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OPTIMIZATION OF GENETIC TRANSFORMATION IN *Spilanthes acmella* Murr BY USING *Agrobacterium tumefaciens* strain EHA 105 CARRYING A BINARY PLASMID VECTOR (pCAMBIA1301)

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ABSTRACT

***Spilanthes acmella* Murr belonging to Asteraceae is used both as an ornamental and medicinal plant. The present study was aimed to optimize *Agrobacterium tumefaciens*–mediated transformation of *S. acmella.* The bacterial density, infection period, co-cultivation temperature and acetosyringone (AS) concentration were evaluated by using leaf explants in transformation. Bacterial culture was added to 50 mL liquid YEP medium supplemented with kanamycin and rifampicin; and grown until it reached the required growth phases (A_{600nm}). Bacterial optical density ranging from A_{600nm} 0.15, 0.53, 1.0 and 1.5 were used. The co-cultivation medium (liquid MS medium) consisted of various concentrations of AS ranging from 100-500 µM. Histochemical analysis of β-glucuronidase *(GUS)* gene expression was carried out in control and transformed leaf explants. Higher bacterial density resulted in more transformation efficiency, but also higher necrosis in explants. Dilution of bacterial suspension reduced necrosis in explants and resulted in higher transformation. The optimized conditions for efficient transformation were: 1:10 dilution of A_{600nm} 0.15 bacterial density, infection period 30 min, cocultivation temperature of 22ºC. The addition of AS into bacterial inoculum at 100 µM resulted in 2.5-fold increase in transformation efficiency. In future, useful genes can be introduced into *S. acmella* by using *A. tumefaciens* carrying a binary vector pCAMBIA 1301.**

Keywords: *Agrobacterium tumefaciens*, *Spillanthes acmella*, *GUS* gene, pCAMBIA 1301 vector, transformation, acetosyringone

INTRODUCTION

Spilanthes acmella Murr belongs to the family *Asteraceae* (Compositae) and is found in tropical and sub-tropical regions all over the world including North Australia, Africa, Malaya, America, India and Sri Lanka. In India, it is confined to South and central parts of Jharkhand (Yadav and Singh, 2010). Of the 35 species of *Spilanthes,* three are reported from India. *S. acmella* is as an ornamental which grows up to 20-50 cm in height. It is mostly grown as annual plant with gold and red flowers without petals. Leaves are ovate. Flowering and fruiting occurs during March-April each year (Savadi *et al*., 2010). The plant possesses several medicinal properties like anti-fungal, anticonvulsant, antidiarrheal, antidiuretic, antiseptic, anti-protozoal, antipyretic, and insecticidal activities (Uthpala and Navaratne, 2020; Lalthanpuii and Lalchhandama, 2020). The plant produces several important secondary metabolites like terpenoids, saponins, sterols, coumarins, flavonoids, polysaccharides and alkylamides (Dallazen *et al.*, 2020). Among these secondary metabolites, spilanthol ((E, E, Z)-2,6,8-decatrienoic acid N-isobutylamide) present in the leaves, flowers, stems and roots has a sturdy pungent taste, shows

astringency and anaesthetic effects (Joshi *et al*., 2020). *In vitro* propagation studies of *S. acmella* have shown more accumulation of spilanthol in root tissue (Singh and Chaturvedi, 2012).

In spite of several genetic transformation methods available for plants, *Agrobacterium* mediated transformation is preferred due to the advantages of simplicity, precision, integration of large size DNA and stable gene expression (Bent, 2000**).** In addition, it has wide host-range to deliver the genes successfully into many agronomically important crops like soybean (Li *et al*., 2017), rice (Ratanasut *et al*., 2017), wheat (Hayta *et al*., 2019). For successful transformation, the optimization of physico-chemical parameters is prerequisite (Kutty *et al.,* 2010). The efficiency of *Agrobacterium*-mediated transformation depends on the plant species, the bacterial strain and the binary vector used. In case of chrysanthemums genetic transformation by *Agrobacterium* was found affected by the strain, binary vectors, and cultivars (Dolgov *et al*., 1997). *Agrobacterium tumefaciens* mediated transformation was previously reported in *S. acmella* by using *A. tumefaciens* strain LBA4404 bearing the binary vector pBI121 *(*Maggini *et al*., 2022). Optimizing the parameters for transformation varies for various plant cultivars, species and even for the *Agrobacterium* strains and type of expression vector used. It is necessary to optimise the physicochemical parameters to achieve high transformation efficiency. An increase in transient expression is expected to yield stable expression of transgenes. The β-glucuronidase *(GUS)* gene is used as a reporter gene as it offers easy detection of expression by histochemical assay (Jefferson, 1987). The present study was aimed to optimize genetic transformation in *Spillanthes acmella* by using *A. tumefaciens* strain EHA 105 containing plasmid vector pCAMBIA1301.

MATERIALS AND METHODS

The experiments were conducted during 2018 to 2022 in the Department of Biotechnology, School of Herbal Studies and Naturo Sciences, Dravidian University, Kuppam, Andhra Pradesh (India). *Agrobacterium tumefaciens* strain EHA 105 containing the binary plasmid vector (pCAMBIA1301) was used as vector system for transformation (Fig. 1) and was obtained from the Directorate of Rice Research, Hyderabad (India). Acetosyringone, *X-Gluc* and kanamycin were procured from Hi-media, Mumbai (India) and rifampicin was procured from Lupin Ltd, Mumbai (India).

The plant *Spillanthes acmella* (Fig. 2) was collected from the Herbal Garden, Dravidian University, Kuppam. *In vitro* grown seedlings of *S. acmella* were produced on MS medium and leaf explants used in transformation studies. The identity of *S. acmella* was authenticated by Dr K. Madhava chetty, Department of Botany, S.V. University, Tirupati, Andhra Pradesh (India).

Agrobacterium strain EHA 105 containing pCAMBIA1301 was grown in liquid YEP medium (Kutty *et al*., 2010) along with antibiotics kanamycin 10 mg L^{-1} and rifampicin 100 mg L^{-1} and incubated at 28ºC for two days. Bacterial density ranging from A_{600nm} 0.15, 0.53, 1.0 and 1.5 were

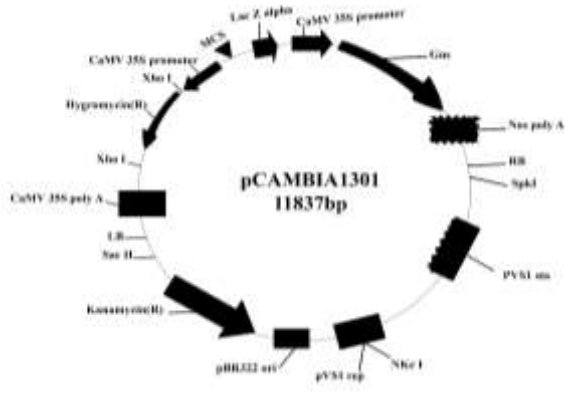

Fig. 1: Restriction map of PCAMBIA1301 vector

Fig. 2: Morphology of *Spilanthes acmella*

used in present study (Spectrophotometer, Hitachi U 2001, Japan). The 15 mL liquid culture was used to infect leaf explants of *S. acmella* explants procured from 2-3 week old seedlings. The explants were co-incubated with bacterial suspension separately at 22ºC for 15, 30 and 60 min.

The explants were placed on co-cultivation medium (Jayaseelan, *et al*., 2017) for two days in dark at 22ºC. The co-cultivation medium comprised of solid MS medium (Murashige and Skoog 1962) supplemented with various concentrations of acetosyringone (0, 100, 200, 300, 400 and 500 µM). Co-cultivation medium without AS served as control. Histochemical analysis of *GUS* gene expression was carried out as per Jefferson (1987). *GUS* positive spots were analysed by using a trinocular microscope (HBio, India). Each treatment was replicated three times and each replicate consisted of at least 10 explants. All the data were subjected to the analysis of variance (ANOVA) 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

The transformation using *A. tumefaciens* strain EHA 105 was assessed at various bacterial loads ranging from A_{600nm} 0.15-1.5 and the infection periods analysed at 15, 30 and 60 min. Of the four bacterial densities tested, the absence of necrotic explants and *GUS*-positive explants (22.5%) were observed at A_{600nm} 0.53, 30 min (Table 1). The transient activity and transformation frequency decreased with increase in bacterial load and infection period. The percentage of *GUS* positive explants from bacterial density at A_{600nm} 0.15 to 1.0 culture increased to 27.2% and decreased at A_{600nm} 1.5 cultures. Transformation frequency decreased as the necrotic damage increased from lower bacterial density (A_{600nm} 0.15) to higher density (A_{600nm} 1.5). This indicated that the bacterial density and infection period have direct effect on transformation efficiency. *Agrobacterium*-mediated transformation is effected by many physicochemical factors. In present study the physicochemical parameters were evaluated by using *GUS* as a reporter gene. The transient expression of *GUS* gene is easily measured from the transformed plant cells (Li *et al*., 2021). Genetic transformation by *A. tumefaciens* EHA-105 was optimized by evaluating physical and chemical factors. Such strategy has earlier resulted in developing efficient method of gene transfer to many plants like tobacco (Kutty, 2010) and cotton **(**Kalbande and Patil, 2016). Expanded *Spillanthes acmella* young green leaves of 4-5 week's age were excised from *in vitro* grown micro-shoots and used as explants for all experiments.

Table 1: The effects of bacterial load and infection periods on transient *GUS* expression and necrotic damage of leaf explants of *S. acmella*

Bacterial density (A_{600nm})	Infection period (min)	No. of spots explant^{-1} (mean± SE)	*GUS* positive explants (%)	Necrotic explants (mean ± SE)	Necrotic explants (%)
0.15	15	0.0 + 0.00^{a}	0.0	0.0 + 0.00^{a}	0.00
	30	1.9 + 0.12^{c}	22.5	0.0 + 0.00^{a}	0.00
	60	1.6 + 0.02^{b}	29.3	1.8 + 0.05^{b}	6.1
0.53	15	10.2 + 0.30^{k}	26.5	3.3 + 0.03^{d}	11.3
	30	7.2 + 0.05^{j}	28.2	2.6 + 0.1^{c}	8.9
	60	6.0 + 0.01^{h}	19.4	4.0 + 0.05^{e}	13.7
1.00	15	6.2 + 0.20^{i}	23.6	8.5 + 0.02^{f}	29.2
	30	5.0 +0.10^{g}	27.2	15.2 + 0.2^{g}	52.2
	60	4.5 + 0.05^{f}	18.2	18.5 + 0.1^{h}	62.5
1.50	15	3.2 + 0.05^{e}	20.5	21.7 + 0.3^{i}	72.1
	30	2.8 + 0.20^{d}	19.3	25.2 + 0.1^{j}	86.6
	60	1.6 + 0.10^{b}	18.7	28.3 + 0.05^{k}	97.2

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

Higher concentrations of *Agrobacterium* were reported to transform recalcitrant plants like orchid (Gnasekaran *et al*., 2014), blackgram (Sainger *et al*., 2015) and tobacco (Niedbała *et al*., 2021). Low bacterial density has previously been used to transform plants such as tobacco **(**Kutty, 2010), common bean (Collado *et al*., 2015), sun flower (Zhang and Finer, 2016) and wheat (Ashrafi-Dehkordi *et al*., 2021). In present study early log phase was found best for transforming *Spillanthes acmella*. Bacterial density at A_{600nm} 0.15 growth phase, 30 min was found to be most effective in producing least necrotic explants and it showed better 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 inoculum was diluted before infection. The transformation efficiency was found at A_{600nm} 0.53 was higher than 1.0 and their mean differences were statistically significant. Necrotic damage was absent in bacterial density at A_{600nm} 0.15 and infection period 30 min; and it was chosen in the further experiments.

Table 2: Effect of various co-cultivation temperatures on transient *GUS* expression of *S. acmella*

Co-cultivation temperature (° C)	No. of spots explant^{-1} (Mean± SE)	*GUS* positive explants (%)
20	40.0 ± 4.13^{b}	28.0
22	68.2 ± 0.4^{c}	75.0
25	32.5 ± 5.5^{a}	43.0

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

The effect of various co-cultivation temperatures on transient *GUS* expression is given in Table 2. The explants were precultured for 2 days, followed by co-cultivated at 3 temperatures (20, 22 & 25ºC). The highest *GUS* positive spots were found at 22ºC with mean value 68.2 and least number of blue spots were observed at 25ºC. This indicates that co-cultivation temperature has direct effect on *Agrobacterium*-mediated transformation. The mean differences in *GUS* positive spots at 20 and 25ºC were significant. The percentage of *GUS* positive explants were approximately 75% at temperature 22ºC and low at 20 and 25ºC (Table 2). After analysing with trinocular microscope, 68.2 ± 0.4 spots explant^{-1} incubated at 22ºC and 40 ± 04.13 and 32.5 ± 05.5 at 20 and 25ºC, respectively. There were several reports on higher transformation efficiency where explants co-cultivated at 22ºC and obtained more number of *GUS* positive spots in *Withania somnifera* Dunal (Mishra *et al*., 2016), *Artemisia Pallens* (Alok *et al*., 2016) and elephant foot yam (Abraham *et al*., 2021). Reports reveal that the size of crown gall tumour decreases when co-cultivation temperature is increased (Braun, 1947). Also, Ti-plasmids were lost in *A. tumefaciens* when the culture was grown over 36 h at elevated temperatures**.** Low temperatures from 20 to 22ºC reportedly promote pilus assembly and is influenced by *VirB* gene at low temperature which is required for conjugal transfer of T-DNA into plant cells (Fullner *et al*., 1996).

In present study the co-cultivation temperature of 22ºC was found effective to get more number of *GUS*-positive spots as well as more number of *GUS*-positive explants. Hence, we chose 22ºC as co-cultivation temperature for further experiments. The results support the earlier reports of efficient T-DNA transfer at 22-25^{0}C as compared to the high temperatures (Salas *et al*., 2001) in common bean (Collado *et al*., 2016).

The effect of acetosyringone (AS) on *Agrobacterium*-mediated transformation was studied using five concentrations (100-500 µM). Different AS concentrations were added separately to the co-cultivation medium, bacterial inoculum and results were shown in Table 3. The AS concentrations of 100 µM in bacterial inoculam gave significantly high number of *GUS* positive spots with mean value of 186.2 ± 0.1. The low *GUS* positive spots were observed at 200-500 µM AS concentration. The number of blue spots per explant at 22ºC were 68.2 without AS and increased to 186.2 on addition of 100 µM AS in bacterial inoculam (Table 3). Other AS concentrations did not show any increase in transient *GUS* expression. It revealed that *Agrobacterium* cells may have been induced to maximum towards virulent stages at 100 µM AS concentration which has also been reported in other plants (Farkhondehtale *et al*., 2022). The optimum concentration of AS for higher transient expression varies based on genotype and cultivar of plant **(**Satish *et al*., 2017).

Table 3: AS in co-cultivation medium and bacterial inoculum and its effect on transient *GUS* expression in *S. acmella*

Concentration of acetosyringone (µM)	Co-cultivation medium		Bacterial inoculums	
	No. of spots (Mean ± SE)	*GUS* positive explants (%)	No. of spots (Mean ± SE)	*GUS* positive explants (%)
0	80.2 ± 0.02[a]	85	95.2 ± 0.3[a]	90
100	158.2 ± 0.5[f]	92	186.2 ± 0.1[f]	95
200	150.3 ± 0.2[e]	89	173.3 ± 0.3[e]	93
300	143.5 ± 0.4[d]	85	152.2 ± 0.1[d]	87
400	136.2 ± 0.5[c]	80	145.0± 0.6[c]	85
500	123.6 ± 0.4[b]	79	133.2 ± 0.2[b]	82

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

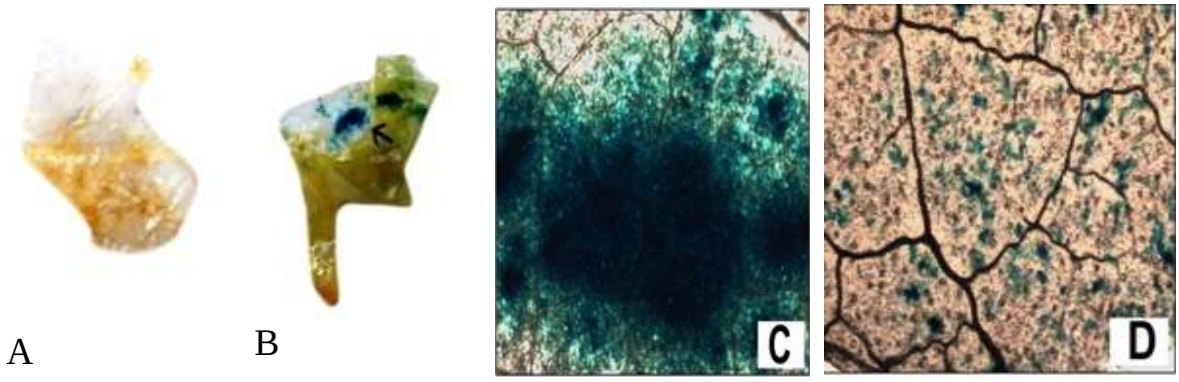

Fig. 3: *GUS* histochemical analysis of *S. acmella;* A) Control, B) Explants co-cultivated at 22°C followed by treatment of 100 µM acetosyringone in bacterial inoculum expressing high level of transient *GUS* activity, C) Close up image of *GUS* positive explant, and D) A microscopic view of *GUS* positive blue spots

The method of Jefferson (1987) was used for *GUS* histochemical assay. The positive results were observed with naked eye and each blue spot was counted using stereomicroscope, irrespective of its size (Fig. 3). The explants co-cultivated at 22ºC followed by treatment with 100 µM AC in bacterial inoculum (Fig. 3B) and control (Fig. 3A). An individual positive explant is shown with magnification (Fig. 3C). A close-up image of microscopic view of *GUS*-positive explants with blue spots are shown in Fig. 3D.

Conclusion: An easy and efficient method of genetic transformation by using *Agrobacterium tumefaciens* strain EHA 105 containing binary plasmid vector (pCAMBIA1301) was optimized by evaluating various important parameters. The transformation efficiency is increased to 2.5 fold. The protocol may help in metabolic engineering of *Spilanthes acmella* to enhance amount of desired bioactive compounds by metabolic engineering.

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