D-5547
[1-6]

RESEARCH ARTICLE Agricultural Science Digest, Volume Issue : ()

An Attempt to Establish an *Agrobacterium*-mediated Transient Expression in *Euphorbia tirucalli* (L.) an Important Medicinal Plant

M. Rajagopal, C.M. Narendra Reddy, V.N. Swetha Prasuna, P.V. Chaithanya Lakshmi, B. Srinivas 10.18805/ag.D-5547

ABSTRACT

Background: Optimization of co-cultivation parameters during *Agrobacterium*–mediated transformation to *Euphorbia tirucalli* evaluated were bacterial density, infection period, acetosyringone (AS) concentration and co-cultivation temperature. Optimized parameters resulted in high transformation efficiency 3 fold increase at transient GUS expression .The optimized conditions were *Agrobacterium tumefaciens* growth phase of A_{600nm} 0.17, infection period of 30 min, addition of acetosyringone (AS) in co-cultivation medium (100 µM) and cocultivation temperature of 25°C.

Methods: Nodal explants were used for transformation**.** Bacterial culture was added to 50 ml of liquid YEP medium with kanamycin and rifampicin and grown until reaching the growth phase (A_{600nm}). Bacterial density ranged from A_{600nm} 0.17, 0.56, 0.8 and 1.2 OD were used in the present study. The co-cultivation medium made of solid MS medium consisted of NAA 2 mg L^{-1} and various concentrations of AS at 0, 50, 100, 200, 400,600 and 800 µM. Histochemical analysis of *gus* gene expression was carried out.

Result: Higher bacterial density resulted in more transformation efficiency, but also higher necrosis in the explants. Dilution of bacterial suspension reduced necrosis in explants and resulted in higher transformation. The transformation efficiency is 72% when the infection process was carried out with acetosyringone in co-cultivation medium (100 µM). Our studies proved that among the optimized conditions, cocultivation temperature and acetosyringone were the critical parameters during *Agrobacterium* mediated transformation.

Key words: *Agrobacterium tumefaciens, Euphoria tirucalli, gus* gene.

INTRODUCTION

Euphorbia tirucalli L., which is also known as a petroleum plant, produces a large amount of phytosterols and triterpenes. It is well known for microbicidal activity against human pathogens (Swapna *et al*., 2011). In East Africa, latex of *Euphorbia tirucalli* (L.) is used against sexual impotence, warts, epilepsy, toothache, hemorrhoids, snake bites, extraction of ecto-parasites and cough among others (Schmelzer and Gurib-Fakim 2008).

Agrobacterium based transformation has advantages in plant transformation due to its simplicity, precession, integration of large size DNA and stable gene expression (Bent 2000). Another advantage of this method is the wide host-range including major crops such as rice, maize, wheat, sugarcane, soybean and cotton. In some plants, it is more difficult to perform *Agrobacterium* mediated transformation (Kutty *et al.,* 2010).

The possibility of gene silencing is due to polyploidy in plant developmental processes (Birch and Bower 2010). Transgene silencing also associates with increasing endoploidy during maturation of differentiated tissues (Jefferson 1987). Reporter genes are commonly used to study the transgene expression. An increase in transient expression of reporter gene indicates an increase in the expression level of transgene. β-glucoronidase (GUS) reporter gene is useful to study the transient expression in plant transformation (Hood *et al.,* 1993). It is an advantage to use GUS reporter system since the expression of *gus* gene in transformed explants can be analysed by using GUS histochemical assay.

Department of Biotechnology, School of Herbal Studies and Naturo Sciences, Dravidian University, Kuppam-517 426, Andhra Pradesh, India.

Corresponding Author: B. Srinivas, Department of Biotechnology, School of Herbal Studies and Naturo Sciences, Dravidian University, Kuppam-517 426, Andhra Pradesh, India.
Email: bathulasrinivas71@gmail.com

How to cite this article: Rajagopal, M., Reddy, C.M.N., Prasuna, V.N.S., Lakshmi, P.V.C. and Srinivas, B. (2022). An Attempt to Establish an *Agrobacterium*-mediated Transient Expression in *Euphorbia tirucalli* (L.) an Important Medicinal Plant. Agricultural Science Digest. DOI: 10.18805/ag.D-5547.

Submitted: 30-12-2021 **Accepted:** 15-08-2022 **Online:** 29-08-2022

Optimizing parameters for transformation varies from various plant cultivars, species and even for *Agrobacterium* strains. Since the transformation efficiency varies based on type of plant, explant and bacterial strains, it is necessary to optimise the parameters. The genetic transformation of *Euphorbia tirucalli* by using *Agrobacterium tumefaciens* strain EHA 105 containing the plasmid vector pCAMBIA1301 is not reported so far.

MATERIALS AND METHODS

The experiments were conducted in the duration of 2010-11 and 2017 to 2018 at the department of Biotechnology, School

of Herbal Studies and Natuoro Sciences, Dravidian University, Kuppam, Andhra Pradesh.

Bacterial strain and vector

Agrobacterium tumefaciens strain EHA 105 containing the binary plasmid vector (pCAMBIA1301) was used as the vector system for transformation (Fig 1).

Bacterial growth conditions

Agrobacterium strain EHA 105 containing pCAMBIA1301 was grown in liquid YEP medium alongwith antibiotics kanamycin 10 mg L^{-1} and 100 mg L^{-1} rifampicin and incubated at 28°C for two days.

Plant material

The plant material of *Euphorbia tirucalli* (Fig 2) was collected from the Herbal garden, Dravidian University, Kuppam, Andhra Pradesh, India. Nodal explants were surface sterilized and inoculated on MS medium supplemented with NAA 2 mg L^{-1} .

Cocultivation

Bacterial density ranged from A_{600nm} 0.17, 0.56, 0.8 and 1.2 OD were used in the present study. A total volume of 15 ml of liquid culture was used to infect nodal explants of *Euphorbia tirucalli* explants from 2-3 weeks old seedlings. The explants were co-incubated with bacterial suspension separately at 25 and 28°C for a period of 15, 30 and 60 min.

Addition of acetosyringene (AS) in co-cultivation Medium

The explants were placed on co-cultivation medium for two days in the dark at 25°C. The co-cultivation medium made of solid MS medium consisted of NAA 2 mg L^{-1} and various concentrations of AS at 0, 50, 100, 200, 400, 600 and 800 µM. Co-cultivation medium without AS was used as control.

GUS histochemical assay

Histochemical analysis of *gus* gene expression was carried out according to Jefferson (1987).

Data analysis

Each treatment in this study consisted of three replicates and each replicate consisted of at least 25 explants. All the data were subjected to Analysis of variance (ANOVA) statistical test using SPSS software version 16.0. The means were compared for significant differences at $p<0.05$ level. All the experiments were repeated at least thrice.

RESULTS AND DISCUSSION

Agrobacterium-mediated transformation is effected by many physical and chemical factors. In the present study both physical and chemical parameters were evaluated by using *gus* as a reporter gene. The transient expression of *gus* gene can be easily measured from the transformed plant cells (Batra and Kumar (2003). Genetic transformation by *Agrobacterium tumefaciens* EHA-105 can be improved by evaluating physical and chemical factors which was resulted in developing an efficient method of gene transfer to several plants like tobacco (Kutty 2010), Sorghum (Indra Arulselvi *et al.,* 2010), Rice (Tripathi *et al.,* 2010).

Bacterial growth phase and infection period

The results of transformation using *Agrobacterium tumefaciens* strain EHA 105 at various bacterial concentrations ranged from A_{600nm} 0.17-1.2 and infection periods analysed at 15, 30 and 60 min were shown in Table 1. Of four different bacterial densities, the highest numbers of GUS-positive spots were found at A_{600nm} 0.17, 30 min (9.33 ± 0.13^{b}). The transient activity and transformation

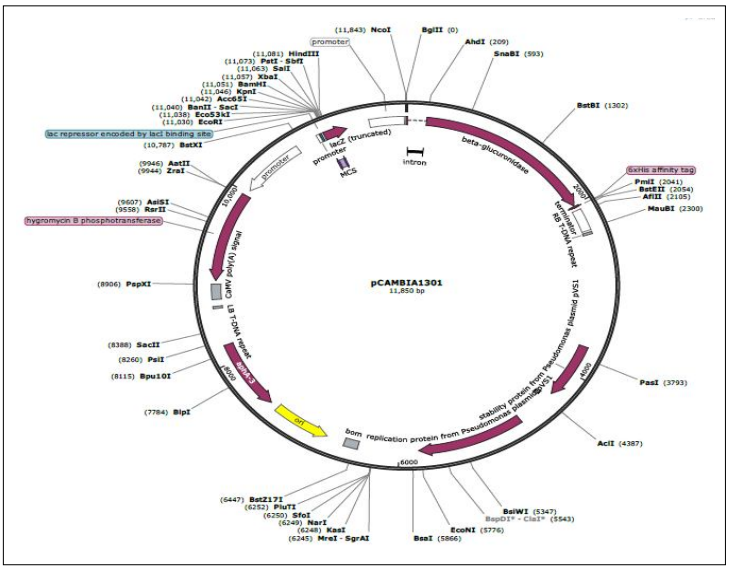

Fig 1: Restriction map of PCAMBIA1301 vector.
(Source:https://www.biocat.com/lp/vectors/maps/201804/pCAMBIA1301%20Map.pdf).

frequency were decreased with increased bacterial concentration and infection period.

The percentage of GUS positive explants from bacterial density at A_{600nm} 0.17 to 0.56 cultures were increased 28.33% and decreased at A_{600nm} 1.2 cultures. Transformation frequency decreases as necrotic damage increases from lower bacterial density (A_{600nm} 0.17) to higher density (A_{600nm} 1.2). This indicates that explants viability has direct effect on transformation efficiency.

Higher concentrations of *Agrobacterium* were used to transform recalcitrant plants such as rice (Chan *et al.,* 1992), Sweet potato (Gonzalez *et al.,* 2008) and pepper (Ismail *et al.,* 2006). Low bacterial density was also used to transform plants such as in Brocolli (Metz *et al* 1995), Wheat (Cheng *et al* 1997) and tobacco (Kutty PC 2010). But in our study early log phase was found to be the best for transforming *Euphorbia tirucalli* L. Bacterial density at A_{600nm} 0.17 growth phase was found to be most effective in producing high transformation efficiency. In order to minimise the necrotic damage of explants, culture was diluted to 1:10 before infection of explants. Similar approach was reported (Chakrabarty *et al.,* 2002), in which bacterial inoculums was diluted before infection. The transformation efficiency was found at A_{600nm} 0.56, 0.8 and their mean differences were statistically insignificant. Necrotic damage was absent in bacterial density at A_{600nm} 0.17 and infection period 30 minutes and it was chosen in this study.

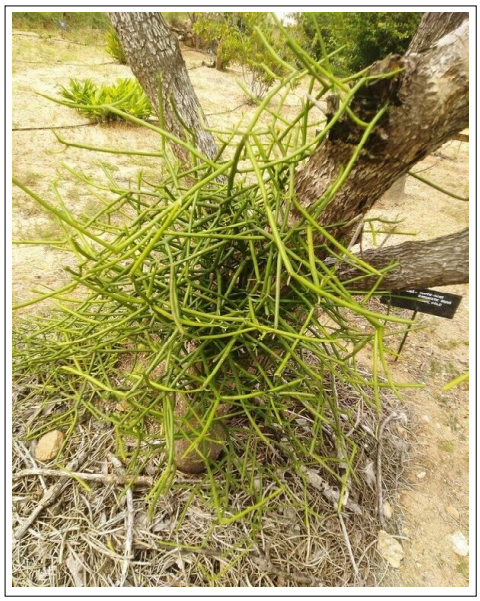

Fig 2: Morphology of *Euphorbia tirucalli.*

Co-cultivation temperature

The effects of different co-cultivation temperatures on transient GUS expression were studied and the result were shown in Table 2. The explants which were precultured for two days followed by co-cultivated at three different temperatures like 22, 25 and 28°C and the highest GUS positive spots were found at 25 and 28°C with the mean value 42 (±05.17[a]) and 39 (±04.13[a]) respectively. There were no visible spots were observed at 22°C. This indicates that co-cultivation temperature has direct effect on *Agro bacterium*-mediated transformation. The mean differences of GUS positive spots at 25 and 28°C were statistically insignificant.

The percentage of GUS positive explants were found to be approximately 40% at both temperature 25 and 28°C (Table 2). After analysing with trinocular microscope, 42 spots were found per explant incubated at 25 and 28°C. There were several reports on higher transformation efficiency where explants Co-cultivated at 22°C and obtained more number of GUS – positive spots (Dillen *et al.,* 1997) (Kutty 2010). Efficient DNA delivery into plant cells at 22 °C was also reported in Sweet potato (Gonzalez *et al.,* 2008), Cotton (Sunil kumar and Rathore 2001) and cauliflower (Chakrabarty *et al.,* 2002). It was reported in the earlier studies that size of crown gall tumour decreased when Co-cultivation temperature was increased (Braun (1947). Another study found that Ti- Plasmids were lost in

Table 1: The effects of bacterial densities and infection periods on transient GUS expression and necrotic damage of the explants.

Bacterial density (A_{600nm})	Infection period (min)	No. of spots (mean±SE)	Percentage of GUS positive explants	Necrotic explants (mean±SE)	Percentage of necrotic explants
0.17	15	0.00±0.00[a]	00.00	0.00±0.00[a]	0.00
	30	9.33±0.13[c]	5.16	0.00±0.00[a]	0.00
	60	0.34±0.22[b]	10.60	1.33±0.33[b]	5.32
0.56	15	1.06±0.11[b]	04.10	1.33±0.33[b]	5.32
	30	2.97±0.11[b]	10.93	0.66 ±0.33[b]	2.64
	60	2.26±0.10[b]	28.33	3.00±0.81[c]	12.0
0.8	15	3.22±0.08[b]	12.33	11. 33±1.45[c]	45.32
	30	3.25±0.05[b]	16.66	12.33±1.45[c]	49.32
	60	2.76±0.08[b]	11.33	15.33±0.88[c]	61.32
1.12	15	1.10±0.18[b]	08.92	14.66±0.88[b]	58.64
	30	0.96±0.14[b]	15.33	20.00±1.15[c]	80.0
	60	0.92±0.14[b]	14.30	21.66±1.20[c]	86.64

Data within the same column followed by the same letter indicated no significance at 5% level.

Agrobacterium tumefaciens when the culture was grown over 36 hours at elevated temperatures (Watson B1975). Low temperatures from 20 to 22°C were found to be promoting pilus assembly and it was influenced by *VirB* gene at low temperature which is required for conjugal transfer of TDNA into plant cells (Fullner *et al.,* 1996).

Table 2: The effects of various co-cultivation temperatures on GUS - positive spots in *Agrobacterium* mediated transformation of *Euphorbia tirucalli* explants.

Co-cultivation temperature (°C)	No. of spots (Mean±SE)	GUS positive explants (%)
22	0.00±0.00[a]	0.00
25	42±05.17[b]	40
28	39±04.13[b]	40

Data within the same column followed by the same letter indicated no significance at 5% level.

Table 3: AS in co-cultivation medium and its effect on transient GUS expression.

Concentration of acetosyringone (µM)	Co-cultivation medium	
	No. of spots (Mean±SE)	GUS positive explants (%)
0	48.33±03.17[a]	38.66
50	59.00±2.49[b]	44
100	163.33±4.36[c]	72
200	62.66±07.13[b]	56
400	60.00±02.44[b]	53.33
600	61.00±02.94[b]	42.66
800	62.33±05.73[b]	41.33

Data within the same column followed by the same letter indicated no significance at 5% level.

Based on the results obtained, Co-cultivation temperature either at 25 or 28°C was found to be equally effective to get more number of GUS-positive spots as well as more number of GUS-positive explants. Hence we have chosen 25°C as co-cultivation temperature and it was introduced in further experiments. We have chosen 25 instead of 28°C since low temperatures are favourable for efficient T-DNA transfer (Salas *et al.,* 2001) compare to high temperatures.

Acetosyringone in cocultivation medium

The effect of Acetosyringone (AS) on *Agrobacterium*-mediated transformation was studied using six different con centrations from 50-800 µM. These different concentrations were added seperately to the Cocultivation medium and results were shown in Table 3. Of six different concentrations of AS 100 µM concentration gave highest number of GUS positive spots with the mean value (163.33±44.36[c]). The lowest numbers of GUS positive spots were found at AS concentration from 200-800 µM. The mean differences between 100 µM and rest of the AS concentrations were statistically significant. The positive explants at 25°C were 40% without AS (Fig 3C) and increased to 72% after the addition of AS 100 µM (Fig 3D). The number of GUS-positive spots were counted using Trinocular microscope and one of such explant with microscopic view was shown in (Fig 3E).

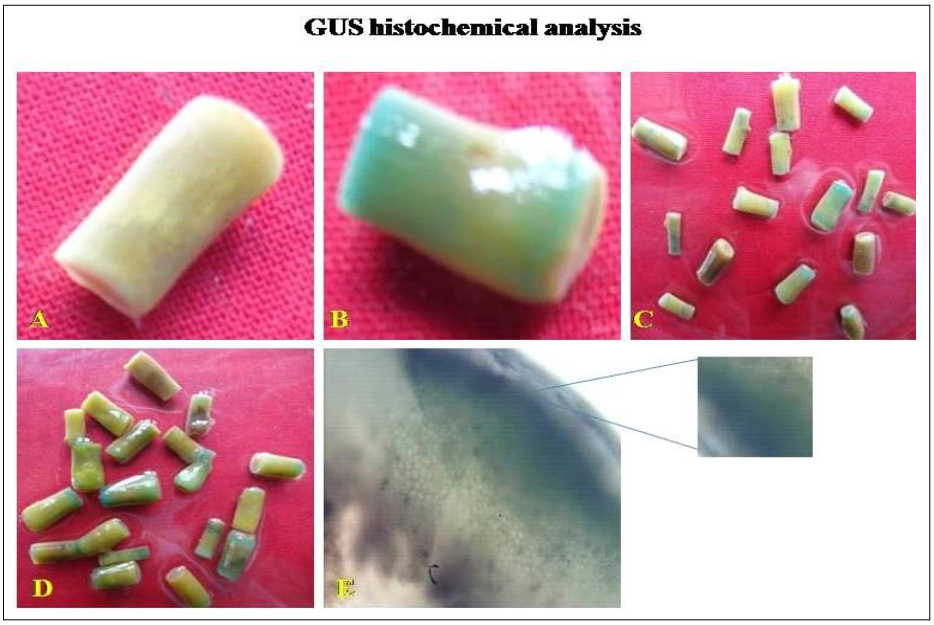

Fig 3: GUS histochemical analysis of *Euphorbia tirucalli L.* explants transformed by *Agrobacterium tumefaciens*. (A) Control (B) GUS positive explant after optimization with cocultivation temperature and AS (C) The explants co-cultivated at 25°C and the number of GUS-positive explants obtained was 15 per 25 explants. (D) The explants co-cultivated at 25°C, AS 100 µM and the number of GUS-positive explants obtained were 18 per 25 explants (E) A close up image of Microscopic view of blue spots of GUS-positive explant.

There was no increase in the number of GUS positive explants after adding AS into the bacterial inoculums (data was not shown). Other concentrations of AS were not shown any increase in the transient GUS expression when compared with AS concentrations at 50 and 200-800 µM. It reveals that *Agrobacterium* cells may have been induced to maximum towards virulent stages at 100µM concentration of AS. The same concentration was also reported in other plants of *Euphorbiaceae* family (Li *et al.,* 2007; Kumar *et al.,* 2010). The optimum concentration of AS for higher transient expression varies based on genotype and cultivar of plant (Gonzalez *et al.,* 2008).

GUS histo chemical analysis

The method of Jefferson (1987) was used for a GUS histochemical assay. The positive results were observed with naked eye and each blue spot was counted using stereo microscope, irrespective of its size (Fig 3). The explants co-cultivated at 25°C and the number of GUS-positive explants obtained was 15 per 25 explants (Fig 3C). Whereas the explants co-cultivated at 25°C and 100 µM AS resulted GUS-positive explants were 18 per 25 explants (Fig 3D). An individual explant of Control (Fig 3A) and GUS positive explant (Fig 3B) were shown with magnification. A close-up image of microscopic view of GUS-positive explants with blue spots were shown (Fig 3E).

pre-cultured explants at room temperature for 2 days showed low transient expression when compared with explants without pre-culture. Increasing the number of days from 2 to 8 days did not show any increased transient GUS expression (data not shown). Similar finding was reported (Cervera M, *et al.,* 1998), where pre-cultured explants of citrus showed decreased transient GUS-expression when compared with explants without pre-culture.

CONCLUSION

An easy and efficient method for *Agrobacterium* -mediated transformation was optimized by evaluating various important parameters. The evaluated parameters were,1:10 dilution of A_{600nm} 0.17 bacterial density, infection period 30 min, addition of AS into cocultivation medium at 100 µM, cocultivation temperature of 25°C and transformation efficiency is increased to 3 fold. Eventually this optimized protocol helps in metabolic engineering of *E.tirucalli* to produce more amount of end product that may be involved in an anti-diabetic activity, anti-inflammatory, toothache, hemorrhoids, snake bites.

ACKNOWLEDGEMENT

The principal investigator Dr. B. Srinivas is grateful for the financial support of Major Research Project (F.No.37- 489/ 2009 (SR) dated 21-12-2009) from UGC, New Delhi, India and thankful to UGC-NonSAP for providing facilities for research experiments. Also thankful to Dr. S.M. Bala chanadran, principal scientist (Biotechnology), Directorate of Rice Research, Hyderabad, India for providing *Agrobacterium tumefaciens* strain EHA 105 containing the pCAMBIA 1301 vector.

Conflict of interest: None.

REFERENCES

Batra, S., Kumar, S. (2003). Agrobacterium-mediated transient GUS gene expression in buffel grass (*Cenchrus ciliaris* L.). Journal of Applied Genetics. 44(4): 449-58.

Bent, A.F. (2000). Arabidopsis in planta transformation. Uses, mechanisms and prospects for transformation of other species. Plant physiology. 124(4): 1540-7.

Birch, R.G., Bower, R.S. (2010). Elliott AR. Highly efficient, 52-sequence-specific transgene silencing in a complex polyploid. Tropical Plant Biology. 3(2): 88-97.

Braun, A.C. (1947). Thermal studies on the factors responsible for tumor initiation in crown gall. American Journal of Botany. 1: 234-40.

Cervera, M., Pina, J.A., Juárez, J., Navarro, L., Pena, L. (1998). Agrobacterium-mediated transformation of citrange: factors affecting transformation and regeneration. Plant Cell Reports.18(3): 271-8.

Chakrabarty, R., Viswakarma, N., Bhat, S.R., Kirti, P.B., Singh, B.D., Chopra, V.L. (2002). Agrobacterium-mediated transformation of cauliflower: Optimization of protocol and development of Bt-transgenic cauliflower. Journal of Biosciences. 27(5): 495-502.

Chan, M.T., Lee, T.M., Chang, H.H. (1992). Transformation of indica rice (*Oryza sativa* L.) mediated by Agrobacterium tumefaciens. Plant and Cell Physiology. 33(5): 577-83.

Cheng, M., Fry, J.E., Pang, S., Zhou, H., Hironaka, C.M., Duncan, D.R., Conner, T.W., Wan, Y. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiology. 1, 115(3): 971-80.

Dillen, W., Clercq, J.D., Kapila, J., Zambre, M., Montagu, M.V., Angenon, G. (1997). The effect of temperature on Agrobacterium tumefaciens mediated gene transfer to plants. The Plant Journal. 12(6): 1459-63.

Fullner, K.J., Lara, J.C. (1996). Nester EW. Pilus assembly by Agrobacterium T-DNA transfer genes. Science. 273(5278): 1107-9.

González, R.G., Sánchez, D.S., Guerra, Z.Z., Quesada, A.L., Valdivia, R.M., Arencibia, A.D., Bravo, K.Q., Caligari, P.D. (2008). Efficient regeneration and Agrobacterium tumefaciens mediated transformation of recalcitrant sweet potato (*Ipomoea batatas* L.) cultivars. Asia Pacific Journal of Molecular Biology and Biotechnology. 16(2): 25-33.

Hood, E.E., Gelvin, S.B., Melchers, L.S., Hoekema, A. (1993). New Agrobacterium helper plasmids for gene transfer to plants. Transgenic Research. 2(4): 208-18.

Indra, Arulselvi, P., Michael, P., Umamaheswari, S., Krishnaveni, S. (2010). Agrobacterium mediated transformation of Sorghum bicolor for disease resistance. International Journal of Pharma and Bio Sciences 1(4): pp.B-281 ref.20. http://www.ijpbs.net/issue-4/Bio-29.pdf.

Ismail, I. Sophia, K., Zhu, H., Zamri, Z., Marzuki, S.N., Hisham, Z.R.S. (2006). T-DNA transfer and Gus expression in Agrobacterium-mediated transformation of C. annum under a Range of in vitro culture conditions. Biotechnology. 5: 257-67.

Jefferson, A.R. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387-405.

Kumar, N., Anand, K.V., Pamidimarri, D.S., Sarkar, T., Reddy, M.P., Radhakrishnan, T., Kaul, T., Reddy, M.K., Sopori, S.K. (2010). Stable genetic transformation of Jatropha curcas *via* Agrobacterium tumefaciens-mediated gene transfer using leaf explants. Industrial Crops and Products. Jul 1, 32(1): 41-7.

Kutty, P.C., Parveez, G.K., Huyop, F. (2010). An easy method for Agrobacterium tumefaciens-mediated gene transfer to Nicotiana tabacum cv. TAPM26. Journal of Biological Sciences.10(6): 480-9.

Li, K., Yang, W.Y., Li, L., Zhang, C.H., Cui, Y.Z., Sun, Y.Y. (2007). Distribution and development strategy for *Jatropha curcas* L. in Yunnan Province, Southwest China. Forestry studies in China. 9(2): 120-6.

Metz, T.D., Dixit, R., Earle, E.D. (1995). Agrobacterium tumefaciens-mediated transformation of broccoli (*Brassica oleracea* var. italica) and cabbage (*B. oleracea* var. capitata). Plant Cell Reports. 15(3): 287-92.

Salas, M., Park, S., Srivatanakul, M., Smith, R. (2001). Temperature influence on stable T-DNA integration in plant cells. Plant Cell Reports. 20(8): 701-5.

Schmelzer, G.H. and Gurib-Fakim, A. (2008). Medicinal plants. In: Plant Resources of Tropical Africa, Backhuys CTA, Wageningen, The Netherlands: Prota Foundation, Pp. 412-415.

Sunilkumar, G., Rathore, K.S. (2001). Transgenic cotton: Factors influencing Agrobacterium-mediated transformation and regeneration. Molecular Breeding. 8(1): 37-52.

Swapna, N.L., Prasad, M.A., Prasad, S.H. (2011). Efficacy of [*Euphorbia tirucalli* (L.)] towards microbicidal activity against human pathogens. International Journal of Pharmacy and Biological Sciences. 2: 12-8.

Tripathi, R.M., Bisht, H.S. and Singh, R.P. (2010). Effect of Acetosyringone and callus age on transformation for *Scutellum* -Derived callus of rice. International Journal of Pharma and Bio Sciences. 1(4): 163-170.

Watson, B., Currier, T.C., Gordon, M.P., Chilton, M.D., Nester, E.W. (1975). Plasmid required for virulence of Agrobacterium tumefaciens. Journal of Bacteriology. 123(1): 255-64.