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Optimization of *Agrobacterium tumefaciens*-mediated Transformation in *Oxalis corniculata* (L.)

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ABSTRACT

Background: Optimization of co-cultivation parameters during *Agrobacterium*-mediated transformation to *Oxalis corniculata* evaluated were bacterial density, infection period, acetosyringone (AS) concentration and co-cultivation temperature. Five fold transformation efficiency is achieved in transient *gus* gene expression after optimizing various parameters. 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 (400 µM) and cocultivation temperature of 22°C.

Methods: *Oxalis corniculata* (L.) 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, 0.56,0.80 and 1.34 OD were used in the present study. The co-cultivation medium made of solid MS medium consisted of BAP 2 mg L^{-1} and NAA 0.5 mg L^{-1} and various concentrations of AS at 0, 50, 100, 200, 400,600 and 800 µM. Histochemical analysis of 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 64% when the infection process was carried out with acetosyringone in co-cultivation medium (400 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, Oxalis corniculata,* GUS gene.

INTRODUCTION

Oxalis corniculata Linn is a medicinally important plant also known as creeping wood sorrel or sleeping beauty. The flora appears to be very delicate, low-growing, herbaceous plant of *Oxalidaceae*. It is expansively found in the regions of tropical and sub-tropical sub-regions of the world. These woody sorrels are grown all over the year (perennial) and with tap rooted herbs. The herb is bushy or mat forming and 0.1-0.5 m tall. The arrangement of foliage is alternate along the stems. The leaves are in the form of tri-foliate with thin, heart shaped; leaflet blades contain a distinctive apical indention. A single long stalk arises from the axils of the leaf, in which three flower stalks extends each one with a single flower. The trifoliate leaves are alternate with slender, heart shaped, leaflet blades having a discrete apical indention.

Oxalis corniculata acquires vital activities like anti-oxidant, anti-cancer, anthelmintic, anti-inflammatory, analgesic, steroidogenic and anti-microbial, anti-amoebic, antifungal, astringent, depurative, di-uretic, febrifuge, cardiorelaxan, stomachic and styptic and many other properties. It has been used to treat headache by the tribal people in koraput district of Odisha and also as a folk medicine to cure skin diseases and in raw form is fine for digestion (Kohli, 1993 and Das *et al*., 1987). It is also has healing properties to cure dysentery, diarrhoea, piles and skin diseases (Arya, 1995 and Kirthikar *et al*., 1935). *Oxalis corniculata* has also showed perceptible antibacterial activity beside *E. coli* (Unni *et al*., 2009).

Metabolic engineering will give an opportunity to enhance the desired product of medicinal plants. Monoterpene biosynthesis is subjected to metabolic engineering and successfully produced *trans*-isopiperitenol in tobacco (Lucker *et al*., 2004). Genetic transformation is most essential method needed to deliver the genes into desired medicinal plants.

Agrobacterium is widely used in plant transformation due to its simplicity, efficient integration of large size DNA

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and stable gene expression (Bent, 2000). β-glucoronidase (GUS) reporter gene is used in many plant transformation studies (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 and it helps in developing an efficient method which leads to the regenerartion of whole plant from the transformed explants. In this study we describe an optimization of *Agrobacterium* mediated transformation in *Oxalis corniculata* (L.). The parameters tested were *Agrobacterium tumefaciens* growth phase, infection period, pre-culture of explants prior to infection, addition of acetosyringone (AS) in co-cultivation medium and in bacterial inoculum and co-cultivation temperature. Optimizing the parameters for transformation is varies among various plant cultivars, species and even for *Agrobacterium* strains. Since the transformation efficiency depends on type of plant, explant and bacterial strains, it is necessary to optimise the parameters. A suitable and efficient method of genetic transformation of *Oxalis corniculata* by using *Agrobacterium tumefaciens* with pCAMBIA1301 plasmid vector is not reported so far.

MATERIALS AND METHODS

The experiments were conducted in the duration of 2010-11 and 2017 to 2019 at the department of Biotechnology, School of Herbal Studies and Natuoro Sciences, Dravidian University, kuppam andhrapradesh.

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 as described by with the following chemicals compositions 10 g of yeast extract, 10 g of peptone and 5 g of Nacl L^{-1}. The culture was incubated at 28°C for two days.

Plant material

The plant material of *Oxalis corniculata* (Fig 2) was collected from the Herbal garden, Dravidian University, Kuppam, Andhra Pradesh, India. Young and green leaves were dissected from *in vitro* grown shootlets and they were used as explants for transformation experiments.

Cocultivation

Bacterial density ranged from OD A_{600nm} 0.17, 0.56, 0.8 and 1.34 were used in the present study. A total volume of 15 ml of liquid culture was used to leaf explants of *Oxalis corniculata* from 2-3 weeks old seedlings. The explants were co-incubated with bacterial suspension at 22, 25 and 28°C. The infection period was carried out for 30 min to evaluate the effects of different cocultivation temperatures on the transformation process.

Addition of Acetosyringene (AS) in co-cultivation medium

The leaf 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 BAP 2 mg L^{-1} and NAA 0.5 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 analysis was done to determine the effects of AS on transformation efficiency.

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 15 explants. All 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 experiments were repeated at least thrice.

RESULTS AND DISCUSSION

Agrobacterium-mediated transformation is effected by many physical and chemical factors. In the current 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 explants (Dundar 2008). Gene delivary by *Agrobacterium tumefaciens* EHA-105 can be improved by evaluating both physical and chemical factors which was resulted in producing a suitable method of gene transfer to several plants such as tobacco (Kutty *et al*. 2010) and groundnut (Ashutosh Vadawale *et al.* 2012).

Bacterial growth phase and infection period

The results obtained after transformation using *Agrobacterium tumefaciens* strain EHA 105 at various bacterial concentrations ranged from A_{600nm} 0.17-1.34 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 (mean value1.17±0.09). The transformation frequency was decreased with increased bacterial density and infection period.

The percentage of GUS positive explants from bacterial density at A_{600nm}0.17 to 0.88 cultures were increased (48.33%) and decreased at A_{600nm} 1.34 cultures. Transformation frequency decreases as necrotic damage increases from lower bacterial density (A_{600nm} 0.17,) to higher density (A_{600nm} 1.34). This indicates that explants viability has direct effect on transformation efficiency. Among the three infection periods used, only explants incubated bacterial density at A_{600nm}0.17 for 30 min gave transformants without necrotic damage (Table 1).

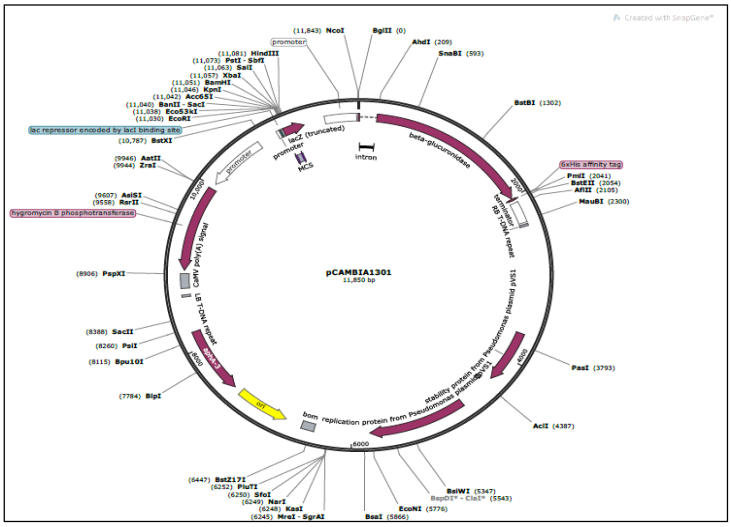


Fig 1: Restriction map of PCAMBIA1301 which is used in the present study. (Source:https://www.biocat.com/lp/vectors/maps/201804/pCAMBIA1301%20Map.pdf).

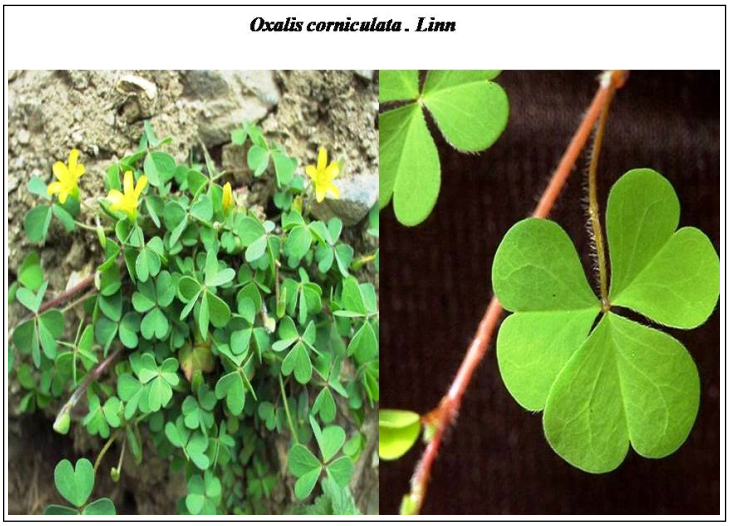


Fig 2: Morphology of *Oxalis corniculta* (A) plant with flowering (B) closeup image of trifoliate leaves.

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

Bacterial density Infection (A_{600nm})	Period (min)	No. of spots (mean±SE)	Percentage of GUS positive explants	Necrotic explants (mean±SE)	Percentage of necrotic explants
0.17	15	0.59±0.03^{a}	0.00	0.00±0.00^{a}	0.00
	30	1.17±0.09^{b}	1.33	0.00±0.00^{a}	0.00
	60	0.51±0.05^{a}	1.66	1.50±0.33^{b}	8.86
0.56	15	3.28±0.13^{a}	4.66	1.66±0.33^{a}	11.11
	30	11.37±0.38^{b}	14.33	2.33±0.33^{a}	15.55
	60	10.39±0.36b	26.66	4.00±0.57^{b}	26.66
0.88	15	10.95±0.25^{a}	39.33	5.33±0.66^{a}	35.55
	30	13.86±0.35^{c}	48.33	5.33±0.88^{a}	35.55
	60	12.22±0.21^{b}	38.33	6.66±0.66^{a}	44.44
1.34	15	5.66±0.31^{b}	23.66	6.00±1.15^{a}	40.0
	30	3.24±0.18^{a}	18.66	9.00±1.45^{ab}	62.22
	60	3.35±0.27^{a}	15.33	12.00±1.15^{b}	80.0

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

Higher bacterial densities of *Agrobacterium* were used to transform recalcitrant plants such as rice, 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) and tobacco (Kutty *et al*., 2010). But in our study early log phase was found to be the best for transforming *Oxalis corniculata* L. Bacterial density at A_{600nm} 0.17 growth phase was found to be most effective in producing high transformation efficiency without necrotic damage. In order to minimise the necrotic damage of explants, culture was diluted to 1:10 before infection of explants. The high transformation efficiency was found at A_{600nm} 0.56, 0.8 and their mean differences were statistically significant. Necrotic damage at A_{600nm} 0.17, 30 min was least hence bacterial density at A_{600nm} 0.17 and infection period 30 min minutes was chosen in this study.

Co-cultivation temperature

The effect of temperature during Co-culture on T-DNA delivery was first reported in dicotyledonary plants (Opabode 2006). The effects of different co-cultivation temperatures on transient GUS expression were studied and the result were shown in Table 2. The explants were co-cultivated at three different temperatures like 22, 25 and 28°C and the highest GUS positive spots were found at 22°C with the mean value (33.33 ±2.02) and less number of GUS positive spots at 25 and 28°C (13.33±1.33), (12.00±1.0) respectively. This indicates that co-cultivation temperature has direct effect on *Agrobacterium* 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 26.66% at temperature 22 and 13.33% at 25 and 28°C. After analysing with trinocular microscope, highest number 33.33 (±2.02) spots were found per explant at 22 and lowest number found at 25 and 28°C 13.33 (± 1.33), 12.00 (±1.0) respectively.

Present study involves three different Co-cultivation temperatures. There were several reports on higher transformation efficiency where explants Co-cultivated at 22°C and obtained high number of GUS-positive spots (Dillen *et al.,* 1997) (Kutty *et al.,* 2010). Efficient DNA delivery into plant cells at 22°C was also reported in Sweet potato (Gonzalez *et al.,* 2008) 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).

Based on the results obtained, Co-cultivation temperature either at 22°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 22°C as co-cultivation temperature and it was introduced in further experiments. Cocultivation temperature at 25 and 28°C resulted in low transformation efficiency. Hence we have chosen 22 instead of 25 or 28°C. It is also supported by the reports that 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 concentrations of AS 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 400 µM concentration gave highest number of GUS positive spots with the mean value (171.00±1.15). The numbers of GUS positive spots were gradually increased at AS concentration from 50-200 µM and decreased from 600-800 µM. The mean differences of the number of blue spots from 50-800 µM were statistically significant (Table 3). The control is without blue sopts (Fig 3A) and the percentages of GUS-positive explants were highest 64.44 at AS concentration 400 µM (Fig 3B). One of the GUS positive explants was shown with magnification (Fig 3C). The number of GUS-positive spots were counted using Trinocular microscope and one of such explant with microscopic view was shown (Fig 3D). It was well documented by Ismail *et al*. (2006) that the optimal concentration of AS for higher transient GUS expression differs based on genotype and cultivars of plant. It reveals that *Agrobacterium* cells may have been induced to maximum towards virulent stages at 400 µM concentration of AS. The higher concentration was also reported in other plants (Li *et al*., 2007 and Kumar *et al*., 2010).

Table 2: The effects of various co-cultivation temperatures on GUS - positive spots.

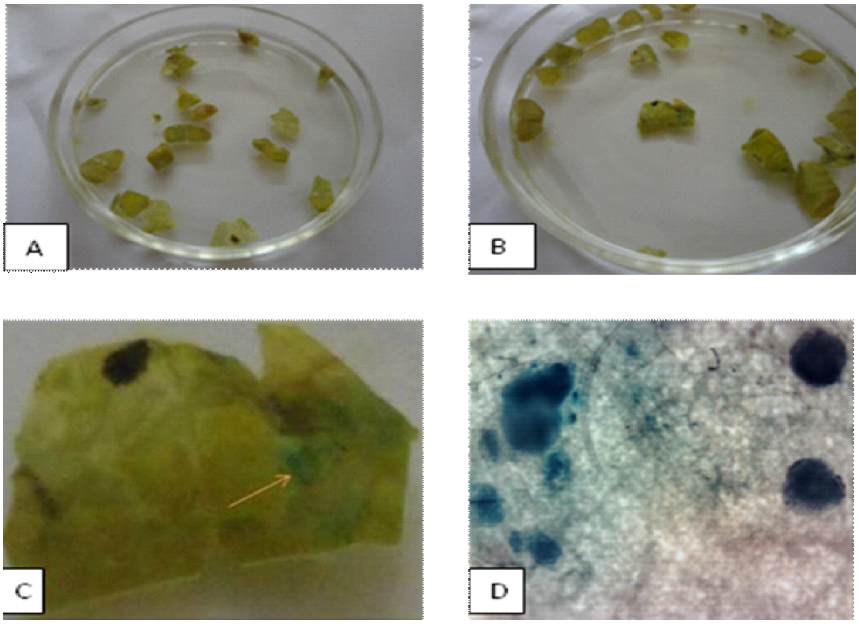
Co-cultivation temperature (°C)	No. of spots (Mean±SE)	GUS positive explants (%)
22	33.33±2.02^{b}	26.66
25	13.33±1.33^{a}	13.33
28	12.00±1.0^{a}	13.33

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	34.66±1.76^{a}	8.88
50	43.33±1.85^{b}	6.66
100	69.00±1.15^{d}	33.33
200	84.33±1.14^{e}	44.44
400	171.00±1.15^{f}	64.44
600	54.00±1.50^{c}	26.66
800	40.66±1.20^{b}	20

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



(A) Control (B) GUS positive explant after optimization with cocultivation temperature 22°C, AS 400 µM and the number of GUS-positive explants obtained were 10 per 15 explants (C) A magnified image of GUS positive explant (D) A close up image of Microscopic view of blue spots of GUS-positive explants.

Fig 3: GUS histochemical analysis of *Oxalis corniculata* leaf explants transformed by *Agrobacterium tumefaciens*.

There was no increase in the number of GUS positive explants after adding AS into the bacterial inoculum (data was not shown).

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 not observed the expansion of leaf explants even after incubating on MS medium for two days. Pre-culturing of explants results in rapid cellular dedifferentiation and new cells which are having weakened pathogen recognition ability (Sangwan *et al*., 1992).

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 (Song and Sink, 2005), tobacco (Sunilkumar *et al.,* 1999) ginger (Suma *et al.,* 2008).

GUS Histo chemical analysis

The method of Jefferson (1987) was used for a GUS histochemical assay. The GUS positive explants were observed with naked eye and each blue spot was counted using stereomicroscope (Fig 3D). The explants co-cultivated at 22°C and 400 µM AS resulted GUS-positive explants were 10 per 15 explants (Fig 3B). The Control (Fig 3A) and a magnified GUS positive explant were shown (Fig 3C). A close-up image of microscopic view of GUS-positive explants with blue spots were shown (Fig 3D).

CONCLUSION

An easy and efficient method for *Agrobacterium tumefaciens* mediated transformation was optimized by evaluating various important parameters. These parameters were,1:10 dilution of A_{600nm} 0.17 bacterial density, infection period 30 min, addition of AS into cocultivation medium at 400 µM, cocultivation temperature of 22°C and transformation efficiency is 64%. Ultimately this protocol helps in metabolic engineering of *Oxalis corniculata* to improve the above traits and produce more amount of desired secondary metabolites.

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Conflict of interest

All authors declared that there is no conflict of interest.

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