Journal of Medicinal Plants Studies
www.PlantsJournal.com

**ISSN 2320-3862**
JMPS 2016; 4(4): 18-24

Received: 06-04-2016
Accepted: 04-05-2016

**Ram Krishna Sahu**
Division of Molecular Oncology, ICPO-ICMR, I-7 Sector 39, Noida, Utter Pradesh, India.

**Rajesh T.S**
Azyme Bioscience Pvt. Ltd., Banaglore, Karnataka, India.

**Pavithra K**
Department of Biochemistry, Dr. GRD College of Science, Bharathiyar University, Coimbatore, T.N. India.

**Correspondence**
**Ram Krishna Sahu**
Division of Molecular Oncology, ICPO-ICMR, I-7 Sector 39, Noida, Utter Pradesh, India.

# Micropropagation and *Agrobacterium*: mediated transformation of *GUS* gene in to *Gymnema sylvestre* antidiabetic plants

**Ram Krishna Sahu, Rajesh T.S, Pavithra K**

**Abstract**
*Gymnema sylvestre* is an herb plant of the tropical forest of Southern and Central India where it has been used as a naturopathic treatment for diabetes for nearly two millennia. Sanskrit Name: Meshasringi, Madhunasini or madhoolika, Hindi: Gurmar, Tamil and malayalam Name Sirukurinchaan, Amudhapushpam, Chakkarakkolli. *Gymnema sylvestre* is a stomachic, diuretic, refrigerant, astringent, and tonic. It has been found to increase urine output and reduce hyperglycemia in both animal and human studies. Gymnemic acids have anti-diabetic, anti-sweetener and anti-inflammatory activities. *Gymnema* is propagated by seed germination. One of the constraints in this conventional propagation is the very short span of seed viability. No alternative mode of multiplication is available to propagate and to conserve genetic stock of this useful plant. Tissue culture offers an effective alternative method for rapid multiplication of desirable clones. *Gymnema* was used for *in vitro* callus induction and *Agrobacterium* mediated transformation experiment. Which showed fast and high growth rate in the MS medium at 0.03mg/l concentration of 2, 4-D. The *Agrobacterium tumefaciense* strain LBA4404 has carrying Ti plasmid pK1W1105 was used for infection of callus. 1µl of 24hr culture of *Agrobacterium* was used for 30 sec. for the infection. Successful delivery of the *GUS* reporter gene was confirmed by 1mM X-gluc as histochemical substrate for staining of the callus. DNA was isolated from the infected callus by using 1.2%SDS (65°C) method and then presence of *GUS* gene was reconfirmed by using *GUS* gene specific marker in PCR amplification technique.

**Keywords:** Gymnema sylvester, *GUS* gene, agrobacterium, transformation, plant tissue culture.

## Introduction
*Gymnema sylvestre* is an herb native to the tropical forest of Southern and Central India where it has been used as a naturopathic treatment for diabetes for nearly two millennia. It's Sanskrit Name: Meshashringi, Madhunashini or madhoolika, Hindi: Gurmar, Tamil and Malayalam Name Sirukurinjan, Amudhapushpam, Chakkarakkolli (Kinghorn *et.al* 1991) [1]. *Gymnema sylvestre* is a large, more or less pubescent woody climber. Its leaves are opposite, usually elliptic or ovate; 1.25 to 2 inches long, and 0.5 to 1.25 inches wide. The leaves are base acute to acuminate, glabrous above and sparsely or densely tomentose beneath. *Gymnema's* small yellow flowers appear in axillary and lateral umbellate cymes with long pedicels. The long, fusiform follicles are terete and lanceolate, up to 3 inches in length. The calyx-lobes are long, ovate, obtuse, and pubescent; the corolla pale yellow, campanulate, valvate; and the corona is single, with five fleshy scales. (figure.1).
*Gymnema sylvestre* is a stomachic, diuretic, refrigerant, astringent, and tonic. It has been found to increase urine output and reduce hyperglycemia in both animal and human studies. *Gymnema* has been used in India for the treatment of diabetes for over 2,000 years. The primary application was for the adult-onset diabetes, a condition for which it continues to be recommended today in India. The leaves were also used for stomach ailments, constipation, water retention, and liver disease (Murakami *et.al.*, 1996) [3]. Gymnemic acids have anti-diabetic, anti-sweetener and anti-inflammatory activities. The antidiabetic array of molecules has been identified as a group of closely related gymnemic acids after it was successfully isolated and purified from the leaves of *Gymnema sylvestre* (Yoshikawa *et. al*., 1992.) [4] *Gymnema* is typically recommended for people who have trouble taming their sweet tooth and who are either borderline diabetic or waging a war against Type II diabetes. When there a high level of blood sugar, the body secretes more insulin, which is the hormone that helps in shuttling blood sugar into the cells of the body.


**Figure.1 Gymnema Sylvester**

**A)***Gymnema sylvestre* a large, woody climber. opposite leaves, elliptic or ovate , The leaves are base acute to acuminate, glabrous above and sparsely or densely tomentose beneath. **B)** fruit of *Gymnema* **C)** It's small yellow flowers appear in axillary and lateral umbellate cymes with long pedicels. The long, fusiform follicles are terete and lanceolate, up to 3 inches in length **D)** The calyx-lobes are long, ovate, obtuse, and pubescent; the corolla pale yellow, campanulate, valvate; and the corona is single, with five fleshy scales

L. predominantly contains proanthocyanidins [21]. In addition, But as too much insulin is produced, the cells become resistant to it a condition known as insulin resistance. When that happens, blood sugar remains high. Many people who have insulin resistance gain weight, no matter what their diet. In such cases, *Gymnema* may help by improving the cells' uptake of blood sugar and helping the body to utilize it (Flores-Riveros, 1993) [5].

*Gymnema* is propagated by seed germination. One of the constraints in this conventional propagation is the very short span of seed viability. No alternative mode of multiplication is available to propagate and to conserve genetic stock of this useful plant. Tissue culture offers an effective alternative method for rapid multiplication of desirable clones. Micropropagation by axillary bud proliferation has proved to be the most reliable method for large-scale production of many forest and medicinal plants. The investigation was undertaken with the aim to optimize *in vitro* conditions for mass (Rama Gopal and Lakshmi Sita *et al.* 2004) [7].

Plant secondary metabolites have enormous potential for research and new drug development. Many secondary metabolites have a complex and unique structure and their production is often enhanced by biotic and abiotic stress conditions. Gymnemic acid ($C_{43}H_{68}O_{14}$), a pentacyclic triterpenoid isolated from the leaves of *Gymnema sylvestre*, exhibits potent inhibitory effect on diabetes. The gymnemic acid content is determined by chromatographic methods: Camag HPTLC system equipped with a sample applicator Linomat IV and TLC scanner and integration software CAT 4.0. In HPLC $C_{18}$ (ODS) reverse phase column; water 486 UV detector; mobile phase, water/methanol (35:65, HPLC grade) + 0.1% acetic acid are used. Sample (20µL) is applied with a flow rate of 1 mL/min and read at 230 nm with UV detector. The production of gymnemic acid is significantly higher in callus treated with 2, 4-dichloro phenoxy acetic acid (2, 4-D) and kinetin (KN). The blue light increases gymnemic acid accumulation upto 4.4-fold as compared with fluorescent light treatment and out of which 2.8 is found in leaves. Gymnemic acid is isolated from callus, grown under stress conditions followed by preparative TLC, simple and reproducible character based on HPTLC and high performance liquid chromatography (Abdul Bakrudeen Ali Ahmed, *et. al*. 2009) [6]. The plant is popularly known as 'gurmar' for its distinctive property of temporarily destroying the taste of sweetness. The complex mixture of the active principles, named gymnemic acids were isolated from *Gymnema* leaves. Recently, the plant has been recognized by natural products industry in North America and Europe and a number of commercial, over-the-counter herbal products are now available that contain varying amounts of *Gymnema*. Hence, it is felt that there is a great need for cultivation of this important medicinal plant (Sairam Reddy *et. al* 2004) [7].

## Materials and Methods

**Sample:** The Whole plant sample was collected from GKVK, UAS campus, Bangalore, Karnataka, India.

### Plant Tissue Culture

### MS media preparation

Plant tissue was cultured in MS media containing macronutrients and micronutrients along with vitamins induces callus induction and hormones which help in the regeneration of root and shoot system in callus (table 1). Media was prepared by diluting 10x macronutrients solution in 100 ml distilled water and hormones in 1ml distilled water. Working media was prepared Adding 10ml macronutrients and 1ml micronutrients in plant tissue culture bottle and other heat stable constituent (e.g. sucrose, vitamins-pyridoxine-nicotinic acid, thiamine-HCL, Inositol, EDTA & $FeSO_4$). Made final volume of medium up to 100ml with distilled water followed by adjusted pH (5.6). Distributed the medium in each culture bottle contains 20ml. Added 0.16g agar in each bottle and

media was sterilized in autoclave on 121 ºC at 15lb pressure for 15min. after sterilization 2-10 µl hormones were added at laminar air flow chamber when media became cool and before it gets solidified. (Gamborg, Murashige *et al*. 1976) [8].

**Table 1:** MS media stock solution

| (Murashige and Skoog medium used for the culture of explant.) | |
|---|---|
| **Constituent's** | **(1X) mg/l** |
| **Macronutrients** | |
| $NH_4NO_3$ | 1650 |
| $KNO_3$ | 1900 |
| $CaCl_2.2H_2O$ | 440 |
| $MgSO_4.7H_2O$ | 370 |
| $KH_2PO_4$ | 170 |
| **Micronutrien** | |
| KI | 0.83 |
| $H_3BO_3$ | 6.2 |
| $MnSO_4.4H_2O$ | 22.3 |
| $ZnSO_4.7H_2O$ | 8.6 |
| $Na_2MoO_4.2H_2O$ | 0.25 |
| $CuSO_4.5H_2O$ | 0.025 |
| $CaCl_2.6H_2O$ | 0.025 |
| Fe-versenate(EDTA) | 43.00 |
| **Vitamins** | **(1X) mg/l** |
| Inositol | 100.0 |
| Nicotinic acid or niacin(vitamin $B_3$) | 0.5 |
| Pyridoxine-HCL | 0.1 |
| Thiamine-HCL | 0.1 |
| **Carbon source** | |
| Sucrose | 30.0g/l pH 5.6 |
| **Hormones** | **(1X)mg/l** |
| BAP | 1.0 |
| NAA | 2.5 |
| 2-4D | 0.5 |
| **Antibiotic stock.** | |
| Cefotoxamine | 200mg/l |

**Surface sterilization of plant sample**

Young leaf from the whole plant sample were collected and washed in running tap water for 2hrs after that leaves were washed in mild detergent for 10min (0.1% triton X100) Followed by washed twice with sterile distilled water for 10min. transfered the leaves in to LAF chamber wash the leaves in 0.05% mercuric chloride solution for 10min followed by twice wash with sterile distilled water. Dried the leaves sample on a sterile filter paper. (S. R. Bhat *et al*. 1995) [9].

**Inoculation of explant and maintenance**

Prepared the leaf disc from leaf sample of *Gymnema Sylvestre*. Leaf disk of *Gymnema* were prepared in laminar air flow chamber by using leaf disk method or sterilized forceps and blade. Transferred the leaf disc in MS media and sealed the bottles through cellophane tape. Kept the bottle in PTC room at 23 ºC for incubation. (Robert B *et al*., 1988) [10].

***Agrobacterium*- Mediated Transformation of *GUS* Gene in to Explant**

*Agrobacterium tumefaciense* LBA4404 containing plasmid pK1W1105 used for the transformation which carry *GUS* gene as a reporter gene got from the GKVK Bangalore, India. In the Vector map of Ti plasmid used in the transformation named pK1W1105 have 21.6Kb size which contains *GUS* reporter gene about 1.8 kb and antibiotics resistance gene.

**Preparation of LB Broth media for *Agrobacterium* culture**

LB broth medium was prepared by adding typtone 5.0g/l, yeast extract 3.0g/l, NaCl 5.0g/l, and Distilled $H_2O$ 1000ml followed by adjusted pH 7.0 and autoclaved the media. After sterilization inoculated the LB broth medium from stock *Agrobacterium* culture. LB broth medium kept for 24 hour incubation for culture of *Agrobacterium* and harvesting.

**Infection of *Agrobacterium tumefaciense* to the Callus**

Half strength MS Liquid medium prepared by using half amount of constituent. 1ml culture of *Agrobacterium* centrifuged in 6000rpm for 5min and 4 °C. Discarded the supernatant and pellet collected in tube. Added 1ml of half strength MS liquid medium in centrifuged tube and mixed well. Scratched the tip of callus through fine sterile needle. Added 1µl of culture in the tip of callus. Sealed the bottle through cellophane tape and kept in the dark condition for two days in the culture room at 23 ºC. (P. Sairam Reddy *et al*. 2004, Guowei Fang *et al*. 1990) [7, 11].

**Identification of *GUS* Gene by Bioassay and PCR**

**Identification of *GUS* gene by bioassay**

**Preparation of staining dye**

Prepare 50mM sodium phosphate buffer solution by adding of mono sodium phosphate and disodium phosphate. Prepared X-Gluc to the 1ml of DMSO solution. Mixed 1ml of DMSO solution to the 9ml of sodium phosphate buffer. Added 10µl of triton 20 and mixed well. After that added ferrocyanide and pinch of ampicillin. Transferred staining dye into centrifuge tubes. (Maureen M. M. Fitch *et al*. 1990, T. Kondo *et al*. 2000) [12, 13].

**Inoculation of callus to the staining dye**

Cut the callus through surgical blade in small pieces. Transferred the callus into centrifuge tubes. Kept all tubes in 37 °C for overnight in the incubator.

**Preparation of microscopic slide**

Callus was removed from the buffer solution followed by Transferred in another tube which contain methanol and kept for ½ hr in room temperature. Cleaned the slide and put drop of water and transferred callus on to the slide and through blade cut very thin section. Put cover slip on to the callus section. Observed in 10X through microscope. (Maureen M. M. Fitch *et al*. 1990, T. Kondo *et al*. 2000) [12, 13].

**Isolation of DNA and amplification of *GUS* gene by PCR**

**DNA Isolation**

SDS extraction buffer was prepared by adding 100mM tris-HCl, 25m M EDTA, 1.2% SDS, and $DH_2O$. DNA was isolated by SDS method 150 mg of *Gymnema* callaus were taken and crushed in pestle and mortar with the addition of 500µl of extraction buffer containing 0.4 to 2% mercaptoethanol and transfer it to 2ml of micro-centrifuge tubes. Incubated in water bath at 65 $^0$C for 20 minutes. 3M Sodium acetate was added followed by kept for ½ hr in ice cold condition. Centrifuged at 10000 rpm for 10 minutes at 4°C Supernatant was transferred to fresh micro-centrifuge tubes and to this added equal volume of ice cold ethanol and kept for 1hr in ice cold temperature. (Which precipitates DNA). Centrifuged at 12000 rpm for 12 minute at 4 $^0$C, to recover the DNA. Pellet was air dried and re-suspended in 50µl of TE buffer. 2µl of RNase A was added and incubate at 37 $^0$C for 30 minutes. Centrifuged at 12000 rpm for 12 minutes at room temperature. Pellet was air dried and then re-suspend it in 50µl 1X TE buffer (DNA isolated is stored at -20$^0$ for further use). 2µl of DNA sample obtained from the above step and 1µl of gel loading dye were mixed and loaded in 0.8% agarose gel. After the gel running it was

observed in UV transilluminator (gel doc). (E.M. Moller *et al*. 1992, Yun-Jiang Cheng *et al*. 2003) [15].

**_GUS_ gene amplification by PCR.**

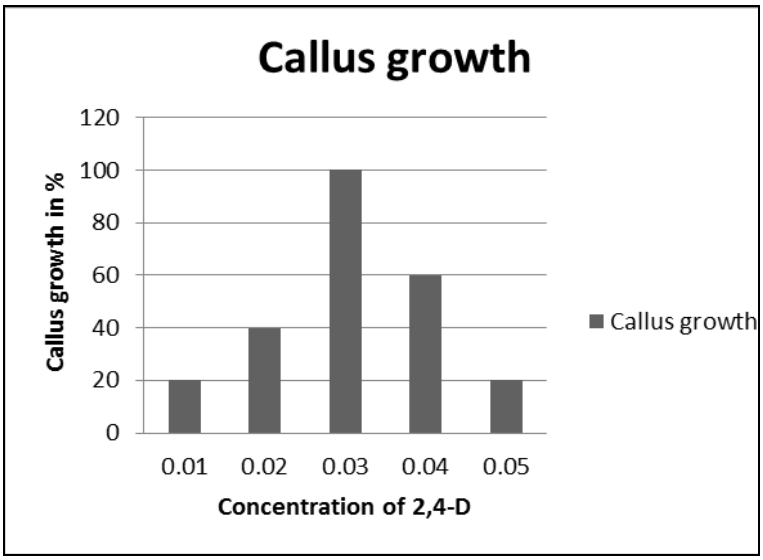
PCR of *GUS* gene was perform using reaction mix in PCR tube contains 12.5µl of 2.5µM 10X Taq buffer, 5.0 µl of 100mM DNTP, 2.0 µl of 3U/ µl Taq polymerase, 1 µl of 10 µM Far ward and reversed primers (Fwd 5`-AAC GGC AAG AAA AAG CAG TC-3` and Rev 5`-GAG CGT CGC AGA ACA TTA CA-3`), and nuclease free water (Genei, Bangalore.) and 2 µl DNA sample. The DNA amplification was subjected to 35 cycles of 5 minute at 94 °C and 1.5 minute at 53 °C and 72 °C. The PCR products were analyzed on a 1% agarose gel.

**Results**

*Invitro* tissue culture studies were carried out to achieve somatic callus induction and successful *agrobacterium* mediated transformation by scratch method in antidiabetic medicinal plant *Gymnema sylvestre*. The Results are documented and discussed in the following pages.

**Table 2:** Effect of different concentration of 2-4D on *in vitro* callus induction from explant of *Gymnema* on MS medium.

| S.No | Concentration of 2,4-D (mg/l) | No of explant inoculated (per bottle) | No of Callus induction (per bottle) | Frequency of Callus induction (%) |
|---|---|---|---|---|
| 1 | 0.01 | 5 | 1 | 20 |
| 2 | 0.02 | 5 | 2 | 40 |
| 3 | 0.03 | 5 | 5 | 100 |
| 4 | 0.04 | 5 | 3 | 60 |
| 5 | 0.05 | 5 | 1 | 20 |




**Graph 1:** Effect of different concentration of 2-4D on *in vitro* callus induction from explant of *Gymnema* on MS medium.

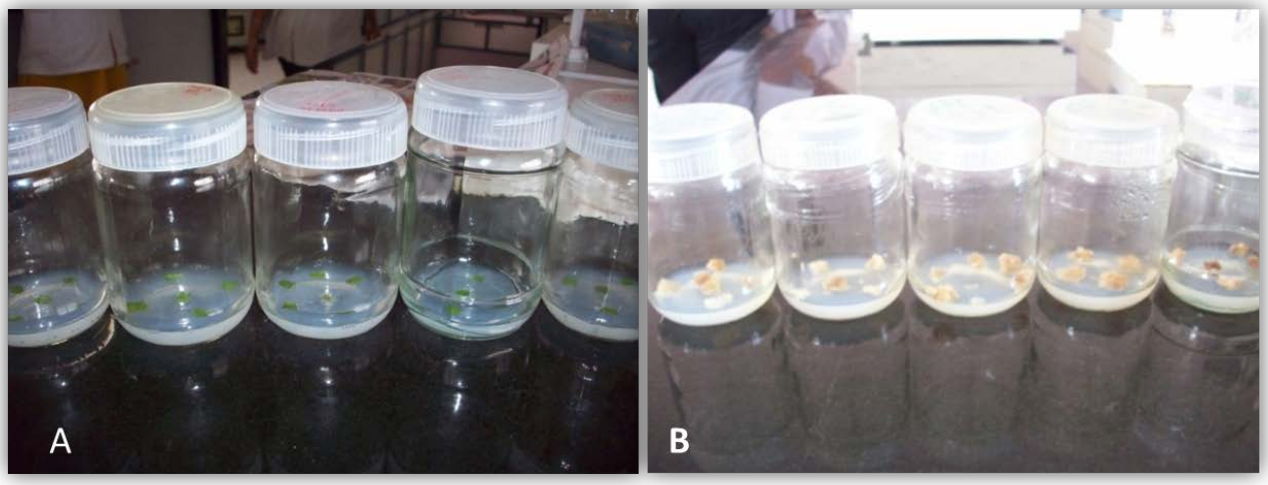



**Figure 2 Callus induction from leaves Sample**

Leaf samples were transferred in MS media containing different concentration of hormone(2-4D) . All explants were grown in MS media with in 15 days of incubation in tissue culture room.

**Callus induction from leaves**

The leaf samples were transferred in MS media containing different concentration of hormone (2-4D) all explants were grown in MS media and it shows maximum growth rate in 0.03mg/l concentration of 2-4D hormones. The results were discussed in detail in the following Table.

***Agrobacterium* mediated transformation of *Gymnema* callus**

The feasibility of any transformation method to deliver a foreign gene can be easily evaluated by studying the expression of any one of reporter gene. In this presence study agrobacterium tumefaciens carrying binary vector pK1W1105 carrying β-glucuronidase (*GUS*) gene was used as a reporter gene. For successful transformation the gene encoding β-glucuronidase has been the most widely used reporter gene for the analysis of plant gene expression in plant transformation system. In order to find out the gene transformation and analysis for *GUS* gene delivery followed by histochemical assay to detect the β-glucuronidase activity in the somatic callus was performed. For all *GUS* assay experiment explants were randomly selected and used for *GUS* staining to detect any visible blue stain. The presence of insoluble blue crystalline product identify the localized action of *GUS* activity after exposure to *GUS* buffer with histochemical substrate 5-bromo 4-chloro 3-indolyl β-glucopyranoside (X-gluc). Transformation was calculated as

$$\frac{\text{No of } GUS \text{ positive plant}}{\text{Total No of assayed}} \times 100$$

To further confirmed the presence of *GUS* product section of *GUS* positive explant were taken and examine under microscope. This infection is done by the scratch method and after infection *Agrobacterium* colonized on the callus.

**Identification of *GUS* gene by Bioassay**

Then *GUS* gene was transferred in callus by insertion of vector into the genome. Which is identified by expression of *GUS* gene protein. It is done by interaction of *GUS* gene protein into X-Gluc which after combination forms blue colour. After keeping callus in staining solution for 24hr it forms blue colour on surface of callus. Then after making slide of callus thin section it shows blue cells which has been transformed.

***GUS* expression analysis in somatic callus culture**

Out of 60 somatic callus pieces transformed with pK1W1105 in the independence experiment, 75% of them (45No.) were randomly selected and assayed for *GUS* expression analysis.

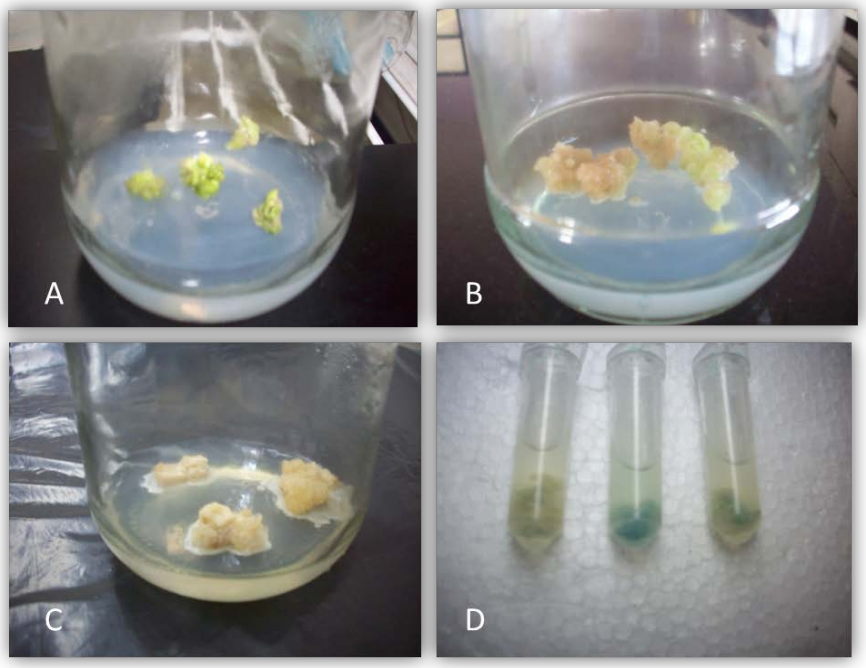



**Figure 3** **A)** Callus growth in first week of Incubation in tissue culture lab **B)** Maximum growth in of callus**,** it shows maximum growth rate in 0.03mg/l concentration of 2-4D hormones. **C)** Agrobacterium Mediated GUS gene transfection by scratch method. D) Confirmation of transferred GUS gene by bioassay using X-gluc substrate.

**Table 3:** *GUS* expression analysis in somatic callus culture

| | **Experiment 1** | **Experiment 2** | **Experiment 3** |
|---|---|---|---|
| **Explant used** | **Callus** | **Callus** | **Callus** |
| Total no of explant employed | 60 | 60 | 60 |
| No of explant taken for *GUS* assay | 50 | 45 | 47 |
| Transformation frequency | 83% | 75% | 78% |

**Histochemical analysis of *GUS* gene in callus**

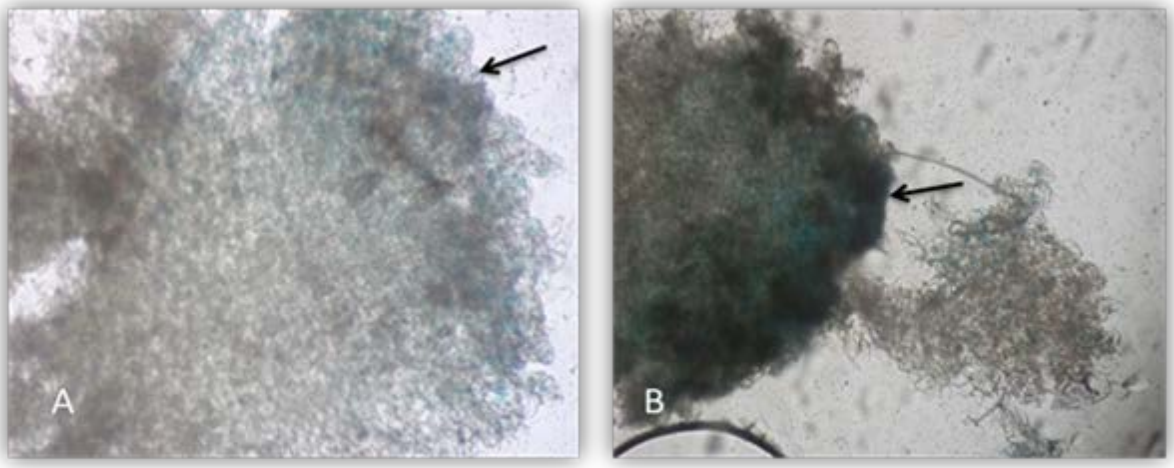



**Figure 4:-**Histochemical GUS expression analysis showing blue colour on somatic callus tissue of *Gymnema* after transformation with agrobacterium culture

**Identification of *GUS* gene by PCR**

**Isolation of DNA**

After successful transformation of agrobacterium with genomic DNA for Identification of *GUS* gene, genomic DNA was isolated from the transformed callus which is done by SDS method. After isolation agarose gel (0.8%) electrophoresis was done which showed DNA band on the gel.

**Amplification of *GUS* gene**

Identification of β-glucuronidase gene, amplification was done in the transformed callus in order to confirmed wheather reporter gene has been delivered successfully and stained with ethidium bromide solution in order to get the amplified visible bands of reporter gene and gel captured under UV illuminator.

After isolation and identification of the DNA, the *GUS* gene was amplified by PCR and then agarose gel (1%) electrophoresis was done which showed band of amplified *GUS* gene on the gel. which confirms the presence of *GUS* gene in the plasmid DNA isolated from the infected callus.

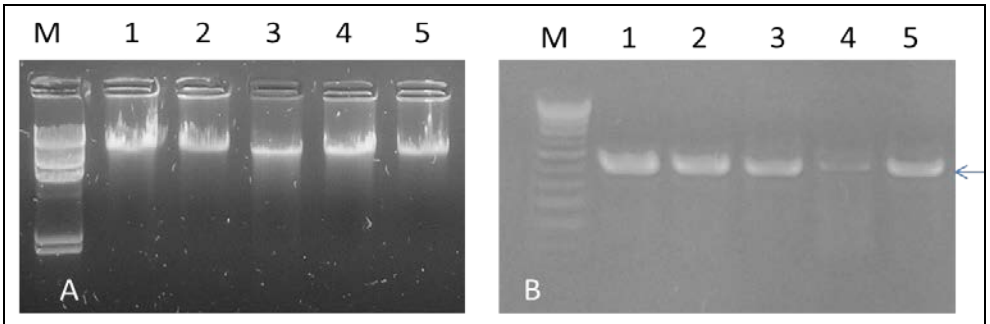



**Figure 5**: DNA Isolation and PCR of GUS gene **A)** genomic DNA was isolated from the transformed callus which is done by SDS method. After isolation agarose gel (0.8%) electrophoresis was done which showed DNA band on the gel. **B)** GUS gene was amplified by PCR and then agarose gel(1%) electrophoresis was done Which showed band of amplified GUS gene on the gel.

**Summary and Conclusion**

In this present study mature leaf explants of one of the widely used medicinal plant *gymnema* was used for in vitro callus induction and Agrobacterium mediated transformation experiment. As reported by other groups leaf explants for the callus. Induction was used which showed fast and high growth rate in the MS medium. Somatic callus induction period & concentration of 2, 4-D leaf explants was found to be best at 0.03mg/6µl. for 3 weeks. But in embryogenic callus induction was started only from 6-8 week (Ashok kumar, *et. al*., 2002).

The explant were employed for agrobacterium mediated transformation by scratch method. The agrobacterium tumefaciens strain LBA4404 has carrying Ti plasmid pK1W1105 was used for infection of callus.1µl of 24hr culture of agrobacterium was used for 30 sec. for the infection. Then after infection it was kept for 2 days in dark room for better growth of the culture and for best incubation.

Successful delivery of the *GUS* reporter gene was confirmed by 1mM X-gluc as histochemical substrate for staining of the callus which showed the blue stains after overnight incubation at 37 ºC.

DNA was isolated from the infected callus by using SDS method and then presence of *GUS* gene was reconfirmed by using *GUS* gene specific marker in PCR amplification technique. The bands which confirm the presence of *GUS* gene

in genomic DNA of infected callus mean that *GUS* gene was successfully transformed in the callus.

In conclusion, we have produced the reliable protocol for the in vitro callus induction followed by successful transformation of *GUS* reporter gene into the leaf callus. Further study focused on to produce the reproducible protocol for plant regeneration. To develop the recombinant protein which help to protect the plant from insect and disease.

**References**


1. Kinghorn AD, Compadre CM. Less common high potency sweeteners. In Alternative Sweeteners, Second Edition, Revised and Expanded, L O'Brien Nabors, Ed., New York, 1991. ISBN 0-8247-8475-8
2. Baskaran K, Kizar AB, Radha SK. Shanmugasundaram ER. Antidiabetic effect of a leaf extract from *Gymnema sylvestre* in non-insulin dependent diabetes mellitus patients. Journal of Ethnopharmacol. 1990; 30(3):295-300.
3. Murakami N, Murakami T, Kadoya M. New hypoglycemic constituents in Gymnemic acid from *Gymnema sylvestre*. Chem pharma bull, Tokyo 1996; 44:469-471.
4. Yoshikawa. Phytochemistry, 31, 237, Chem. Abst 1991, 114, 58873, 03562, 1989, 110,191267, 1992.
5. Flores-Riveros JR. Insulin down-regulates, expression of the insulin responsive glucose Transporter (GLUT4) gene. Effects on Transcription and Mrna Turnover 1993; 90:512-516.
6. Ahmed ABA, Rao AS, Rao MV. In Vitro Production of Gymnemic Acid from *Gymnema sylvestre* (Retz) R. Br. Ex Roemer and Schultes Through Callus Culture Under Abiotic Stress Conditions. Protocols For *In Vitro* Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, 2009, 93-105.
7. Reddy PS, Rama G, Sita GL. *In vitro* multiplication of *Gymnema sylvestre* R.Br. An important medicinal plant, Department of Microbiology and Cell Biology, article17, Indian Institute of Science, 2004.
8. Gamborg OL, Murashige T, Thorpe TA. Plant tissue culture media**,** *In Vitro* Cellular & Developmental Biology – Plant 1976; 12(7):473-478.
9. Bhat SR, Chandel KPS, Malik SK. Plant regeneration from various expiants of cultivated Piper species, Plant Cell Reports 1995; 14(6):398-402.
10. Robert BH, Joyce F, Nancy H, Jeanne N, Stephen GR, Robert TF. Leaf disc transformation**,** Plant Molecular Biology Manual 1988; A5 1-9:63-71.
11. Fang G, Grume R. *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants, Plant Cell Reports 1990, 9(3):160-164.
12. Maureen MMF, Richard MM, Dennis G, Slightom JL**,** Sanford JC. Stable transformation of papaya via microprojectile bombardment, Plant Cell Reports 1990; 9(4):189-194
13. Kondo T, Hasegawa H, Suzuki M. Transformation and regeneration of garlic (*Allium sativum* L.) by Agrobacterium-mediated gene transfer. Plant Cell Reports 2000; 19(10):989-993.
14. Moller EM, Bahnweg G, Sandermann H, Geiger HH. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues, Nucleic Acids Research 1992; 20(22):6115-6116.
15. Cheng y, Guo W, Yi H, Pang X, Deng X. An efficient protocol for genomic DNA extraction from Citrus species. Plant Molecular Biology Reporter 2003; 21(2):177-178.
16. Llamoca-Zárate RM, Campos AFP, Landsmann J. Establishment and Transformation of Callus and Cell Suspension Cultures of the Prickly-Pear (*Opuntia ficus-indica).*