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# *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *CISSAMPELOS PAREIRA* LINN: FACTORS AFFECTING ON TRANSIENT GUS EXPRESSION

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## ABSTRACT

Optimization of co-cultivation parameters during *Agrobacterium*–mediated transformation to *Cissampelos pareira* evaluated were bacterial density, infection period, wounding and pre-culture of explants, acetosyringone (AS) concentration and co-cultivation temperature. Optimized parameters resulted in high transformation efficiency at transient GUS expression 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 (50µM) and in bacterial growth culture (800µM) and co-cultivation temperature of 25$^{o}$C. 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 was increased 6 fold when the infection process was carried out with acetosyringone in co-cultivation medium (50µM) and in bacterial culture (800µM).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, Cissampelos pareira, gus* gene.**

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# INTRODUCTION

*Cissampelos pareira* Linn belongs to the family menispermaceae is a climbing herb, known as ambastha or laghupatha in Indian traditional medicine[1]. It is commonly distributed throughout topical and sub topical India, ascending up to an altitude of ca 2000 m[2, 3]. This plant exhibits anti-diabetic activity in hydro-alcoholic extract of leaves[4]. The paste made out of the roots is used in fistula; purities, skin disorders and snake poison externally. Internally roots are useful in anorexia, indigestion, abdominal pain, gastric disorders, diarrhoea and dysentery[5]. The roots show significant antibacterial activity against Gram-positive organisms than against Gram-negative strains[6].Traditionally the plant is reported for its blood purifying and anti-inflammatory properties in India[7]. Astringent, mild tonic, diuretic, stomachic, analgesic, antipyretic and emmenagogue properties are also reported in roots[1, 8]. It is also used in cough and as it purifies breast milk, it is used in various disorders of breast milk secretion[9]. It is a potent diuretic[10]. This plant is frequently prescribed for cough, dyspepsia, dropsy, urino-genital troubles such as prolapsed uteri, cystitis, haemorrhage and menorrhagia and calcular nephritis[11]. Genetic transformation is prerequisite to identify the genes responsible for the medicinal property followed by genetic manipulations. So far there were no reports on *Agrobacterium*- mediated transformation of *Cissampelos pareira*. *Agrobacterium* based transformation has advantages in plant transformation due to its simplicity, precision, integration of large size DNA and stable gene expression[12]. 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[13]. The difficulties in the transformation can be overcome by optimizing physical and chemical conditions which can lead to increase in the transformation efficiency[14]. 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[15, 16]. This phenomenon is to protect against viruses attack and transposons[17], another possibility of gene silencing is due to polyploidy in plant developmental processes[18]. Transgene silencing also associates with increasing endoploidy during maturation of differentiated tissues[19]. 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[20]. It is an advantage to use GUS reporter system since the expression of *gus* gene in transformed explants can be easily detected at an early stage of the transformation. The most effective co-cultivation parameter can be identified by using *gus* gene expression where its shows maximum expression. Evaluation of several physical and chemical conditions helps to produce an easy and reproducible protocol. In this study, we describe an optimization of *Agrobacterium* mediated transformation to *Cissampelos pareira 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, speices and even for *Agrobacterium* strains. It is essential to optimize co-cultivation parameters for each protocol of different type of plants, explants and bacterial strains used in the transformation.

# MATERIALS AND METHODS

Bacterial strain and vector: *Agrobacterium tumefaciens* EHA-105[21] containing the binary plasmid vector (pCAMBIA1301) was used as the vector system for transformation (Fig 1).

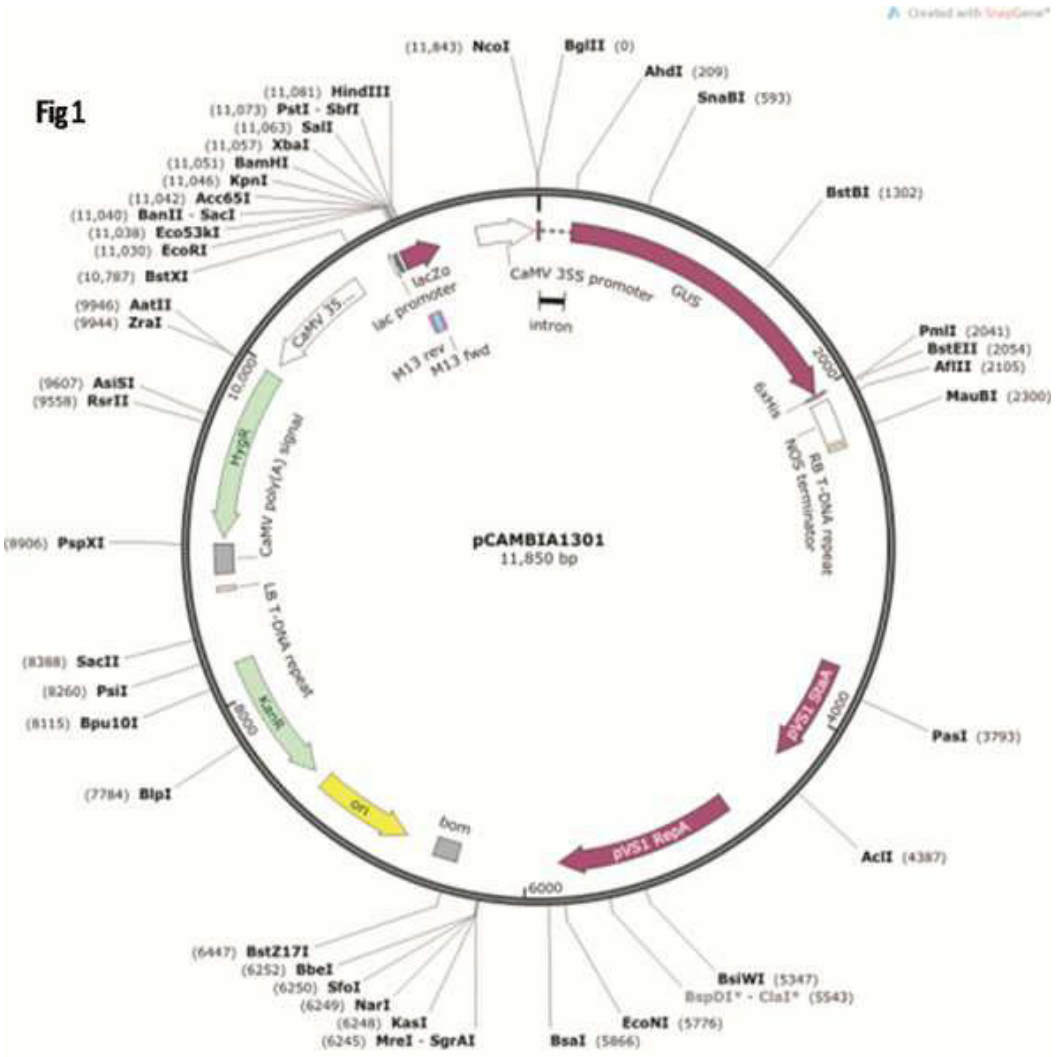



**Figure 1**
***Restriction map of plasmid vector (pCAMBIA 1301)***

Bacterial stock culture of *Agrobacterium tumefaciens* strain EHA-105 harbouring pCAMBIA1301 was kindly provided by Dr.K.Veluthambi Professor, Department of Biotechnology, Madurai kamaraj university, Madurai, 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 with following composition[22] 10g yeast extract, 10g peptone and 5g NaCl $L^{-1}$. All the chemicals used in this study were purchased from Hi media, India unless otherwise specified. The medium also has 10 mg $L^{-1}$ rifampicin and 100mg $L^{-1}$ 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$^{o}$C for two days.

***(ii) Plant material***

Seeds of *Cissampelos pareira* Linn 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. Then 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$^{o}$C under a 16/8hrs 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 10 mg L$^{-1}$ rifampicin and 100mg L$^{-1}$ kanamycin and incubated at 28$^{o}$C for 2 days. A single colony was inoculated into 2ml of YEP broth and grown for overnight at 28$^{o}$C with 200 rpm. 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, 0.56, 0.8 and 1.2. A total volume of 15 ml of liquid culture was used to infect *Cissampelos pareira* leaf explants from 2-3 weeks old seedlings. The explants were co-incubated with bacterial suspension at 25$^{o}$C. The infection period was carried out at same temperature of the above and allowed to last for 15, 30 and 60 minutes 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 papers to remove excess bacterial suspension. The explants were then placed on co-cultivation medium (MS-agar medium with NAA 2mg L$^{-1}$) and maintained in a tissue culture chamber at 25$^{o}$C in dark for two days. After incubation the explants were gently rinsed using sterile distilled water to remove excess agar followed by GUS histochemical analysis. This 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$^{o}$C. After co-cultivation process, the explants were subjected to GUS histochemical analysis. The most effective treatments/parameters were used in subsequent evaluation experiments.

***(vi) The effect of pre-culture***

Pre-culture process consisted of incubating wounded leaf explants in MS medium for 2 days (Sunil Kumar et al. 1999) at room temperature before the infection process, whereas leaves without pre-culture were used as explants in control experiments. Pre-cultured explants and the control explants were co-incubated with bacterial suspension and later co-cultivated as in the conditions mentioned earlier. GUS histochemical analysis was conducted on explants after 2 days to determine the most effective treatments/parameters.

***(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 co-cultivation medium made of solid MS medium consisted of NAA 2mg $L^{-1}$ 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. 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 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 effects of AS on transformation efficiency.

***(x) The effects 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 Jefferson (1987), Suma et al. (2008). 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 5-bromo-4 chloro-3 indolyl β-D-glucuronide (X-gluc) 0.5g $L^{-1}$. 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

***(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 at $A_{600nm}$ 0.56, 60 min (mean value 2.98±0.22) followed by 0.8, 30 min (3.28±0.26) and 60 min (3.09±0.26) and their mean differences are statistically insignificant. The lowest GUS activity found at $A_{600nm}$ 1.7 culture with the infection periods of 30min (mean value 1.44±0.12), 60 min (1.38±0.14) and $A_{600nm}$ 1.2 culture, 60 min (1.34±0.06) and their mean differences were statistically insignificant. The number of GUS-positive spots from low bacterial concentration ($A_{600nm}$ 0.56) and higher bacterial concentration ($A_{600nm}$ 0.8) were same and their mean difference are 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 0.8 cultures were increased and decreased at $A_{600nm}$ 1.2 culture. Transformation frequency decreases as necrotic damage increases from lower bacterial density ($A_{600nm}$ 0.17, 30min) to higher density ($A_{600nm}$ 1.4, 60 min). This indicates that explants viability has direct effect on transformation efficiency. Bacterial concentration $A_{600nm}$ 0.17 was chosen in spite of having low transformation frequency when

compared with other higher bacterial densities, since it has least amount of necrotic damage and it was used in further experiments.

**Table 1**

***Effects of bacterial concentrations and infection periods on transient GUS expression and explants viability Data within the same column followed by the same letter indicated no significance at 5% level***

| Bacterial density ($A_{600nm}$) | Infection period (min) | No. of spots (mean ± SD) | Percentage of GUS positive explants | Necrotic explants (mean± SD) | Percentage of necrotic explants |
|---|---|---|---|---|---|
| 0.17 | 15 | $0.00\pm0.00^{a}$ | 0.00 | $0.00\pm0.00^{a}$ | 0.00 |
| | 30 | $1.44\pm0.12^{b}$ | 9.72 | $0.33\pm0.47^{ab}$ | 1.37 |
| | 60 | $1.38\pm0.14^{b}$ | 11.11 | $2.66\pm0.47^{bc}$ | 11.08 |
| 0.56 | 15 | $2.17\pm0.10^{e}$ | 5.55 | $3.00\pm0.81^{c}$ | 12.50 |
| | 30 | $2.61\pm0.10^{f}$ | 12.49 | $6.00\pm0.81^{d}$ | 25.00 |
| | 60 | $2.98\pm0.22^{g}$ | 23.61 | $4.66\pm1.24^{cd}$ | 19.41 |
| 0.8 | 15 | $2.10\pm0.06^{de}$ | 9.72 | $4.66\pm1.24^{cd}$ | 19.41 |
| | 30 | $3.28\pm0.26^{g}$ | 20.83 | $7.0\pm0.81^{d}$ | 25.92 |
| | 60 | $3.09\pm0.10^{g}$ | 26.38 | $11.33\pm1.24^{e}$ | 47.20 |
| 1.2 | 15 | $1.66\pm0.17^{bc}$ | 9.71 | $13.0\pm1.63^{e}$ | 54.16 |
| | 30 | $1.84\pm0.10^{cd}$ | 13.88 | $20.0\pm2.16^{f}$ | 83.33 |
| | 60 | $1.34\pm0.06^{b}$ | 11.11 | $21.33\pm1.69^{f}$ | 88.87 |

***(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, 25 and 28$^{o}$C and the highest GUS positive spots were found at 25 and 28$^{o}$C with the mean values (140±9.79) and (136.33±5.31) respectively. There was low number of GUS-positive spots in the explants co-cultivated at 22$^{o}$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 or 28$^{o}$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 or 28$^{o}$C followed by 22$^{o}$C. The explants incubated at 25$^{o}$C exhibited clear and visible blue spots (Fig 2B).

**Table 2**

***The effects of various co-cultivation temperatures on transient GUS expression***

| Co-cultivation temperature($^{0}$C) | No. of spots (Mean±SD) | GUS positive explants (%) |
|---|---|---|
| 22 | $117.66\pm3.68^{a}$ | $2.77^{a}$ |
| 25 | $140\pm9.79^{b}$ | $11.10^{b}$ |
| 28 | $136.33\pm5.31^{b}$ | $11.11^{b}$ |

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

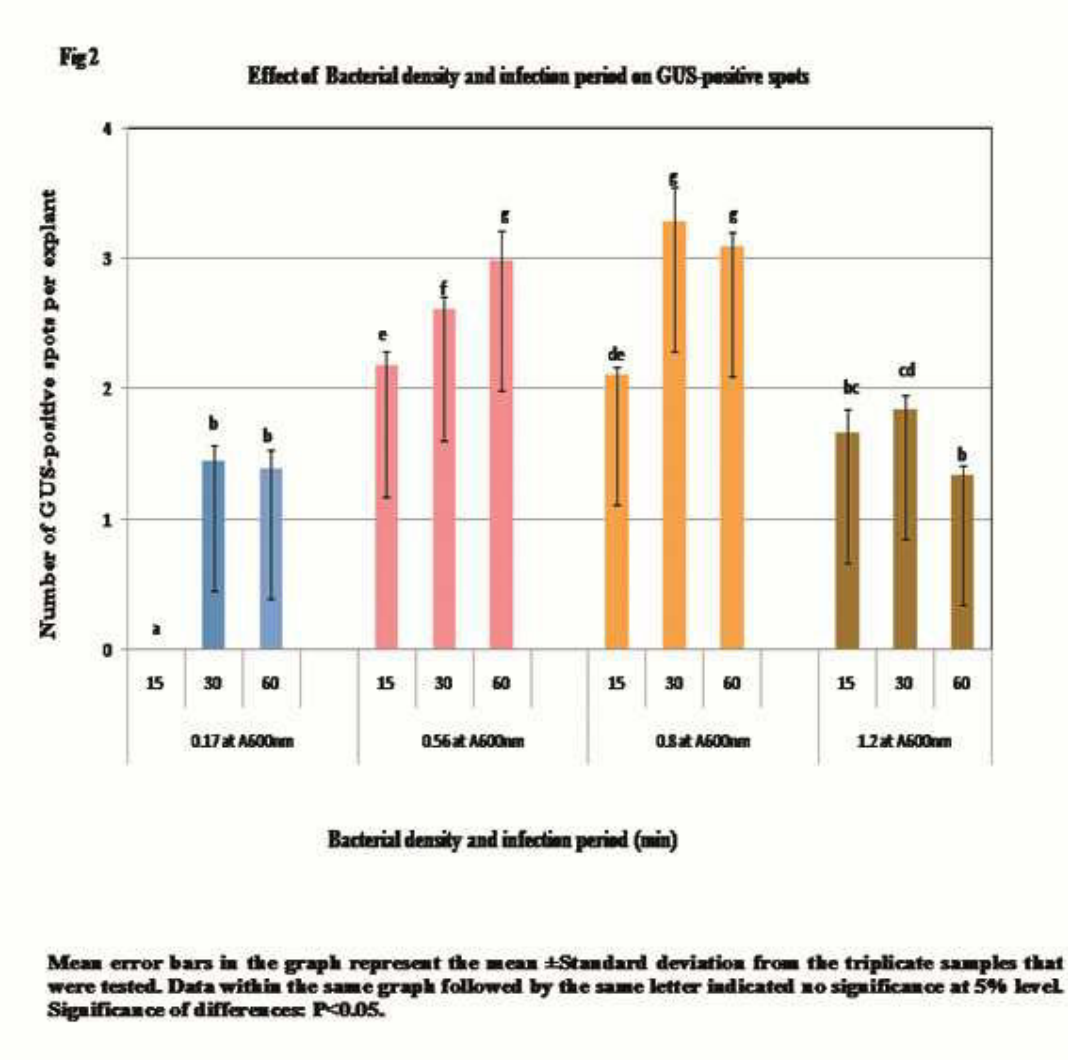



**Figure 2**

***The effect of bacterial density on GUS-positive spots in Agrobacterium mediated transformation of Cissampelos pareira L. 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. Vertical bars indicate the SD from three experiments***

Based on the results obtained, Co- cultivation temperature either at 25 or 28$^{o}$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$^{o}$C as co- cultivation temperature and it was introduced in further experiments.

### *(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 50-800µM were added to Co-cultivation medium and results were shown in Table 3. Of six different concentrations of AS 50µM concentration gave highest number of GUS-positive spots with the mean value (723.33±54.36). The lowest numbers of GUS positive spots were found at AS concentration from 100- 800µM and control medium without AS and the mean differences between 50µM and rest of the AS concentrations were statistically significant. The percentages of GUS-positive explants were highest at AS concentration 50µM and gave 78.56%. The number of GUS-positive spots increased only at AS concentration 50µM and the remaining concentrations of AS (100-800µM) did not show any increase and the mean differences among the AS concentrations except 50µM were statistically insignificant. The explants

with higher number of GUS-positive spots were shown in Fig 2C and the control has not shown any blue spots (Fig 2A). The number of GUS-positive spots were counted using Trinocular microscope and one of such explant with microscopic view was shown in Fig 2D. There was an increase in the number of GUS-positive spots when the explants incubated at AS concentration 50µM (Fig 2C) when compared with explants co-cultivated at 25$^{o}$C without AS in co-cultivation medium. Similar studies were done by introducing various concentration of AS (50-800µM) into bacterial inoculums and results were shown in Table.3. The highest GUS-positive spots were found at AS concentration 800µM with the mean value (870.33±9.46) followed by control with AS 50µM in Co-cultivation medium. The low numbers of GUS-positive spots were found from 100-600µM. The mean difference between AS (50µM) in Co-cultivation medium and AS (800µM) in Bacterial inoculums were statistically significant. The mean differences among the AS concentrations in bacterial inoculum from 100-600µM were statistically insignificant. This indicated that higher concentration of AS (800µM) in bacterial inoculums is favourable to get higher transient GUS expression. The transformation efficiency at co-cultivation temperature 25$^{o}$C was 146.0 GUS-positive spots per explant and it was increased 6 fold when the infection process was carried out with AS at concentration 50µM in co-cultivation medium and 800µM in bacterial culture. The percentage of GUS-positive explants were analysed in AS concentrations from 50-800µM where 800µM gave 78.78% followed by control 76.18%.This indicated that inclusion of AS (800µM) in Bacterial inoculum increases GUS-positive spots but no change in the percentage of GUS-positive explants when compared with control.

**Table 3**
***Acetosyringone 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 | 146.00±4.32$^{a}$ | 10.18 | 667.66±23.47$^{d}$ | 76.18 |
| 50 | 723.33±54.36$^{b}$ | 78.56 | 375.00±5.35$^{b}$ | 70.17 |
| 100 | 144.66±9.39$^{a}$ | 20.83 | 356.33±5.79$^{ab}$ | 71.42 |
| 200 | 143.33±4.78$^{a}$ | 27.77 | 363.66±6.59$^{ab}$ | 69.04 |
| 400 | 138.00±1.63$^{a}$ | 33.32 | 344.33±9.39$^{a}$ | 59.52 |
| 600 | 176.66±7.58$^{a}$ | 35.18 | 445.33±11.11$^{c}$ | 61.90 |
| 800 | 174.33±7.58$^{a}$ | 39.21 | 870.33±9.46$^{e}$ | 78.78 |

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

### *(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 (89±5.35) 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 also decreased to low (76.33±6.59) when compared to control without pre-culture and wounding (726.0±55.98).This indicated that pre-culture and wounding had negative effect on transient GUS-expression.

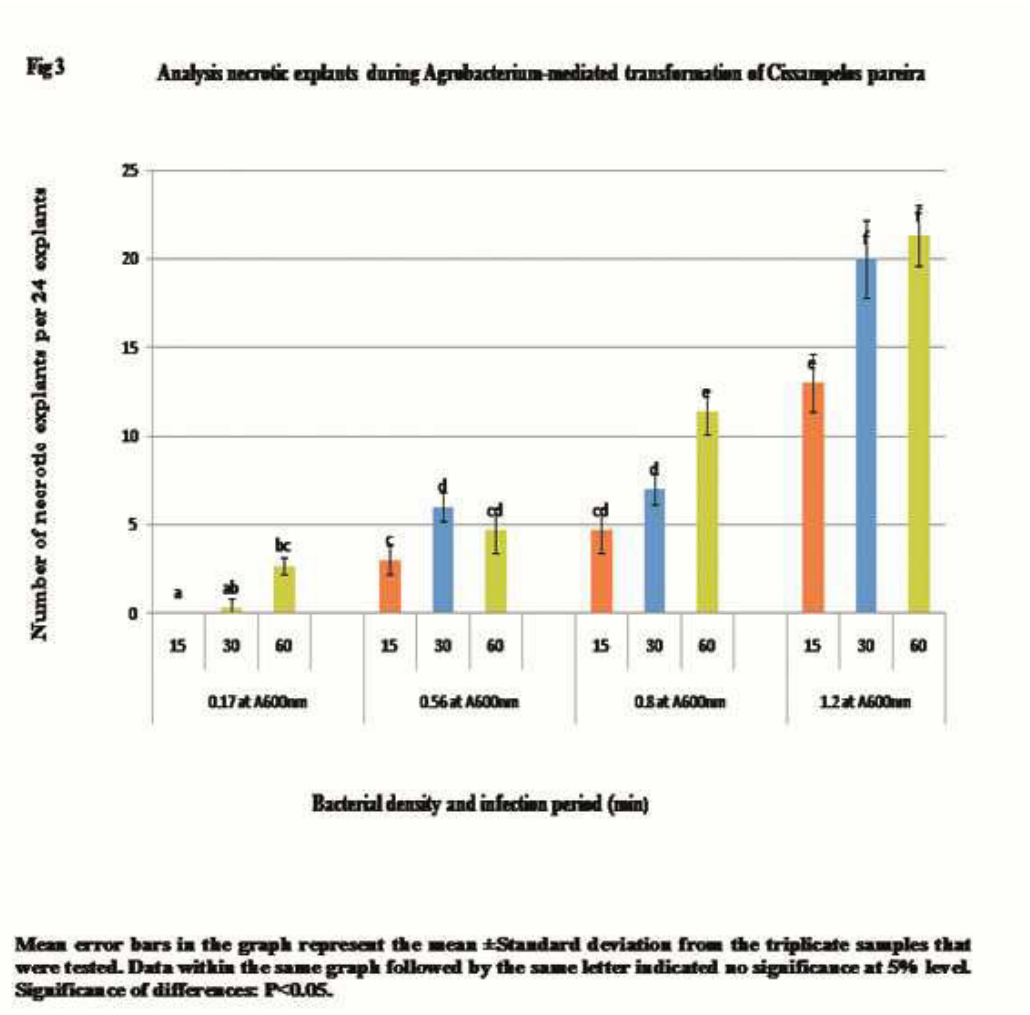



**Figure 3**

***The analysis of necrotic explants in Agrobacterium mediated transformation of Cissampelos pareira L 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.***

# 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[23, 24]. 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*[14] ,*Sorghum* [25], *Rice*[26] and *Groundnut*[27], 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 like leaf, inter node etc, different strains of *Agrobacterium* and type of plant cultivars could influence the transformation of plants[28, 29]. Higher concentrations of *Agrobacerium* were used to transform recalcitrant plants such as rice[30], Sweet potato[31], and pepper[32]. Low bacterial density was also used to transform plants such as in

Brocolli[33], Wheat[34] and tobacco[14]. But in our study early log phase was found to be the best for transforming *Cissampelos pareira* 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[35], 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. The high transformation efficiency was found at $A_{600nm}$ 0.56, 0.8 and their mean differences were statistically insignificant. Necrotic damage at $A_{600nm}$, 30 minutes was least hence bacterial density at $A_{600nm}$ 0.17 and infection period 30 minutes was chosen in this study. The effect temperature during Co-culture on T-DNA delivery was first reported in dicotyledonary plants[36]. Present study evaluated three different Co-cultivation temperatures. There were several reports on higher transformation efficiency where explants Co-cultivated at $22^0$C and obtained more number of GUS – positive spots[37, 14]. Efficient DNA delivery into plant cells at $22^0$C was also reported in Sweet potato[31], Cotton[38] and cauliflower[35]. It was reported in the earlier studies that size of crown gall tumour decreased when Co-cultivation temperature was increased[39]. Another study found that Ti-Plasmids were lost in *Agrobacterium tumefaciens* when the culture was grown over 36 hours at elevated temperatures[40]. Low temperatures from 20 to $22^o$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 T-DNA into plant cells[41]. But in our study, Co-cultivation temperature at 25 and $28^o$C resulted in same and high transformation efficiency. We have chosen 25 instead of $28^o$C since low temperatures are favourable for efficient T-DNA transfer[42] compare to high temperatures. Addition of AS (50µM) 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 50µM. It reveals that *Agrobacterium* cells may have been induced to maximum towards virulent stages at 50µM concentration of AS. The influence of AS in bacterial inoculum was also investigated, the concentration at 800µM was found to be most effective while lower concentration of AS resulted in low level of transient GUS-expression when compared to control. This result indicated that at high concentration of AS may have a positive effect on transient GUS- expression. The optimum concentration of AS for higher transient expression varies based on genotype and cultivar of plant[31]. Therefore it is recommended to consider the cultivar and its responses and sensitivity of AS to achieve high transformation efficiency. This is due to no induction of rapid cellular dedifferentiation. We have also observed the size of explants after incubation on MS agar medium which was found that there was no expansion of leaf explants even after incubating for two days. Pre-culturing of explants results in rapid cellular dedifferentiation and new cells which are having weakened pathogen recognition ability[46]. This reveals that new cells are not formed from the leaf explants (data not shown) as a result susceptibility of *Agrobacterium* is highly reduced. Pre-cultured explants prior to infection increases transient GUS expression and it was reported in many plants like Sour Cherry[43], tobacco[44] ginger[45]. 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. 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[47], 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 expression when compared with non- wounded explants[43, 44]. Wounding induces cell division and newly synthesized cell wall was found to be essential for the productive attachment of

*Agrobacterium* transformation.[48] Wounding also induces the expression of Vir genes[49, 50] and activation of Vir genes is required for the T-DNA transfer.[51,52,53] In the current study the effect of wounding on pre-cultured explants was studied and it was found that transient GUS expression was further decreased when compared with explants pre-cultured at room temperature for 2 days and control without pre-culture and wounding. This may be due to the requirement of specific concentration of acetosyringone (AS). Since explants were already exposed to optimized concentration of AS and explants subjected for wounding results in increasing of AS several fold that may disturb the optimum concentration of AS provided in the co-cultivation medium. Wounding of tobacco explants produces several folds AS is well documented.[54] According to [14], 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 seven times lower than that was obtained without pre-culture. These pre-cultured explants were further analysed after wounding where the transformation frequency was reduced that was similar to pre-culture treatment.

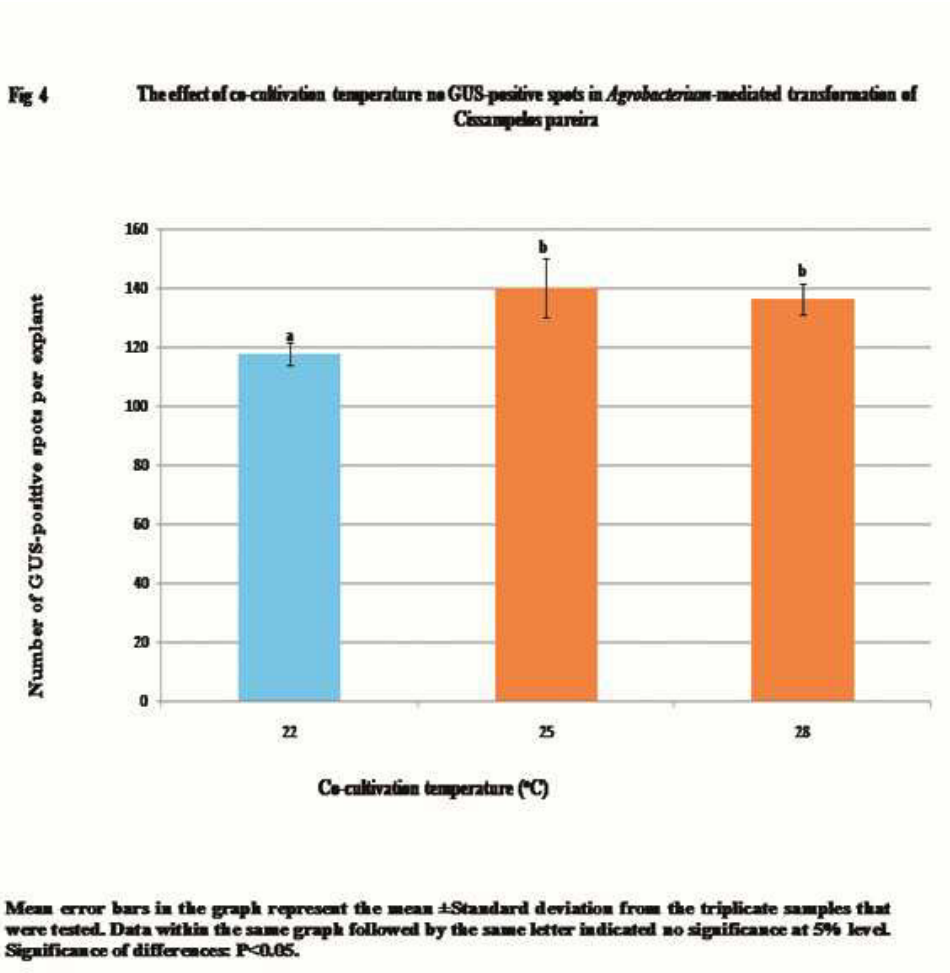



**Figure 4**

***The effect of co-cultivation temperature on GUS-positive spots in Agrobacterium mediated transformation of Cissampelos pareira L. 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. Vertical bars indicate the SD from three experiments.***

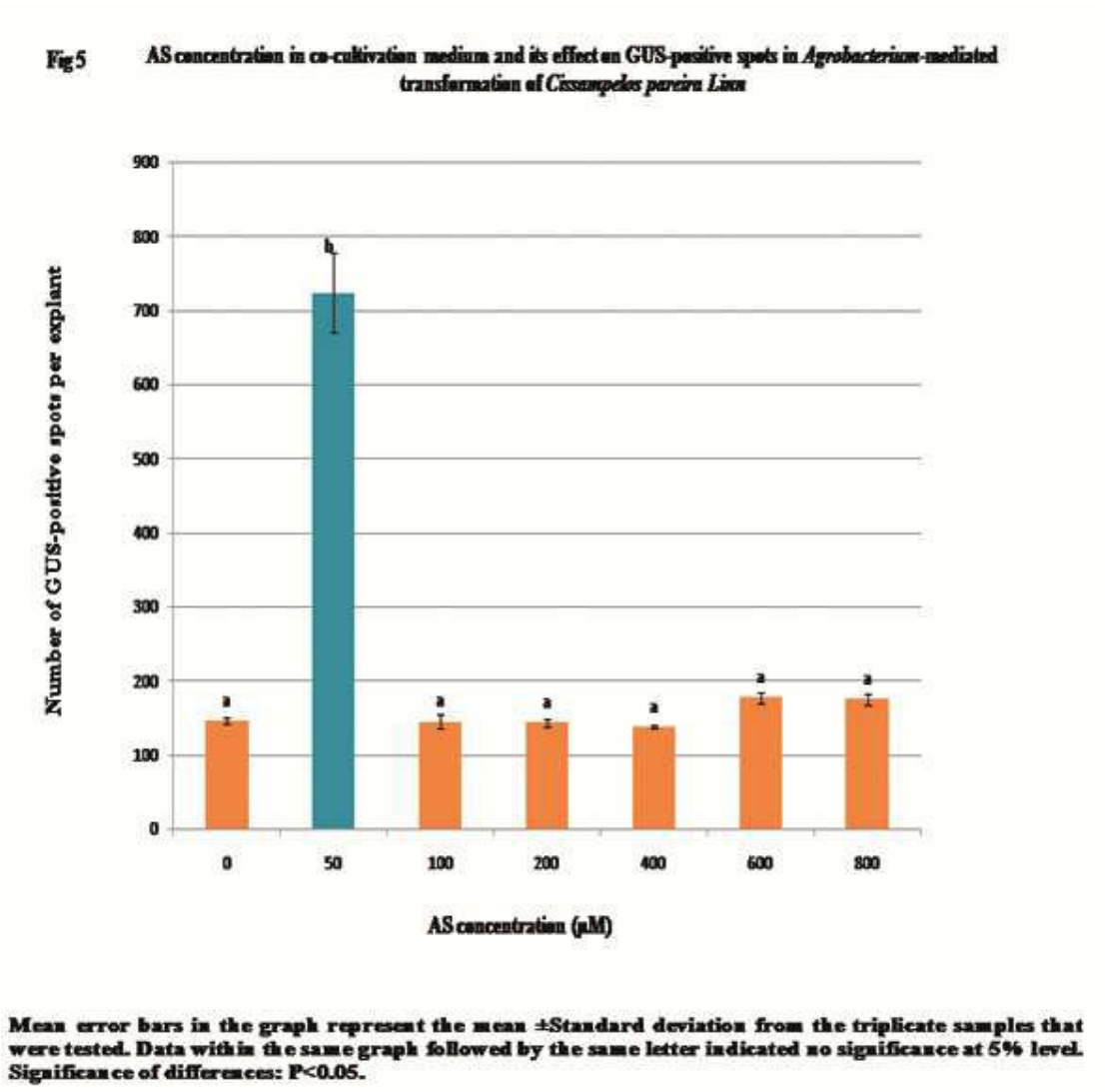
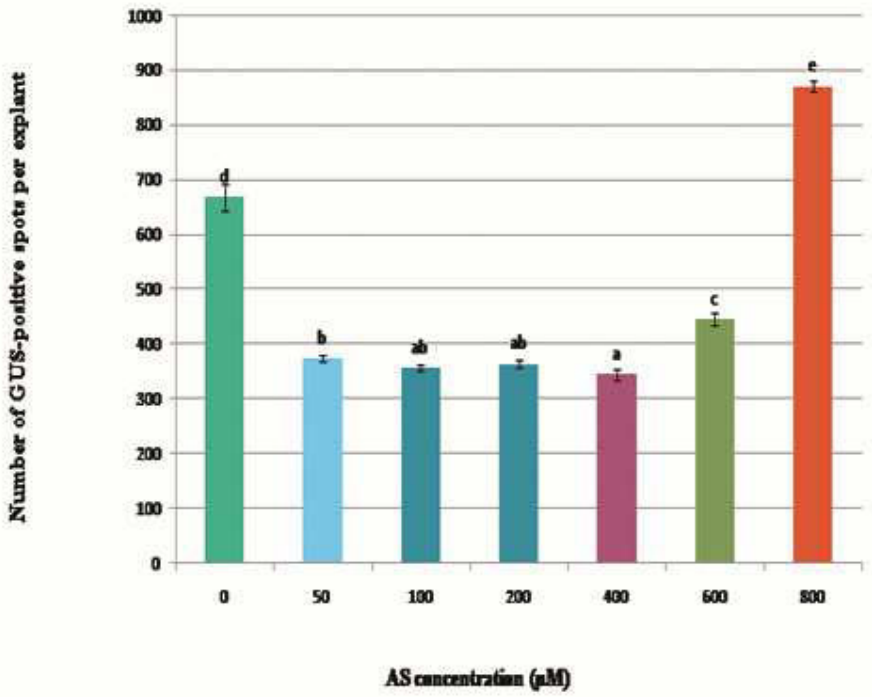



**Figure 5**

***AS concentration in co-cultivation medium and its effect on GUS-positive spots in Agrobacterium mediated transformation of Cissampelos pareira L. 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. Vertical bars indicate the SD from three experiments.***

Fig 6 AS concentration in bacterial inoculum and its effect on GUS positive spots in *Agrobacterium*-mediated transformation of *Cissampelos pareira Linn*




Mean error bars in the graph represent the mean ±Standard deviation from the triplicate samples that were tested. Data within the same graph followed by the same letter indicated no significance at 5% level. Significance of differences: P<0.05.

**Figure 6**

***AS concentration in bacterial inoculum and its effect on GUS-positive spots in Agrobacterium mediated transformation of Cissampelos pareira L. 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. Vertical bars indicate the SD from three experiments.***

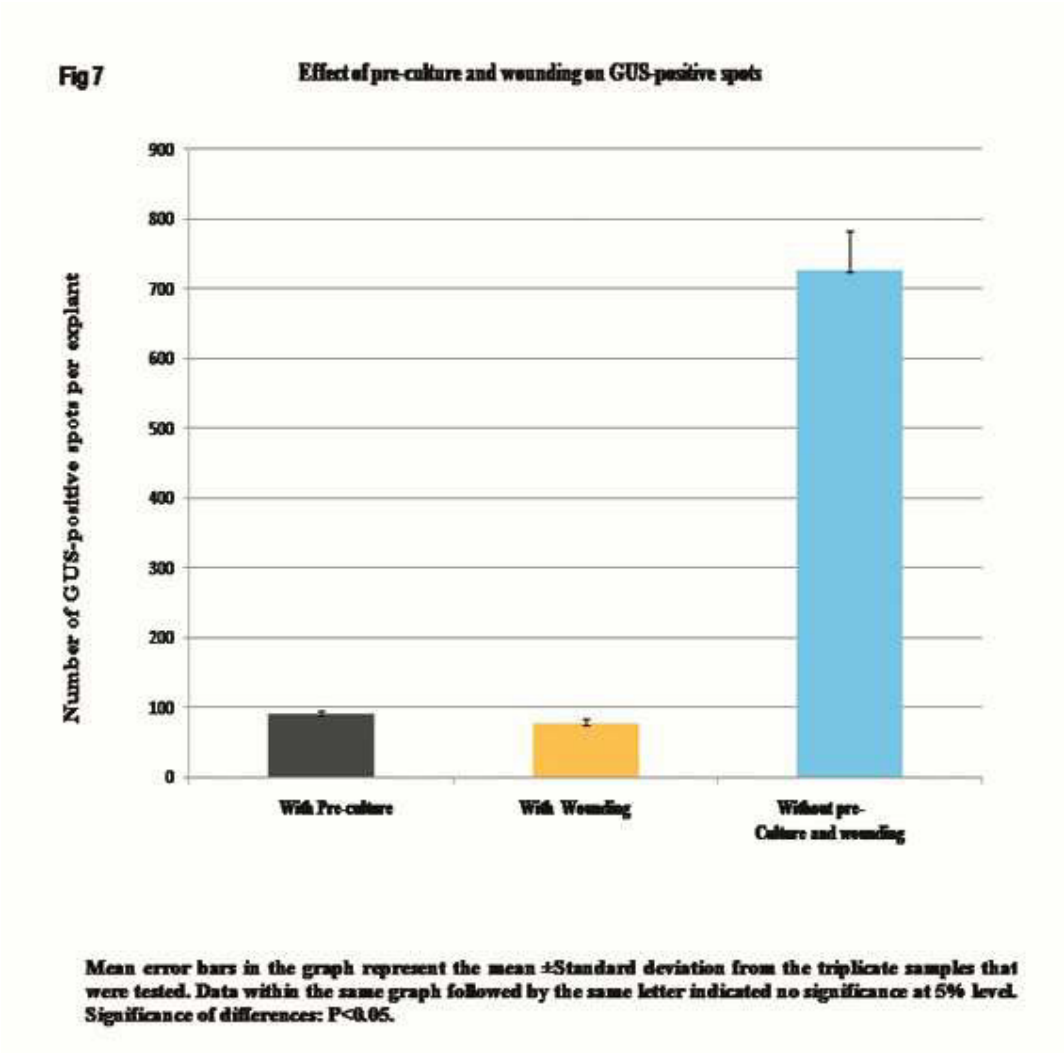



**Figure 7**

***The effect of pre-culture and wounding on GUS-positive spots in Agrobacterium mediated transformation of Cissampelos pareira L. 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. Vertical bars indicate the SD from three experiments.***

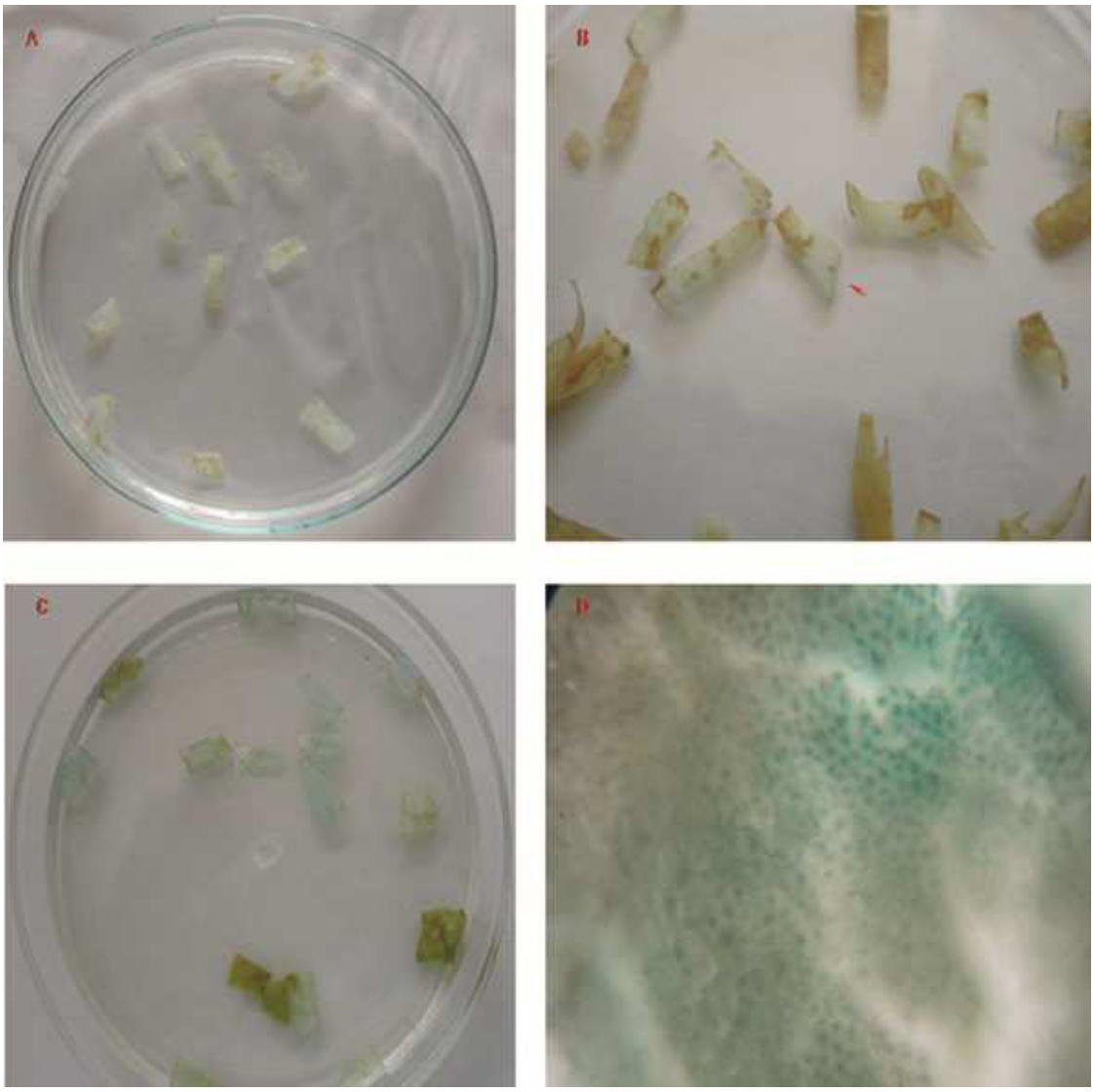



**Figure 8**

***GUS histochemical analysis of Cissampelos pareira L. explants transformed by Agrobacterium tumefaciens. (A) Control (B) Explants co-cultivated at 25°C expressing moderate level of transient GUS activity and a Gus positive spot was shown with arrow mark.(C) Explants in co-cultivation medium at AS concentration 400µM expressing high level of transient GUS expression.(D) A close up image of GUS-positive spots found in the explant in C.***

## 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 co-cultivation medium at 50µM, addition of AS into bacterial inoculums at 800µM and co-cultivation temperature of 25°C. Since *C.pareira* 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 *C.pariera* to produce more amount of end product that may be involved in a anti-diabetic activity, anti-inflammatory and also controlling

diarrhoea and dysentery, cough, dyspepsia, dropsy, urino-genital troubles.

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