OPTIMIZATION OF FACTORS AFFECTING THE *Agrobacterium tumefaciens*-MEDIATED TRANSFORMATION OF *Eucalyptus saligna*[1]

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ABSTRACT – This study aimed to evaluate the effect of factors that may affect the genetic transformation of cotiledonary explants of *Eucalyptus saligna* mediated by EHA105 strain of *Agrobacterium tumefaciens*. The vector pBI121 carrying *gus* gene under control of 35S CaMV promoter was used. The effect of the following factors was evaluated: explant pre-culture, use of different antibiotics and presence of acetosyringone (AS) in co-culture media. An antioxidant solution was also used during excision, containing ascorbic acid (250mg.L^{-1}), citric acid (25mg.L^{-1}) and PVP-40 (1g.L^{-1}). Pre-culture of the explants before the co-culture with bacteria was done over a 4-day period in MS culture medium supplemented with 4.4µM BAP and 2.7ìM NAA. After the co-culture period, three concentrations of kanamycin (12.5; 25 and 50mg.L^{-1}) combined with 300mg.L^{-1} Augmentin® in the culture medium were tested The influence of the antibiotic was also evaluated by keeping the explants in a medium containing 50mg.L^{-1} Km and 300mg.L^{-1} Augmentin® or 500mg.L^{-1} cefotaxime. It was concluded that Augmentin® stimulates organogenesis, that a Km concentration of 12.5mg.L^{-1} allows selection of explants transformed with *gus* gene and, finally, the addition of AS (50ìM) to the liquid and solid co-culture media has a positive effect on *gus* gene expression. Moreover, the use of an antioxidant solution during cotyledon excision is dispensable and the pre-culture of the explants has no effect on bud regeneration or *gus* gene expression. A transformation efficiency of 1.5% was reached.

Keywords: Acetosyringone; Antibiotic; Kanamycin

OTIMIZAÇÃO DE FATORES QUE AFETAM A TRANSFORMAÇÃO GENÉTICA DE Eucalyptus saligna MEDIADA POR Agrobacterium tumefaciens

RESUMO – Esse trabalho teve como objetivo avaliar o efeito de vários fatores que podem afetar a transformação genética de explantes cotiledonares de Eucalyptus saligna *mediante co-cultura com a cepa EHA105 de* Agrobacterium tumefaciens. *Foi utilizado o vector pBI121 carregando o gene repórter* gus *sob controle do promotor 35SCaMV. Foi estudado o efeito dos fatores a seguir: pré-cultura dos explantes, uso de diferentes antibióticos e presença de acetosiringona (AS) nos meios de co-cultura. Uma solução contendo ácido ascórbico (250mg.L^{-1}), ácido cítrico (25mg.L^{-1}) e PVP-40 (1g.L^{-1}) foi utilizada no momento da excisão dos explantes. A pré-cultura dos explantes antes da co-cultura com a bactéria foi de 4 dias, em meio de cultura MS suplementado de 4,4µM BAP e 2,7ìM ANA. Após a co-cultura três concentrações de canamicina (12,5; 25 e 50mg.L^{-1}) combinadas com 300mg.L^{-1} de Augmentin® no meio de cultura foram testadas. O efeito do Augmentin® (300mg.L^{-1}) nos explantes foi comparado ao da cefotaxima (500mg.L^{-1}), em meio contendo 50mg.L^{-1}de Km. Foi concluído que Augmentin® estimula a organogênese, em comparação com a cefotaxima, que a concentração de 12,5mg.L^{-1}*

de Km permite a seleção de explantes transformados com o gene gus *e, finalmente, a adição de AS (50ìM) aos meios de co-cultura líquido e sólido tem efeito positivo sobre a expressão do gene* gus*. O uso de antioxidante não é necessário e a pré-cultura dos explantes antes da inoculação com* A. tumefaciens *não tem efeito na regeneração de gemas nem na expressão do gene* gus*. A eficiência de transformação foi de 1,5%.*

Palavras-Chave: Acetosiringona; Antibiótico; Canamicina.

1. INTRODUCTION

Eucalyptus genus is economically important worldwide and represents one of the main sources of biomass (Girijashankar, 2011). In 2014, the total area planted with *Eucalyptus* in Brazil was approximately 5.6 million ha (SNIF, 2015) of which the main part was intended for paper and pulp production. In southern Brazil this industry relies primarily on plantations of *E. saligna* Smith and *E.dunnii* Maiden for cellulose supply (Fett-Neto et al., 2001). *E. saligna* is also one of the species with great potential for production of veneers and plywood panels for outdoor use (Iwakiri et al., 2013).

By conventional breeding methods, genetic improvement of forest trees is limited, due to their long breeding cycles, high level of heterozygosity and large segregated populations (McRae and Van Staden, 1999). Biotechnological methods are important as they reduce the long time needed for breeding. Several techniques of genetic transformation have already been applied to *Eucalyptus* species and *A. tumefaciens*-mediated protocols have been the most followed (Girijashankar, 2011). The biolistic method was used less frequently, but some reports indicate that it can be successfully applied, for example recently in the case of *E. camaldulensis* plants (Mendonça et al., 2013).

Dibax et al. (2010) established an *A. tumefaciens*-mediated protocol for genetic transformation of *E. saligna* leaf explants, with an efficiency of transformation of 0.5% (1:200), indicating the need for an optimized protocol. Various factors may affect the success of plant transformation by indirect methods, some of them acting on bacteria virulence, others on the bud regeneration process. In the first case, the use of phenolic compounds that improve *Agrobacterium* virulence and then gene transfer into plant cells is important for transformation of some plant species. The co-culture period is also a crucial factor (Seong and Song, 2008). In the second case, a pre-culture period and treatment of explants with antioxidant compounds may improve the regeneration process (Seong and Song, 2008; Li et al., 2011). Kanamycin is an aminoglycoside antibiotic used to select the tissues transformed with *npt*II (kanamycin resistance) gene. Its concentration is species-dependent and must be tested in each case. On the other hand, the antibiotics used to eliminate the agrobacteria after the co-culture can inhibit (Ogawa and Mii, 2005) or promote organogenesis (Danilova and Dolgikh, 2004).

This study aimed to evaluate the effect of some of these factors that may affect bud regeneration or transformation efficiency of *E. saligna* cotyledonary explants, such as explant treatment with an antioxidant solution, explant pre-culture, type of antibiotic used to eliminate the *Agrobacterium* after the co-culture and addition of acetosyringone into the co-culture media.

2. MATERIAL AND METHODS

Plant material and *in vitro* culture. Cotyledons of *E. saligna* plantlets cultured *in vitro* were used as an explant source twelve days after sowing. The explants were cultured in Petri dishes (2 x 10 cm), containing 25 ml of culture media, sealed with PVC film and kept at 25 ± 2ºC under fluorescent tubes providing a cold white light with an irradiance of 47µmol m^{-2} s^{-1} and a 16 h photoperiod. All culture media had their pH adjusted to 5.8 prior to autoclaving at 120 °C for 20 min.

Genetic transformation of *E. saligna*. The protocol followed in this study was the one published by Dibax et al. (2010). The *Agrobacterium tumefaciens* strain used for the genetic transformation of *E. saligna* was EHA105 (Hood et al., 1993) containing the binary vector pBI121. This vector carried the â-glucuronidase reporter gene (*gus*), under control of the CaMV35S constitutive promoter, and the neomycin phosphotransferase selection marker gene (*nptII*) under control of *nos* promoter. Entire cotyledonary explants were immersed in a bacterial solution of A_{600} = 0.6 for 30 min in an orbital shaker (120 rpm). They were then cultured for five days on MS (Murashige and Skoog, 1962) medium with half concentrations of potassium

and ammonium nitrates, 2.7 ìM NAA, 4.4 ìM BAP and 30 gL^{-1} sucrose (Dibax et al., 2005). After this period, explants were transferred onto the selective medium (the same medium, supplemented with Km and cefotaxime (Cx) or Augmentin, according to the experiment). The histochemical assay for â-glucuronidase activity (Jefferson, 1987) was performed on 15 explants of each treatment five and twelve days after the bacterial inoculation. Explants with one or more blue region were considered *gus* positive.

Use of an antioxidant solution during explant excision. The treatments were: (a) explants in a solution containing ascorbic acid (250 $mg.L^{-1}$), citric acid (25 $mg.L^{-1}$) and polyvinylpyrrolidone (PVP-40) (1$g.L^{-1}$) at pH 5 (Tournier et al., 2003); (b) explants in autoclaved water. The explants were then co-cultured with the bacteria as described above. After co-culture on solid medium, the explants were transferred onto the same medium, supplemented with 250 $mg.L^{-1}$ Cx and 50 $mg.L^{-1}$Km and subcultured every 15d in the dark. Each treatment consisted of four replicates of 50 cotyledons. The experiment was repeated once. The percentages of oxidized explants, explants forming calluses and forming buds were evaluated after 60 days.

Pre-culture of cotyledonary explants. The pre-culture consisted of four days on MS culture medium supplemented with 4.4 µM BAP and 2.7 ìM NAA before co-culture with the bacterial suspension. The control was not pre-cultured. The cultures were kept in the dark. Each treatment consisted of four replicates of 50 explants, except in some cases indicated below. The experiment was repeated once. *Gus* gene expression was observed after 5 d and oxidized explants, callus formation, bud regeneration after 60 d.

Kanamycin concentration. After the co-culture period, the explants were cultured for 60 d on the same medium, supplemented with 300 $mg.L^{-1}$ Augmentin®- Sandoz (amoxicilin/clavulanic acid - 1g/125mg) and Km (12.5, 25 and 50 $mg.L^{-1}$) in the dark and for another 60 d period under light. Subcultures on fresh medium were carried out every 15 d. Each treatment consisted of five replicates of 20 explants.

Histochemical â-glucuronidase assay and PCR analysis were performed five and 180 days after explant infection. The experiment was repeated twice.

Bud regeneration in the presence of Augmentin or cefotaxime. At the end of the 5d-co-culture, the explants were transferred to the same medium supplemented with 50 $mg.L^{-1}$ Km and Augmentin® (300 $mg.L^{-1}$) or Cx (500 $mg.L^{-1}$). Petri dishes were kept in the dark for 60 d, being subcultured on fresh medium every 15 d. After this period, the same evaluations were carried out as indicated above. Contamination of the explants with *A. tumefaciens* was also recorded. The treatments were composed of six replicates, with 20 explants each. The experiment was repeated once.

Acetosyringone and transformation efficiency. During the co-culture of the explants with the bacteria, 50 ìM of acetosyringone (AS) was added to the culture media, according to the following treatments: 1- addition to the liquid co-culture medium; 2- to the solid co-culture medium; 3- to both co-culture media; 4- without addition and 5- control not inoculated with the bacteria. After the co-culture period the explants were transferred to the same culture medium supplemented with Km (50 $mg.L^{-1}$) and Augmentin®(300 $mg.L^{-1}$). The culture conditions and evaluation factors were those described above. Each treatment consisted of five replicates of 20 explants. Histochemical â-glucuronidase assay was carried out after 5 and 12 d. The experiment was repeated once.

DNA extraction and PCR. DNA was extracted from three leaves of putatively transformed shoots 180 d after transformation and from control material not inoculated, and processed according to the protocol of Doyle and Doyle (1987). Putative transformed shoots were PCR-screened to detect the presence of *gus* reporter gene. Each reaction (25 µl) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM of each dNTP, 1.0 U of *Taq* polymerase, 50 ng DNA and 10 ìM of each oligonucleotide specific to the *gus* gene. Invitrogen's Taq DNA polymerase recombinant kit was used for DNA amplification. The following primer pair was used in the PCR assay: 5'-CAGCGCGAAGTCTTTATATACCG-3' and 5'-ATGCGTCACCACGGTGATATCG-3'. The samples were submitted to the following amplification programme in a thermocycler (Applied Biosystems, Veriti, 96 Well Thermal Cycler): 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, 72 °C for 7 min and 4 °C until application in the gel. After electrophoresis on 1.5% agarose (p/v) gel containing ethidium bromide (0.5 µg), the PCR products were visualized under UV radiation.

Experimental design and statistical analysis. In all experiments statistical design was totally randomized. Bartlett´s test for homogeneity of variance was applied and means compared by Tukey's multiple range test using ASSISTAT software (Silva and Azevedo, 2009).

3. RESULTS

Effect of the use of an antioxidant solution during explant excision. There was no difference between treatments (Table 1). Oxidation rate was high (83.2 and 87.2% for antioxidant and water treatment, respectively) after 60 d, indicating that the treatment was not efficient in controlling tissue oxidation. Callogenesis was observed in 62.8 and 63.6% of surviving explants and bud regeneration was low (Table 1).

Effect of explant pre-culture on transformation efficiency. The 4 d pre-culture did not affect the regeneration process and the results of oxidation and callogenesis were similar to those obtained in the previous experiment (results not shown). However, some bud regeneration was observed in both treatments (1.6 and 1.2% respectively for treated and untreated explants). Expression of the *gus* gene reached 20% of the explants in both cases (data not shown).

In the present study, pre-culture was not used in the following experiments.

Table 1 – Effect of treatment of cotiledonary explants of *Eucalyptus saligna* with an antioxidant solution* during excision and before inoculation with *Agrobacterium tumefaciens*. Mean of two experiments, evaluated after 60 d of culture.

Tabela 1 *– Efeito do tratamento dos explantes cotiledonares de* ***Eucalyptus saligna*** *com uma solução antioxidante* durante a excisão e antes da inoculação com* ***Agrobacterium tumefaciens****. Média de dois experimentos, avaliados após 60 d de cultura.*

Treatment	Oxidiz edexplants (%)*	Explantswith callus (%)*	Explants with buds (%)*
Antioxidant	83.2 a	62.8 a	2.4 a
Water	87.2 a	63.6 a	2.0 a
CV (%)	3.75	3.88	7.04

Means followed by the same letter within a column do not differ significantly by Tukey´s test at the 0.05 level. Culture medium: MS N/2 supplemented with 2.7 µM NAA and 4.4 µM BAP, 250 mg.L^{-1} Cx and 50 mg.L^{-1} kanamycin.*Ascorbic acid (250 mg.L^{-1}), citric acid (25 mg.L^{-1}) and PVP-40 (1 g.L^{-1}) at pH 5. CV= coefficient of variation.

Effect of kanamycin concentration in the selection of transformed tissues. Oxidation rate and callus formation were similar in the three treatments (12.5, 25 and 50 mg.L^{-1} Km) 60 days after inoculation (Table 2). However, on a medium containing 12.5 mg.L^{-1} Km, a higher number of explants formed buds with a higher number of buds per explant than in the other treatments and, after 180 d in the same medium, more than 15 buds were counted in 12% of explants.

Every shoot that developed from an explant on selection medium was considered as an event and molecular analysis was carried out in order to detect the presence of escapes. PCR of 13 events, 180 d after inoculation, revealed the presence of the *gus* reporter gene in three of them (Figure 1). In this experiment, the efficiency of transformation was 1.5% (3/200), three times superior to that obtained by Dibax et al. (2010).

Effect of two antibiotics on bud regeneration in cotyledonary explants. In the presence of Augmentin, the oxidation rate of the explants was lower, while callus and bud formation were higher than in the presence of Cefotaxime (Cx) (Table 3). Some contamination with *A. tumefaciens* was still observed, mainly when Cx was used (Table 3).

Effect of acetosiringone (AS) on transformation efficiency. The analysis of transient expression of the *gus* gene, performed 5 d after the initiation of the co-culture, showed the effect of AS (Figure2). It was noted that AS positively affected this expression when added to liquid and solid media and 80% of the explants expressed the *gus* gene (T3). Twelve days after initiation of the co-culture, surprisingly the highest number of explants expressing the *gus* gene was observed in explants treated with liquid co-culture medium containing AS (50%) (Figure 2). At this time, expression is already stable.

4. DISCUSSION

In *Eucalyptus* species, tissue browning frequently induces a reduction of growth and may lead to explant death. Several antioxidants may be used to avoid this phenomenon. PVP is a polyamide that impedes the oxidation and polymerization of phenolic compounds (Zhou et al., 2010). Ascorbic acid does not act directly on polyphenoloxidases but avoids the browning of

Table 2 – *Gus* gene expression and organogenesis in *Eucalyptus saligna* cotyledonary explants in selective medium containing different concentrations of kanamycin. Mean of two experiments.

Tabela 2 – *Expressão do gene* ***gus*** *e organogênese em explantes cotiledonares de* ***Eucalyptus saligna*** *em meio de seleção contendo diferentes concentrações de canamicina. Média de dois experimentos.*

			60 days afterinoculation			180 days after inoculation	
Km*Gus* gene ($mg.L^{-1}$)	Oxidized Expression	Explants explants	Explants with callus	Mean number of with buds	Explants buds/	Necrose with buds	
	(%)*	(%)	(%)	(%)	explant**	(%)	(%)
12.5	40	31.99 a	67.7 a	24.0 a	3.90 a	12	88
25	47	37.29 a	59.9 a	16.0 b	2.53 b	1	99
50	33	37.96 a	63.9 a	14.6 b	1.62 c	0	100
CV (%)		26.3	20.1	8.2	17.06		

Means followed by the same letter in the column do not differ statistically by Tukey´s test at the 0,05 level.

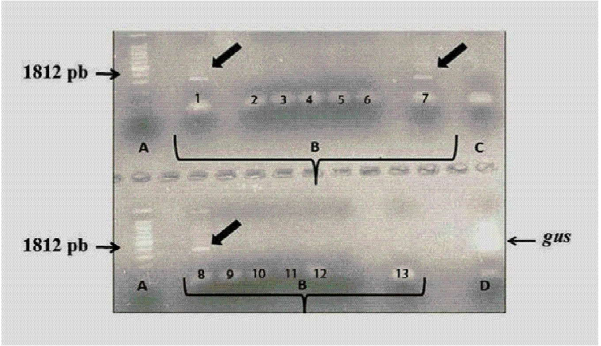

Figure 1 – Polymerase chain reaction amplification (|PCR) of *gus* gene from a sample of putatively transformed shoots of *Eucalyptus saligna*. A. Molecular weight marker (Ladder 100 pb). B. Events 1 to 13: transformed with the *gus* gene and regenerated in the presence of kanamycin (arrows: *gus* gene). Events 1 to 7 with 12.5 $mg.L^{-1}$ Km and event 8 to 13 with 25 $mg.L^{-1}$. C. Negative control. D. Positive control: pBI121 plasmid.

Figura 1 – *Eletroforese dos produtos do PCR de DNA de* ***Eucalyptus saligna****. A. Marcador de peso molecular (100 pb). B. Eventos 1 a 13: transformados com o gene* ***gus*** *e regenerados na presença de canamicina (setas: gene* ***gus****). Eventos 1 a 7 com 12.5 $mg.L^{-1}$ Km e eventos 8 a 13 with 25 $mg.L^{-1}$. C. Controle negativo. D. Controle positivo: plasmídeo pBI121.*

the cultures by reducing oxidized substrates (Ahmad et al., 2013). It is a high spectrum antioxidant (Ali and Alqurainy, 2006; George and Davies, 2006; Suaréz et al., 2010). On the other hand, the antioxidant effect of citric acid is due to its ability to act as a chelating agent which traps ions that increase oxidation, especially copper. Copper is a component of polyphenoloxidases, but it also catalyses the reaction of inactivation of ascorbic acid by molecular oxygen (Nawar, 1996; Raju and Bawa, 2006). AC may therefore optimize the antioxidant effect of other compounds.

The antioxidant solution applied in this work was successfully used by Tournier et al. (2003) during the explant excision from *E. grandis* x *E. urophylla* leaves. For *E. tereticornis,* PVP (500 $mg.L^{-1}$) was efficient in controlling tissue oxidation (Subbaiah and Minocha, 1990). However, *E. saligna* material did not respond to this treatment and other concentrations of the antioxidants or their addition to the culture medium should be tested. Soaking of explants of *Musa laterita* in an antioxidant mixture of citric and ascorbic acids (50 or 100 $mg.L^{-1}$) prior to their culture was effective in limiting phenolic exudation (Dayarani and Dhanarajan, 2013). The addition of reduced glutathione (25 mg.L) and ascorbic acid (10 mg.L) to culture medium was also beneficial for the growth of *Jatropha curcas* shoots (Misra et al., 2010).

In the present study, a 4 d pre-culture of explants before the inoculation of the bacteria had no effect on *gus* gene expression or bud regeneration. However, in explants of *E. grandis* × *E. urophylla,* a 2 d pre-culture affected positively the expression level of the *gus* gene (Alcantara et al., 2011). In the case of hypocotyl segments of *E. camaldulensis*, a 3 d pre-culture of the explants on B5C medium is followed by a 2 d co-culture with the bacterium (Ho et al., 1998). Moralejo et al. (1998) also recommend a pre-culture of 4 to 6 d in order to increase the expression level of *gus* gene in cotyledons and hypocotyls of *E. globulus*. For *E. tereticornis,* a pre-culture of cotyledons and hypocotyls is also beneficial (Prakash and Gurumurthi, 2009). The difference between these and our results may be due to the type of explant (leaf/cotyledon/hypocotyl) or to the eucalyptus species or even to the composition of culture medium used during pre-culture, which was different in every case.

Table 3 – Effect of Augmentin® and cefotaxime on indirect organogenesis in cotyledonary explants of *Eucalyptus saligna* inoculated with *Agrobacterium tumefaciens*, after 60 days of culture. Mean of two experiments.

Tabela 3 *– Efeito de Augmentin e cefotaxima na organogênese indireta de explantes cotiledonares de* ***Eucalyptus saligna*** *inoculados com* ***Agrobacterium tumefaciens*** *aos 60 dias de cultura. Média de dois experimentos.*

Treatment	Oxidiz explants (%)*	Explants with callus (%)*	Explants with buds (%)*	Contamination by *A. tumefaciens* (%)*
Augmentin®(300 mg.L^{-1})	56.3 b	80.8 a	46.6 a	18.3 b
Cefotaxime(500 mg.L^{-1})	79.1 a	64.1 b	10.0 b	40.0 a
CV (%)	10.4	4.1	25.7	19.7

Means followed by the same letter in the column do not differ by Tukey´s test at the 0.05 level. Culture medium: MS N/2 supplemented with 2.7 µM NAA, 4.4 µM BA and 50 mg.L^{-1} Km.

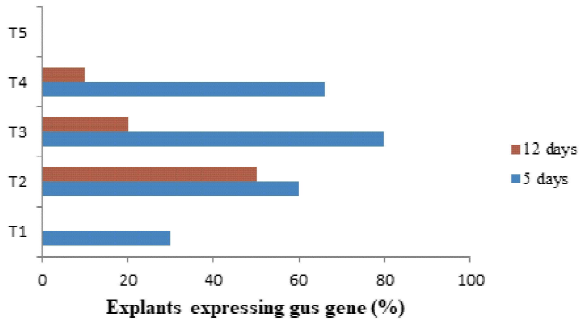

Figure 2 – Percentages of *Eucalyptus saligna* cotyledonary explants expressing the *gus* gene after 5 and 12 days of co-culture with *Agrobacterium tumefaciens* in the presence or absence of acetosyringone (AS). T1: AS (50 µM) in solid co-culture medium. T2: AS in liquid co-culture medium. T3: AS in solid and liquid co-culture media. T4: control without AS. T5: non-inoculated control.

Figura 2 *– Percentagens de explantes cotiledonares de* ***Eucalyptus saligna*** *expressando o gene* ***gus*** *após 5 e12 dias de co-cultura com* ***Agrobacterium tumefaciens****. T1: AS (50 µM) no meio de co-cultura sólido. T2: AS no meio de co-cultura líquido. T3: AS nos meios de co-cultura sólido e líquido. T4: Controle sem AS. T5: Controle não inoculado.*

Kanamycin is added to media after the co-culture period in order to select tissues transformed with a construction containing *npt*II gene. The concentration of 12.5 mg.L^{-1} was considered appropriate for cotyledonary tissues of *E. saligna*, as it allowed the selection of some events. The response of *Eucalyptus* to this kind of antibiotic varies with the species (Gonzales et al., 2000). For *E. camaldulensis*, 9 mg.L^{-1} Km was sufficient to select transgenic shoots (Mullins et al., 1997; Quisen et al., 2009) while, for *E. tereticornis*, Prakash and Gurumurthi (2009) established an efficient protocol using 40 mg.L^{-1}, and reaching 14.4 to 21.2% of transformation efficiency.

Augmentin added to the co-culture media was more efficient for bud regeneration than Cefotaxime. The same effect of Augmentin on bud regeneration was described for cotiledonary explants of *E. camaldulensis* (Quisen et al., 2009) co-cultured with the strain C58C1 of *A. tumefaciens*, where the antibiotic concentration was reduced from 300 mg.L^{-1} to 150 mg.L^{-1} after 15 d and then to 100 mg.L^{-1} after another 15 d. The number of buds per explant (5.4) was similar to the number obtained in the present study. In peanut (*Arachis hypogea*), a combination of 200 mg.L^{-1} Cx and the same concentration of Augmentin® was recommended to counteract the bacteria growth and to enhance the bud formation (Tiwari and Tuli, 2012). Augmentin is a mixture of amoxicillin and clavulanic acid. The former is an inhibitor of bacterial cell wall synthesis and the latter is an inhibitor of â-lactamase which protects amoxicillin from inactivation by â-lactamase. Cx also is a â-lactam antibiotic. The effect of antibiotics used to eliminate *A. tumefaciens* varies with plant species and explant type (Nauerby et al., 1997). In maize embryogenic callus, Cx enhanced the morphogenesis, increased root and shoot length (Danilova and Dolgikh, 2004). Tambarussi et al. (2015) studied the effect of several concentrations of timentin, cefotaxime and carbenicillin on shoot regenerationfrom teak explants (*Tectona grandis* L.). They observed that 300 mg L^{-1} Cx stimulated this regeneration process while 500 mg L^{-1} inhibited it. The authors attributed this response mainly to the interaction between the endogenous hormonal concentrations and the product of â-lactam antibiotic breakdown that may affect, positively or negatively, the shoot regeneration. In the present study, the same concentration of cefotaxime (500 mg L^{-1}) inhibited shoot regeneration from callus.

Acetosyringone (AS) is a phenolic compound involved in plant-pathogen recognition. The mechanism of activation

of *vir* genes by these compounds is well known in the case of *A. tumefaciens* (Lee et al., 1995). In the present study, the addition of AS (50 µM) to the liquid and solid co-culture media had a positive effect on *gus* gene expression. The concentrations used by other authors vary in each case and sometimes are superior to 50 µM. For example, Jha et al. (2011) tested three concentrations (100, 200 and 400 µM) during the co-culture of caulinar apexes of *Pennisetum glaucum* and obtained the highest transformation frequency (5.79%) with 400 µM. On the other hand, Dutta et al. (2013) added 200 µM AS to the liquid medium for one day-pre-culture of *Leptadenia pyrothnica* explants and found 14% of *gus* positive explants. For *Agrobacterium*-mediated transformation of *Cassia occidentalis*, the concentration of acetosyringone was the critical parameter during the co-cultivation process (Rajagopal et al., 2014).The number of *gus*-positive spots per explant was increased 6 fold when the infection process was carried out with acetosyringone in the co-culture medium (400ìM) and in bacterial suspension (50ìM) (Rajagopal et al., 2014).

5.CONCLUSIONS

Some progress in transformation efficiency was reached in this study, especially by using Augmentin instead of Cefotaxime and incorporating acetosyringone into co-culture media. The use of 12.5 $mg.L^{-1}$ Km is efficient for transformed material selection and transformation efficiency was increased in comparison with that reached by Dibax et al. (2010) for the same species.

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