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FACTORS INFLUENCING *AGROBACTERIUM*-MEDIATED TRANSIENT EXPRESSION OF *uidA* IN *CASSIA OCCIDENTALIS* LINN EXPLANTS

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ABSTRACT

Optimization of co-cultivation parameters during *Agrobacterium*–mediated transformation to *Cassia occidentalis* (Linn) evaluated were bacterial density, infection period, wounding and pre-culture of explants, acetosyringone (AS) concentration and co-cultivation temperature. Some of the parameters optimized and resulted in high transformation efficiency were as follows; *Agrobacterium tumefaciens* growth phase of A_{600nm} 0.17, infection period of 30 min, addition of acetosyringone (AS) in co-cultivation medium (400µM) and in bacterial growth culture. 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 at co-cultivation temperature 25^{o}C was 193 GUS-positive spots per explant and it was increased 6 fold when the infection process was carried out with acetosyringone in co-cultivation medium (400µM) and in bacterial culture (50µM).Our studies proved that among the optimized conditions; the concentration of acetosyringone was the critical parameter during co-cultivation process in *Agrobacterium* mediated transformation.

KEY WORDS: ***Agrobacterium tumefaciens, Cassia occidentalis, gus*** **gene.**

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INTRODUCTION

Cassia occidentalis L called as Kasmard in Sanskrit, Kasondi in Hindi and Coffee Senna in English belongs to family *Caesalpiniaceae*. It is distributed throughout India and in most tropical countries. It is an ayurvedic plant with huge medicinal importance. Leaves of *C. occidentalis* plant have ethnomedicinal importance like paste of leaves is externally applied on healing wounds, sores, itch, cutaneous diseases, bone fracture, fever, ringworm, skin diseases and throat infection. A previous pharmacological investigation shows that *C. occidentalis* leaf extracts have several activities of antibacterial[1,2], anti-malarial[3],antimutagenic[4,5],antiplasmodial[6],anticarcinogenic[5],hepatoprotective[7], anti-anxiety, anti-depressant[8], and anti-diabetic [9].Earlier workers have revealed various constituents from this plant[10,11,12].Metabolic engineering will give an opportunity to enhance the desired product of medicinal plants and such manipulations leads to the accumulation of isoflavonoid in Alfalfa[13].Genetic transformation is prerequisite to identify the genes responsible for the medicinal property by using genetic manipulations. To our knowledge, genetic transformation of *Cassia occidentalis* is not yet reported. *Agrobacterium* based transformation has advantages in plant transformation due to its simplicity, precession, integration of large size DNA and stable gene expression[14]. 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[15]. The difficulties in the transformation can be overcome by optimizing physical and chemical conditions which can lead to increase in the transformation efficiency[16]. In some of the plants the integration of foreign gene along with T-DNA may not give stable expression due to the phenomena of gene silencing[17,18]. This phenomenon is to protect against viruses attack and transposons[19]. Another possibility of gene silencing is due to polyploidy in plant developmental processes[20]. Transgene silencing also associates with increasing endoploidy during maturation of differentiated tissues [21].

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 [22]. 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 and it helps in developing an efficient protocol. In this paper, we describe an optimization of *Agrobacterium* mediated transformation to *Cassia occidentalis* L. The co-cultivation conditions tested are *Agrobacterium tumefaciens* growth phase, infection period, wounding of the explants, pre-culture of explants prior to infection, addition of acetosyringone (AS) in co-cultivation medium and in bacterial inoculum and co-cultivation temperature. 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.

MATERIALS AND METHODS

Bacterial strain and vector: *Agrobacterium tumefaciens* EHA 105 [23]containing the binary plasmid vector (pCAMBIA1301) was used as the vector system for transformation (Fig 1). Bacterial stock culture of *Agrobacterium tumefaciens* strain EHA 105 harbouring pCAMBIA1301 was kindly provided by Dr. S.M. Balachandran, principal scientist (Biotechnology), Directorate of Rice Research, Hyderabad, India. This vector was in the size of 11.850 kb and antibiotic selection markers were rifampicin for bacteria and hygromycin for plants. This vector also has *E.coli gus A*

reporter gene as a visual marker in transformed plant tissues.

(i) Bacterial growth conditions

Agrobacterium strain EHA 105 containing pCAMBIA1301 was grown in liquid YEP medium as described by [24] with following composition, 10g yeast extract, 10 g peptone and 5g $NaClL^{-1}$. All the chemicals used in this study were purchased from Hi media, India unless otherwise specified. The medium also has $10mgL^{-1}$ rifampicin and 100 mg L^{-1} kanamycin. Bacto agar (1.5% w/v) was used for solid medium. The pH of the medium was adjusted to 7.0 and autoclaved. The culture was incubated at 28°C for two days.

(ii) Plant material

Seeds of *Cassia occidentalis* L were collected from field trips conducted in various parts of Andhra Pradesh and Karnataka states. Seeds were surface sterilized, washed with tap water for several times and soaked the seeds in Tween-20(1%) for 5 minutes. These seeds were washed with running tap water for 3 – 4 times. Seeds were immersed in 70% Ethanol for 1-2 min and washed with sterile water for several times. They were immersed in 0.1% $HgCl_2$ for 30 to 60 seconds and washed with sterile distilled water for several times. Sterilized seeds of 5-6 were placed on sterile filter paper in culture test tubes with sterile tap water for the development of 2-3 week old seedlings. Test tubes with 5 seeds per tube were incubated in tissue culture chamber at 18°Cunder a16/8 hour's light/dark photoperiod with light intensity of 12.16µmol/m^2/sec. After an incubation period of 2-3 weeks, young and green leaves were harvested from in vitro grown seedlings and they were washed thoroughly with sterile distilled water for several times before used as explants for all transformation experiments.

(iii)Preparation of Bacterial inoculums

Agrobacterium tumefaciens strain EHA 105 was streaked onto solid YEP medium containing $10mgL^{-1}$ rifampicin and 100mg L^{-1} kanamycin and incubated at 28°C for 2 days. A single colony was inoculated into 2ml of YEP broth and grown for overnight at 28°C with 200rpm. This culture was added to 50 ml of liquid YEP medium with kanamycin and rifampicin and grown until reaching desired growth phase (A_{600nm}).Bacterial culture of 1.5 ml from this medium was harvested by centrifugation at 5000g for 5 min and pellet was resuspended in 15 ml of MS liquid medium. This dilution was done to avoid the loss of viability of explants during transformation.

(iv) The effect of bacterial growth phase and infection period

In order to evaluate the effects of *Agrobacterium tumefaciens* growth phase and Bacterial density ranged from A_{600nm} 0.17, 0.49, 0.97 and 1.4 OD were used in the present study. A total volume of 15 ml of liquid culture was used to infect *Cassia occidentalis* leaf explants from 2-3 weeks old seedlings. The explants were co-incubated with bacterial suspension at 25°C. The infection period was carried out at same temperature of the above and allowed to last for 15, 30 and 60min separately in order to evaluate the effects of different infection periods on the transformation process. After the infection process, explants were recovered from infection medium and blotted on sterile filter paper to remove excess bacterial suspension. The explants were then placed on co-cultivation medium (MS-agar medium with BAP 2mg L^{-1} and NAA 0.5mg L^{-1}) and maintained in a tissue culture chamber at 25°C in the dark for two days. After incubation the explants were gently rinsed using sterile distilled water to remove excess agar followed by GUS histochemical analysis. The most effective treatments/ parameters were used in subsequent evaluation experiments.

(v) The effect of wounding procedure

The effects of wounding the explants on transformation efficiency were evaluated by comparing the wounded explants with unwounded explants. The wounding procedure involved cutting the margins of the leaves without removing the petiole and making small pricks (~6 to 10 times) on the surface of the leaves using sterile head pin. Unwounded leaves were used as control explants. The

wounded and unwounded explants were co-incubated with *Agrobacterium tumefaciens* inoculum from A_{600nm} 0.17culture, over 30 min exposure and co-cultivated for two days in dark at 25°C. The co-cultivation period from 4-10 days did not show any positive effect on transformation efficiency. GUS histochemical analysis was carried out after every optimization of parameters and included the best parameter in further experiments.

(vi) The effect of pre-culture

Pre-culture process consisted of incubating wounded leaf explants in MS medium for 2 days [25] at room temperature before the infection process, whereas leaves without pre-culture were used as explants in control experiments.
The GUS histochemical analysis was performed after co-incubation with bacterial culture, control explants and pre-cultured explants for two days to find out the best optimized condition.

(vii) The effect of acetosyringone

Two separate experiments were conducted to evaluate the effects of adding acetosyringone (AS) in co-cultivation medium and in bacterial inoculum on transformation efficiency.

(viii) The effect of AS in co-cultivation medium

The explants were placed on co-cultivation medium for two days in the dark at 25°C. The solid co-cultivation medium consists of MS medium supplemented with 2mg L^{-1} BAP, 0.5mg L^{-1} NAA and various concentrations of AS at 0, 50, 100, 200, 400, 600 and 800µM. Co-cultivation medium without AS was used as control. GUS histochemical analysis was done to determine the effects of AS on transformation efficiency.

(ix) The effect of AS in bacterial inoculum

Overnight culture 2ml of *Agrobacterium tumefaciens* strain EHA 105 was used to inoculate 50ml of YEP medium supplemented with 100mg L^{-1} kanamycin and 10mg L^{-1} rifampicin and various concentrations of AS at 0, 50,100,200,400, 600 and 800µM. Cultures were grown at 28°C until it reaches desired bacterial density. Cells of 1.5ml were pelleted by centrifugation and were resuspended in 15ml of MS liquid medium. Then, 5ml of the suspension was later used for the infection of leaf explants. After co-cultivation period, the explants were subjected to GUS histochemical analysis to determine the effect of AS on transformation efficiency.

(x) The effect of co-cultivation temperatures

The infected leaves were co-cultivated in the dark at different co-cultivation temperatures of 22, 25 and 28°C. The most effective co-cultivation temperatures were determined by using GUS histochemical analysis.

(xi) GUS histochemical analysis

Histochemical analysis of *gus* gene expression was carried out according to [22, 26]. Explants were incubated overnight at 37°C in 100mM sodium phosphate buffer (pH 7.0) containing 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide, 10mM Na_2 EDTA, 0.5% (v/v) Triton X-100 and 0.5g L^{-1} 5-bromo-4 chloro-3 indolyl β-D-glucuronide (X-gluc). After incubation, the explants were cleared and fixed in 95% (v/v) ethanol + 1% (v/v) acetic acid. Each blue spot indicating GUS activity was counted using trinocular microscope (Model microscope BM 2000, China), irrespective of its size.

(xii) Data analysis

Each treatment in this study consisted of three replicates and each replicate consisted of at least 8 explants. All data were subjected to Analysis of Variance (ANOVA) statistical test using SPSS software version 11.5.The means were compared for significant differences at $p<0.05$ level. All experiments were repeated at least thrice.

RESULTS AND DISCUSSION

(i) 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 in all three infection periods of 15, 30 and 60min at A_{600nm} 0.97 culture with the mean values (1.40±0.20), (1.20±0.10) and (1.16±0.15) respectively. The lowest GUS activity found at A_{600nm}1.4 culture with the infection periods of 15, 30 60min with the mean values (0.60±0.20), and (0.60±0.25) and (0.43±0.15) respectively. The number of GUS-positive spots from low bacterial concentration (A600nm0.17) to higher bacterial concentration (A600nm0.97) was same and their statistical difference is insignificant. The transient activity and transformation 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 0.97 cultures were increased and decreased at A_{600nm}1.4 culture. Transformation frequency decreases as necrotic damage increases from lower bacterial density (A_{600nm} 0.17, 60min) to higher density (A_{600nm} 1.4). This indicates that explant viability has direct effect on transformation efficiency. Bacterial concentration A600nm 0.17 was chosen in spite of having a low transformation frequency when compared with other higher bacterial densities, since it has no necrotic damage and it was used in further experiments. *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 [27, 28]. 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 ripe fleshy fruit [29], tobacco [30], apple [31], pear [32] and [33]. Present study focussed on the physical and chemical factors like bacterial density, Co-cultivation temperature, Acetosyringone, Pre-culture and Wounding. There are many reports on growth phase of *Agrobacterium* which can affect the plant transformation. Apart from growth phase, different plant materials (leaf, internodes etc.), different strains of *Agrobacterium* and type of plant cultivars could influence the transformation of plants [34, 35]. Higher concentrations of *Agrobacerium* were used to transform recalcitrant plants such as rice [36], Sweet potato [37], and pepper [38]. Low bacterial density was also used to transform plants such as in Brocolli [39], Wheat [40] and tobacco [16].But in our study early log phase was found to be the best for transforming *Cassia occidentalis* 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 by [41], in which bacterial inoculums was diluted before infection. If high bacterial density was used aseptically for recalcitrant plants, transformation and viability of the plants can be improved with short infection period, dilution of inoculums and Anti-necrotic pre-treatment of explants could be adapted. In spite of early log phase used in this study but transformation efficiency in terms of GUS expressing spots could not show any difference when compared with other bacterial concentrations and their mean differences were statistically insignificant. Necrotic damage at A_{600nm}, 30minutes was completely absent hence bacterial density at A_{600nm} 0.17 and infection period 30minutes was chosen in this study.

(ii) Co-cultivation temperature

The effects of different co-cultivation temperatures on transient GUS expression were studied and the results were shown in Table 2.The explants were co-cultivated at 22,25and 28^{o}C and the highest GUS positive spots were found at 25^{o}C with the mean values (193±6.16) followed by 22^{o}C (170.33±7.76).There were no GUS- positive spots in the explants co-cultivated at 28^{o}C.This indicates that co-cultivation temperature has direct effect on *Agrobacterium* mediated transformation. The mean differences of GUS positive spots at 22 and 25^{o}C were statistically significant. The percentage of GUS positive explants were analysed at 22, 25 and 28^{o}C, highest GUS positive explants found at 25^{o}C

followed by 22°C and no GUS positive explants were observed at 28°C. The explants incubated at 25°C exhibited clear and visible blue spots (Fig 2A).Based on the results obtained, Co-cultivation temperature at 25°C was found to be most 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. The effect of temperature during Co-culture on T-DNA delivery was first reported in dicot plants [42]. Present study evaluated three different Co-cultivation temperatures. There were several reports on higher transformation efficiency where explants Co-cultivated at 22°C and obtained more number of GUS – positive spots[43, 16]. Efficient DNA delivery into plant cells at 22°C was also reported in Sweet potato[37], Cotton[44] and cauliflower[41]. It was reported in the earlier studies that size of crown gall tumour decreased when Co-cultivation temperature was increased[45]. Another study found that Ti-Plasmids were lost in *Agrobacterium tumefaciens* when the culture was grown over 36 hours at elevated temperatures[46]. Low temperatures from 20 to 22°C, were found to promote pilus assembly and it was influenced by Vir B gene at low temperature which is required for conjugal transfer of T-DNA into plant cells[47].But in our study, Co-cultivation temperature at 25°C resulted in high transformation efficiency, even though 19°C was found optimal for T-DNA delivery [48].

(iii) Addition of Acetosyringone in Co-cultivation Medium

The effect of Acetosyringone (AS) on *Agrobacterium* mediated transformation was studied using six different concentrations of AS from 50µM- 800µM were added to Co-cultivation medium and the results were shown in Table3. Of six different concentrations of AS, 400µM concentration gave highest number of GUS-positive spots with the mean value (902±42.76). The lowest numbers of GUS positive spots were found at AS concentration from 50µM - 200µM, 600µM - 800µM and control medium without AS and the mean differences between 400µM and rest of the concentrations were statistically significant. The percentages of GUS-positive explants were highest at AS concentration 400µM and gave 56.25%. The number of GUS-positive spots increased only at AS concentration 400µM and the remaining concentrations of AS (50-200µM and 600-800µM) did not show any increase and the mean differences among the AS concentrations except 400µM were statistically insignificant. The explants with higher number of GUS-positive spots were shown in Fig 2B and one of such explant shown in Fig 2D and the control has not shown any blue spots (Fig 2C). The number of GUS-positive spots were counted using trinocular microscope and one of such explant with microscopic view was shown in Fig 2E. There was an increase in the number of GUS-positive spots when the explants incubated at AS concentration 400µM (Fig 2B) when compared to explants co-cultivated at 25°C without AS in co-cultivation medium. Similar studies were done by introducing various concentration of AS (50µM- 800µM) into bacterial inoculums and results were shown in Table.3. The highest GUS-positive spots were found at AS concentration 50µM with the mean value (1132.33±58.45) followed by control with AS 400µM in Co-cultivation medium. The low numbers of GUS-positive spots were found from 100 -800µM. The mean difference between AS (400µM) in Co-cultivation medium and AS (50µM) in Bacterial inoculums were statistically significant. The mean differences among the AS concentrations in bacterial inoculum from 100 - 800µM were statistically insignificant. This indicated that higher concentration of AS (>50µM) in bacterial inoculums is unfavourable to get higher transient GUS expression. The transformation efficiency at co-cultivation temperature 25°C was 193 GUS-positive spots per explant and it was increased 5 fold when the infection process was carried out with AS at concentration 400µM in co-cultivation medium and 50µM in bacterial culture. The percentage of GUS-positive explants were analysed in AS concentrations from 50 – 800µM where 50µM gave 71.78% followed by control 58%.This indicated that inclusion of AS (50µM) in Bacterial inoculum increases GUS-positive explants as well as GUS-positive spots.

Addition of AS (400µM) into Co-cultivation medium gave higher GUS expression than the control Co-cultivation medium without AS. Other concentrations of AS were not shown any increase in the transient GUS expression when compared with AS concentration at 400µM. It reveals that *Agrobacterium* cells may have been induced to maximum towards virulent stages at 400µM concentration of AS. The influence of AS in bacterial inoculum was also investigated, the concentration at 50µM was found to be most effective while higher concentration of AS resulted in reduced transient GUS-expression. This result indicated that at high concentration of AS may have a detrimental effect on transient GUS- expression. The optimum concentration of AS for higher transient expression varies based on genotype and cultivar of plant [37]. Therefore it is recommended to consider the cultivar and its responses and sensitivity of AS to achieve high transformation efficiency.

(iv) Pre-culture and wounding

The influence of pre-culture and wounding on transient GUS expression were analysed prior to infection with *Agrobacterium tumefaciens* (Fig 3). The explants pre-cultured for two days were compared with explants without pre-culture. The GUS-positive spots were low (46.66±3.39) in pre-cultured explants when compared with explants that were infected directly. The effect of wounding on transient GUS expression was also evaluated by comparing wounded explants with non-wounded explants. The GUS-positive spots were decreased very low (44.33±4.98) when compared to control without pre-culture and wounding (1122.67±54.62).This indicated that pre-culture and wounding had negative effect on transient GUS-expression. Pre-cultured explants prior to infection increases transient GUS expression and it was reported in many plants like Sour Cherry [49], tobacco [25] ginger [26]. But in the present study, pre-cultured explants at room temperature for 2 days showed low transient expression when compared with explants without pre-culture. It was further investigated to study the effect of wounding on pre-cultured explants; it was found that transient expression further decreased when compared with explants pre-cultured at room temperature for 2 days and control without pre-culture and wounding. Similar founding was reported by [50], where pre-cultured explants of citrus showed decreased transient GUS-expression when compared with explants without pre-culture. The explants with wounding were found to be more effective in transient GUS expression when compared with non-wounded explants [49, 25]. Wounding induces cell division and newly synthesized cell wall was found to be essential for the productive attachment of *Agrobacterium* transformation [51]. Wounding also induces the expression of Vir genes [52, 53] and activation of Vir genes is required for the T-DNA transfer [54, 55, 56]. According to [57], wounded tobacco cells had 10 fold more AS than uninjured cells. According to[16], tobacco explants pre-cultured on MS medium at room temperature for two days showed an increase in the size of explants. Similar approach was used in the present study where the explants did not show any increase in the size of explants even after incubating on MS medium from 2- 5 days at room temperature. These pre-cultured explants reduced the transformation frequency to almost half that obtained without pre-culture. These pre-cultured explants were further analysed after wounding where the transformation frequency was further reduced. In overall, 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 co-cultivation medium at 400µM addition of AS into bacterial inoculums at 50µM and co-cultivation temperature of 25^{o}C. We are currently optimizing the regeneration of transformed explants into whole plant. Success in the regeneration of these transformed explants will give an opportunity to characterize the genes responsible for the medicinal property of this plant and eventually that helps in metabolic engineering.

Table 1

The Effects of bacterial concentrations and infection periods on transient GUS expression and necrotic damage of the explants.

Bacterial density (A600nm)	Infection period (min)	No. of spots (mean ±SD)	Percentage of GUS positive explants	Necrotic explants (mean±SD)	Percentage of necrotic explants
0.17	15	0.00±0.0[a]	0.00	0.00±0.00[a]	0.0
	30	1.43±0.0[c]	4.00	0.00±0.00[a]	0.0
	60	1.36±0.50[c]	1.66	0.66±0.47[b]	2.74
0.49	15	0.56±0.15[b]	6.33	2.00±0.81[b]	8.33
	30	1.46±0.30[c]	8.33	3.33±0.47[b]	13.87
	60	1.46±0.15[c]	8.00	4.00±0.81[b]	16.66
0.97	15	1.40±0.20[c]	9.33	3.33±0.94[b]	13.87
	30	1.20±0.10[c]	11.33	5.00±0.81[b]	20.83
	60	1.16±0.15[c]	10.00	7.66±1.24[c]	31.91
1.4	15	0.60±0.20[b]	7.33	17.00±2.44[d]	70.83
	30	0.60±0.25[b]	5.66	18.33±1.24[de]	76.83
	60	0.43±0.15[b]	2.00	20.00±1.63[f]	83.33

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

Table 2

The effects of different co-cultivation temperatures on transient GUS expression

Co-cultivation temperature(^{o}C)	No. of spots (Mean±SD)	GUS positive explants (%)
22	170.33±7.76[b]	11.11
25	193.00±6.16[c]	16.66
28	0.00±0.00[a]	0.00

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

Table 3

AS in co-cultivation medium and bacterial inoculum and its effect on transient GUS expression

Concentration of acetosyringone(µM)	Co-cultivation medium		Bacterial inoculum	
	No. of spots (Mean±SD)	GUS positive explants (%)	No. of spots (Mean±SD)	GUS positive explants (%)
0	192.33±8.17[a]	13.88	898.0±48.92[b]	58.0
50	195.00±9.62[a]	10.41	1132.33±58.45[c]	71.78
100	181.00±9.79[a]	22.91	145.66±7.36[a]	23.07
200	173.33±9.46[a]	18.75	134.0±6.53[a]	25.63
400	902±42.76[b]	56.25	144.66±9.84[a]	38.45
600	175.66±7.76[a]	22.91	152.0±6.16[a]	33.33
800	174.66±8.33[a]	18.75	146.33±7.25[a]	28.19

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

Figure 1
Restriction map of plasmid vector (PCAMBIA 1301)

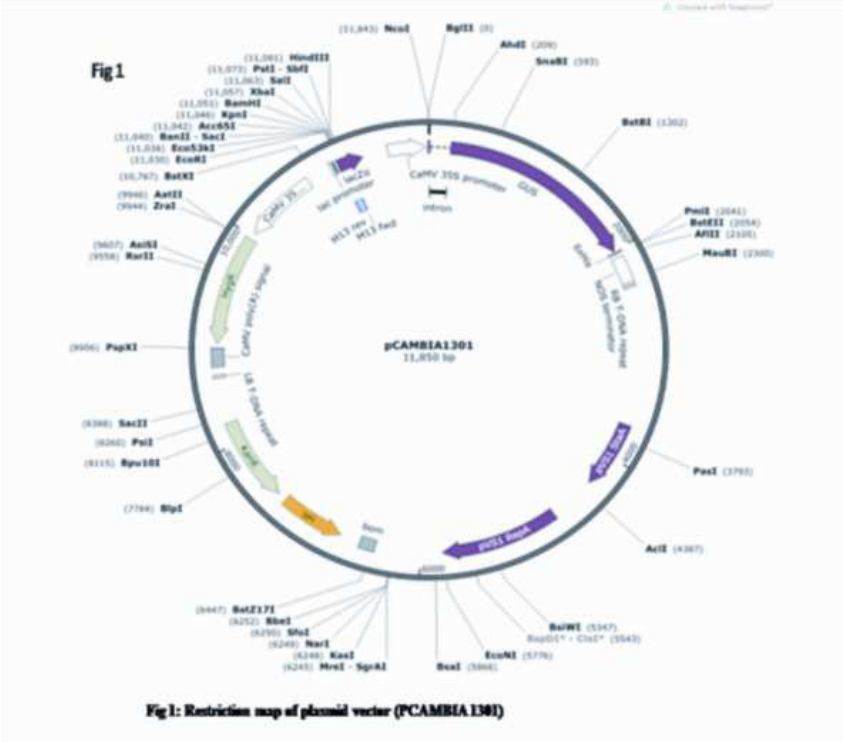


Figure 2
The Effect of bacterial density on GUS-positive spots in Agrobacterium mediated transformation of Cassia occidentalis explants. Transformation is represented as the total number of GUS-positive spots per explant. Transient GUS expression was analysed with respective to different bacterial densities and infection periods. A vertical bar indicates the SD from three experiments.

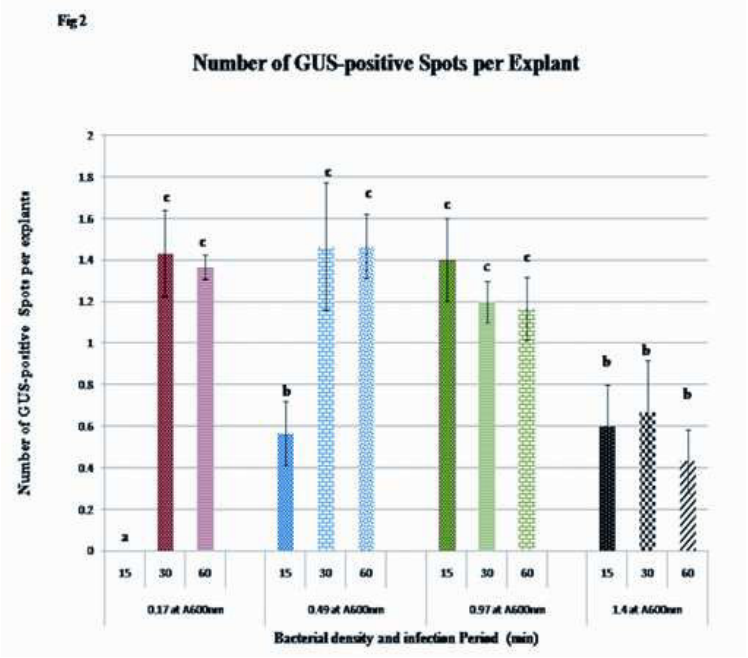


Figure 3

Analysis of necrotic explants in Agrobacterium mediated transformation of Cassia occidentalis explants. Necrotic damage represented as number of necrotic explants per 24 explants. Necrotic damage was analysed with respective to various bacterial concentrations and infection periods. Vertical bars indicate the SD from three experiments.

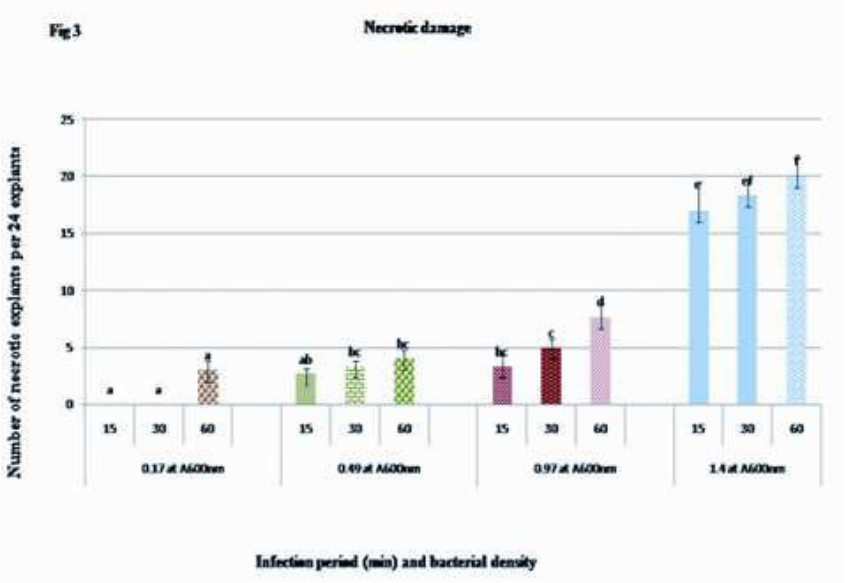


Figure 4

The Effect of co-cultivation temperature on GUS-positive spots in Agrobacterium mediated transformation of Cassia occidentalis explants. Transformation is represented as the total number of GUS-positive spots per explant. Transient GUS expression was analysed with respective to different co-cultivation temperatures. A vertical bar indicates the SD from three experiments.

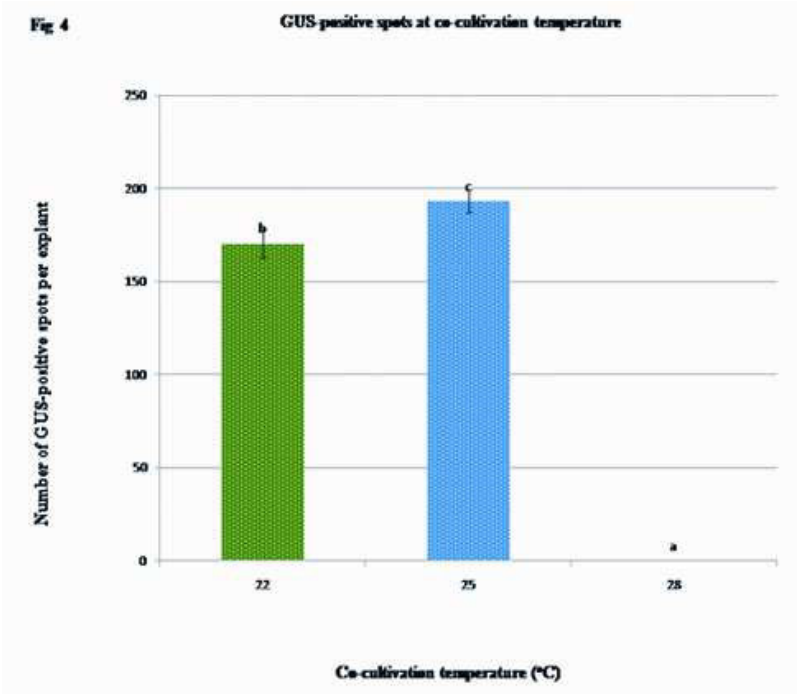


Figure 5

AS concentration in co-cultivation medium and its effect on GUS-positive spots in Agrobacterium mediated transformation of Cassia occidentalis explants. Transformation is represented as the total number of GUS-positive spots per explant. Transient GUS expression was analysed with respective to various concentrations of AS. A vertical bar indicates the SD from three experiments.

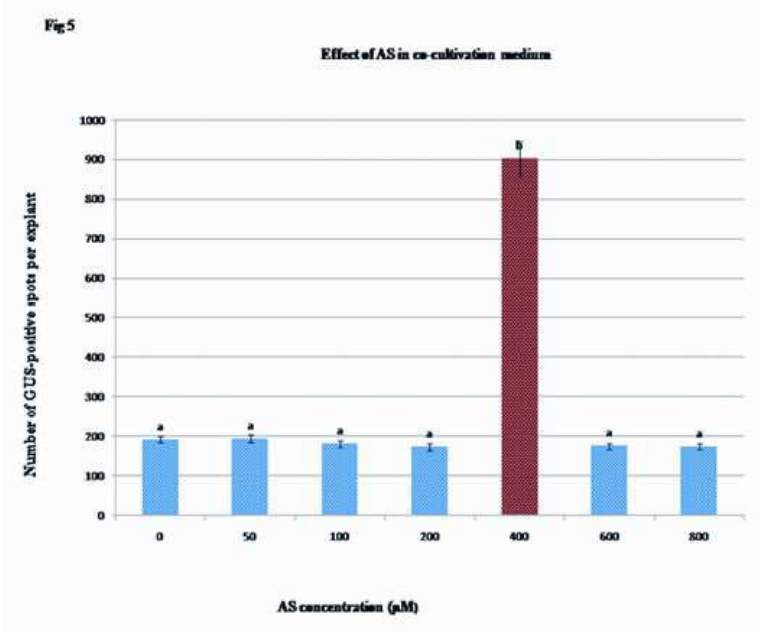


Figure 6

AS concentration in bacterial inoculum and its effect on GUS-positive spots in Agrobacterium mediated transformation of Cassia occidentalis explants. Transformation is represented as the total number of GUS-positive spots per explant. Transient GUS expression was analysed with respective to various concentrations of AS. A vertical bar indicates the SD from three experiments.

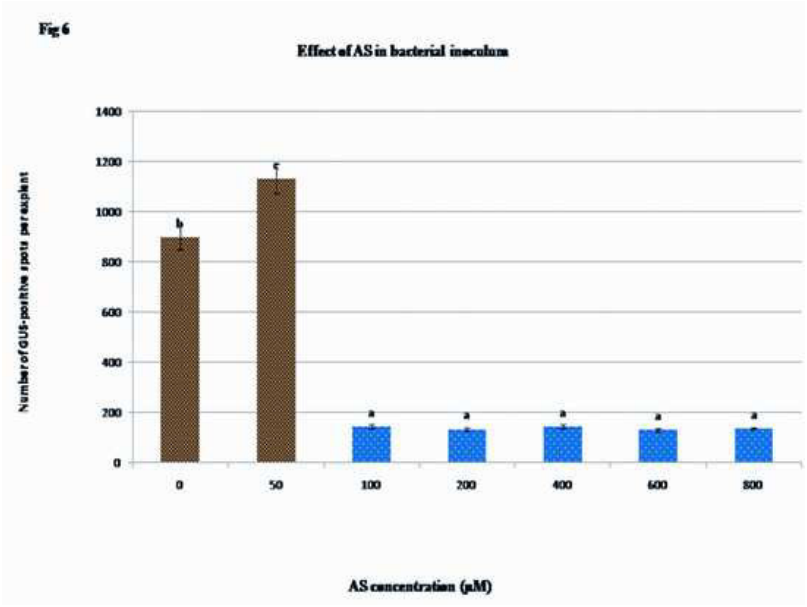


Figure 7

The Effect of pre-culture and wounding on GUS-positive spots in Agrobacterium mediated transformation of Cassia occidentalis explants. Transformation is represented as the total number of GUS-positive spots per explant. Transient GUS expression was analysed with respective to pre-culture, wounding and control without pre-culture and wounding. A vertical bar indicates the SD from three experiments.

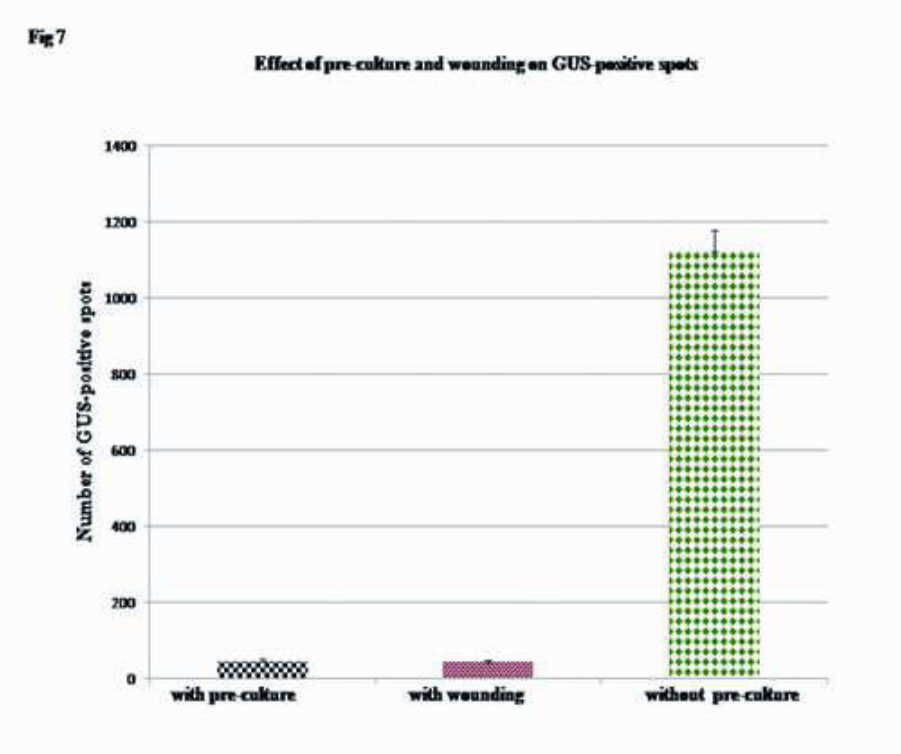
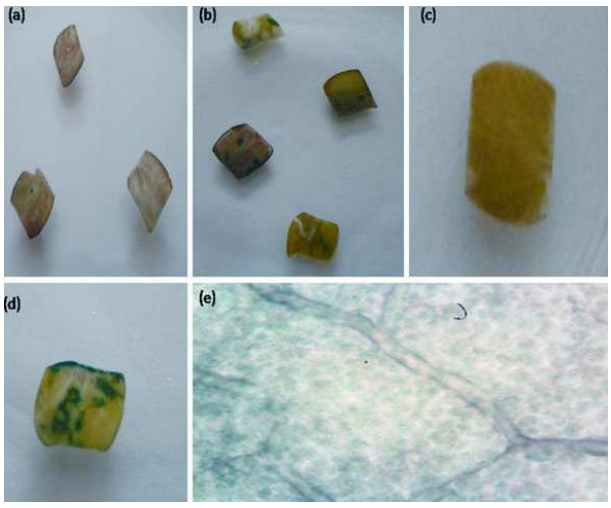


Figure 8

GUS histochemical analysis of Cassia occidentalis explants transformed by Agrobacterium tumefaciens. (A) Expalnts co-cultivated at 25^{o}C expressing moderate level of transient GUS activity.(B) Explants in co-cultivation medium at AS concentration 400µM expressing high level of transient GUS expression.(C) Uninfected control explant.(D)Infected explant in co-cultivation medium at AS concentration 400µM.(E) A close up image of GUS-positive spots found in the explant in D.



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 50µM in co-cultivation medium, addition of AS 800µM in bacterial inoculum and co-cultivation temperature of 25^{o}C. Since *Cassia occidentalis* L has been reported for high medicinal values against various diseases, one can characterize the gene(s) involved in controlling the synthesis of active compounds by delivering anti-sense gene will require a suitable transformation method. Eventually that helps in metabolic engineering of *Cassia occidentalis* L to produce more amount of end product that may be involved in an anti-diabetic activity, anti-carcinogenic, antibacterial, hepatoprotective, anti-anxiety and anti-depressant and also healing wounds, sores, itch, cutaneous diseases, anti-mutagenic, bone fracture, fever, ringworm, skin diseases and throat infection.

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