

The data on Agrobacterium tumefaciens mediated transformation of Gymnema sylvestre presented here is the first attempt to transform this plant using leaf and nodal explants. In our efforts we could able to obtain
transformed callus from leaf explants, but despite of repeated attempts, callus failed to induce organogenesis. Influence of various parameters on *Agrobacterium*-mediated transformation was evaluated. Pre-culture of the explants has been reported to influence transformation frequency. In the present study for the transformation of *Gymnema sylvestre* 2 days of pre-culture has been found to be optimum period. Prior to co-cultivation with *Agrobacterium*, explants were cultured for a period varying from 1 day to 4 days. Shorter duration of pre-culture less than 2 days resulted more transformants than longer duration. This could be due to the secretion of phenolics or other *vir* gene inducing compounds by wounded tissue into surrounding media (Stachel et al. 1985). Explant pre-culture has been reported to be a useful parameter in *Agrobacterium*-mediated transformation in several plant species (Karthikeyan et al. 1996; Lawrence and Koundal 2000; Priya and Shivendra 2009; Guo et al. 2010). The explants were pre-cultured for less than 2 days followed by infection for less than 5 min. This was followed by a co-cultivation for two days in the dark which gave rise to maximum transient transformation frequency. Prolonged infection time and co-cultivation lead to bacterial overgrowth and resulted in the necrosis and death of the explants. Optimal co-cultivation duration was critical for plant transformation, and different co-cultivation times appear to be specific to different species, varying from 1 to 7 days (Vergauwe et al. 1998; Suzuki et al. 2001).

The complete elimination of bacteria after co-cultivation is a necessary step during the transformation process, the bacteriostatic antibiotic cefotaxime was used at an optimum concentration of 250 mg l⁻¹ in the selection media (Figure-7.3). The detection of transformed cells or tissue in a mixture of transformed and non-transformed cell is an important factor during selection process. The use of antibiotic-resistance selection marker, in the vector along with the desired gene, gives convenient and effective selection system. Kanamycin up to 75 mg l⁻¹ in the selection media was used to discriminate between transformed
and non-transformed cells, below this concentration kanamycin was found to be ineffective and higher than the 75 mg l\(^{-1}\) concentration was found to be detrimental (Figure-7.2). In most of the studies kanamycin at concentrations between 50-100 mg l\(^{-1}\) was found to safe for the plant cells (Niu et al. 2000; Barik et al. 2005; Sandhya and Kamlesh 2007; Pandey et al. 2010; Ntui et al. 2010; Guo et al. 2010). The response of explants to bactericidal antibiotics has been observed in many other plant species also (Meurer et al. 1998; Tang et al. 2004; Yan and Wang 2007; Guo et al. 2010; Ntui et al. 2010). A decline in transformation efficiency with increase in bacterial cell density has been reported in other plant species also (Orlikowska et al. 1995; Saini and Jaiwal 2007; Yongxue, 2010), that has been attributed to variation with regard to plant species, explanted tissue, duration of co-cultivation and mode of regeneration.

The GUS assay followed by the PCR analysis using \textit{gusA} primers of callus and regenerates after 3-4 cycles of selection, indicating that the T-DNA of the binary plasmid vector was present in the genome of the transgenic plants and confirmed the stable transformation. This clearly showed that \textit{Gymnema sylvestre} is amenable to transformation either by indirect mode using \textit{Agrobacterium tumefaciens} or by direct mode by microprojectile bombardment method (Figures-7.8, 7.9 & 7.10). Transformation depends upon a complex interplay of many factors including genotype, explant source, media nutrients, growth regulators, gelling agents, culture environment, \textit{Agrobacterium} strains, infection and co-cultivation methods, and selection scheme. Excessive growth of \textit{Agrobacterium} with an increase in co-cultivation period has been reported in other plants as well (Cervera et al. 1998; Saima and Bushra 2004; Wang and Zu 2007; Srivastava et al. 2009; Shilpa et al. 2010). As in the present study, a co-cultivation period of 2 days was adopted in other plant species (Holford et al. 1992; Li et al. 2004; Saima and Bushra 2004; Uranbey et al. 2005; Shilpa et al. 2010). The transformed plants reported here were the product of a limited set of manipulations of some of these factors. Improvement of the system will
require optimization of multiple factors, among the most important is an improvement of regeneration. High levels of regeneration are critical for increasing transformation rates.

7.3.3. Transformation with *Agrobacterium rhizogenes*

Apart from indirect genetic transformation using *Agrobacterium tumefaciens* and direct gene transfer by biolistics method, in the present study, *Gymnema sylvestre* was transformed with *Agrobacterium rhizogenes* strain A4 for hairy root induction. Despite of 4-5 attempts hairy roots were not induced even after regular sub culture for 3 months. This failure might be attributed to a number of parameters like explant type, genotype of bacterial strain and construct structure, chemical and physical factors and signal molecules.

In conclusion, a simple transformation protocol was developed for *Gymnema sylvestre*. Kanamycin upto 75 mg l$^{-1}$ in the selection media was evaluated as optimal to discriminate between transformed and non-transformed cells, cefotaxime 250 mg l$^{-1}$ was evaluated as an optimum concentration for suppressing the over growth of *Agrobacterium tumefaciens* in the culure, 2 days of pre-culture and 2 days co-cultivation have been found to be optimum for high efficiency transformation of nodal and explants by indirect genetic transformation using *Agrobacterium tumefaciens*. Similarly, for direct gene transfer by biolistics, 1100 psi pressure and 9 cm target cell/tissue distance from launching plate have been standardized for better transformation efficiency compared to other trials, lthat produced highest GUS positive calli with intense blue colour.

This is a preliminary attempt to transform *Gymnema sylvestre* and an important first step which offers the opportunity to transfer foreign genes into *Gymnema sylvestre* with the view to investigate the secondary metabolic pathways and metabolic engineering for over production of secondary metabolites.