Plant Cell Tiss Organ Cult (2009) 99:287–298
DOI 10.1007/s11240-009-9603-1

ORIGINAL PAPER

Studies on genetic transformation of *Theobroma cacao* L.: evaluation of different polyamines and antibiotics on somatic embryogenesis and the efficiency of *uidA* gene transfer by *Agrobacterium tumefaciens*

Thiago E. R. Silva · Luciana C. Cidade · Fátima C. Alvim · Júlio C. M. Cascardo · Marcio G. C. Costa

Received: 3 July 2009 / Accepted: 6 September 2009 / Published online: 17 September 2009

Abstract In order to develop a more efficient genetic transformation system for cacao somatic embryos, the effects of polyamines and β-lactam antibiotics on somatic embryogenesis, hygromycin as selective agent, and different factors affecting *uidA* gene transfer have been evaluated. The polyamines putrescine, spermidine, and spermine significantly improved secondary somatic embryogenesis in cacao. Spermine at 1,000 μM provided the best responses, increasing 6.7× the percentage of embryogenic callus and 2.5× the average number of embryos per embryogenic callus. The β-lactam antibiotics timentin and meropenem, used for *Agrobacterium tumefaciens* counter-selection, had a non-detrimental effect on secondary somatic embryogenesis, depending on their concentration, whereas the commonly used β-lactam cefotaxime inhibited it, irrespective of the tested concentration. Hygromycin showed a strong inhibitory effect on secondary somatic embryogenesis of cacao, impairing completely the embryo production at 20 mg l^{-1}. Following the criterion of GUS activity, the best conditions for T-DNA transfer into cotyledon explants from primary somatic embryos of cacao were a sonication of the explants for 100 s, a 20-min incubation period in *Agrobacterium* solution, an *Agrobacterium* concentration of 1.0 (OD_{600}), and cocultivation of the explants on tobacco feeder layers. These findings will have important implications for studies on functional genomics of cacao.

T. E. R. Silva · L. C. Cidade · F. C. Alvim · J. C. M. Cascardo · M. G. C. Costa (✉)
Center for Biotechnology and Genetics, Biological Sciences Department, State University of Santa Cruz, Ilhéus, BA 45662-000, Brazil
e-mail: mcosta@labbi.uesc.br

Keywords Cocoa · Putrescine · Spermidine · Spermine · Beta-lactam antibiotics · SAAT

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
DKW	Driver and Kuniyuki medium
ED	Embryo development medium
GUS	β-Glucuronidase
hptII	Hygromycin phosphotransferase II
PCG	Primary callus growth medium
SAAT	Sonication-assisted *Agrobacterium*-mediated transformation
SCG-2	Secondary callus growth medium
SE	Somatic embryogenesis
TDZ	Thidiazuron
WPM	Woody plant medium

Introduction

Theobroma cacao L. (cacao) is a tropical perennial tree, native to rainforest of the Amazon basin, which has been cultivated since pre-Columbian times (Hurst et al. 2002). Mayas used its seeds (cocoa beans) in ritual drinks and also as currency. Today, cacao seeds are the sole source of cocoa powder and butter, which are important ingredients used in the chocolate, pharmaceutical, and cosmetic industries. Cacao is grown as a component of complex agroecosystems in several developing countries, providing both economic and ecological benefits to the farmers and the producing countries (Wood and Lass 1985; Lobão 2007).

Despite its importance, cocoa production is seriously affected by a number of pests and diseases, with average annual losses estimated as much as 40% worldwide (Fulton 1989). Genetic improvement of cocoa for disease resistance through conventional sexual hybridization has yielded only limited success. It is mainly due to the narrow genetic base and prolonged generation time of the crop (Kennedy et al. 1987). Consequently, biotechnological approaches such as genetic transformation provide a powerful means for introducing new traits that are difficult to obtain via traditional breeding. Genetic transformation provides also a valuable tool for functional genomics studies of cacao candidate resistance genes.

A reliable protocol for genetic transformation and generation of transgenic cacao plants has been established only recently (Maximova et al. 2003). This protocol employs *Agrobacterium*-mediated transformation of cotyledon segments from primary somatic embryos (SEs), originally obtained from floral tissue explants, followed by regeneration of secondary SEs. Despite this recent achievement, cacao transformation still remains inefficient. The main problems are the low transformation efficiency and low numbers of regenerated transgenic plants.

Because the transformation method developed by Maximova et al. (2003) has a potential to consistently and reproducibly generate transgenic cacao plants, the objective of the present study was to improve this method by the evaluation of different factors affecting somatic embryogenesis and transformation efficiencies in cacao. Specific variables analyzed included type and concentration of polyamines and β-lactam antibiotics, hygromycin concentration, sonication-assisted *Agrobacterium*-mediated transformation (SAAT), incubation period in *Agrobacterium* solution, *Agrobacterium* concentration, and composition of the cocultivation medium, which have not been studied in cacao yet. This study enabled us to optimize the cacao transformation protocol for application in functional analysis of candidate resistance genes using the elite cacao clone 'TSH 565'.

Materials and methods

Plant material

Unopened immature flower buds about 4–5 mm long from the field-grown elite cacao clone 'TSH 565' were periodically collected early in the morning at CEPEC/CEPLAC (Centro de Pesquisas da Comissão Executiva do Plano da Lavoura Cacaueira, Ilhéus, Bahia, Brazil). The immature flowers were stored in sterile water on ice during transportation.

Agrobacterium strain and plasmid

The disarmed *Agrobacterium tumefaciens* strains EHA105 (Hood et al. 1993) containing the plasmid pCAMBIA1301 (http://www.cambia.org.au) was used in experiments of *Agrobacterium* transformation. This plasmid contains the chimeric genes *uidA* (*GUS*int) and hygromycin phosphotransferase II (*hptII*), driven by the CaMV 35S promoter.

Tissue culture and *Agrobacterium* transformation

Immature flower buds were surface-sterilized by immersion in 70% (v/v) ethanol, for 1 min and 30 s, followed by 20 min in a 2.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween-20 and then five rinses in sterile distilled water. Explants were prepared according to the methodology described by Li et al. (1998). Briefly, the flower buds were sliced perpendicular to their longitudinal axis at a position 1/3 of the flower length from the base using a sterile scalpel blade. The petal tissues were extracted through the opening at the cut end using a sharp sterile forceps. Petal bases were used as explant source.

Petal base explants were placed into Petri dishes containing 30 ml of PCG medium (Li et al. 1998), with the abaxial surface in contact with the medium. Cultures were maintained in the dark at 27 ± 2°C for 2 weeks, then transferred for another 2 weeks in a secondary callus growth medium (SCG-2). PCG medium contained the DKW basal salt (Driver and Kuniyuki 1984) supplemented with 250 mg l^{-1} glutamine, 200 mg l^{-1} myo-inositol, 20 g l^{-1} glucose, 2 mg l^{-1} 2,4-D, 0.005 mg l^{-1} TDZ, and 2 g l^{-1} phytagel, pH 5.8. SCG-2 medium consisted of Woody Plant Medium (WPM) and Gamborg's vitamins, 20 g l^{-1} glucose, 2 mg l^{-1} 2,4-D, 0.05 mg l^{-1} BAP, and 2.2 g l^{-1} phytagel, pH 5.7. Somatic embryos were induced by transfer of floral tissue-derived calli from SCG-2 medium to embryo development (ED) medium. ED medium was composed of DKW salts and vitamins, 30 g l^{-1} sucrose, 1 g l^{-1} glucose, and 2.0 g l^{-1} phytagel, pH 5.7. Cultures were subcultured every 2 weeks in ED medium under the same incubation conditions described above. During the ED culture period, a large number of somatic embryos developed.

Secondary somatic embryogenesis was induced as described by Maximova et al. (2002). Briefly, mature primary embryos with light yellow developed cotyledons were selected for secondary embryogenesis. The cotyledons were cut with a scalpel into approximately 4-mm^2 pieces and subcultured for 2 weeks in SCG-2 medium for callus induction. Thereafter, the explants were transferred to ED medium and sub-cultured every 2 weeks for induction of secondary somatic embryos. The regenerated

embryos were grown to maturity and their cotyledons excised and cultured for tertiary and quaternary embryo production as described by Maximova et al. (2002).

Agrobacterium-mediated transformation was performed as described by Maximova et al. (2003). Cotyledons pieces from primary somatic embryos were mixed in *Agrobacterium* solution (OD adjusted to 0.5 at 600 nm), sonicated for 30 s at 20 KHz and 80% amplitude (Sigma Ultrasonic Processor, USA), followed by 10 min of incubation at 25°C and 50 rpm agitation, and then blotted dry with sterile filter paper to remove the excess bacteria. The explants were transferred to a modified SCG medium, for a 2 days co-cultivation period at 25 ± 2°C in the dark. Following co-cultivation, the explants were transferred to fresh solid SCG medium containing 20 mg l^{-1} hygromycin (Sigma Chemical Co., USA) for *hptII* selection and 500 mg l^{-1} cefotaxime (Sigma Chemical Co., USA) for *Agrobacterium* counter-selection. After 1 week of incubation in SCG medium at 27 ± 2°C in the dark, transient expression of GUS was assayed histochemically by staining the explants with X-GLUC (Sigma Chemical Co., USA), according to Jefferson et al. (1987).

Factors evaluated

Polyamines

In order to evaluate the effect of polyamines on regeneration of secondary somatic embryos, cotyledon explants were cultured on SCG-2 and ED media containing different concentrations (1, 100, and 1,000 μM) of putrescine (Put), spermidine (Spd), and spermine (Spm). Polyamines were filtered through 0.22 μm filter (Fischer Scientific) and then separately added to the culture media after autoclaving and cooling.

β-Lactam antibiotics

Three β-lactam antibiotics, cefotaxime sodium salt (Cef; Ceftriax IM, Sigma Pharma, Brazil), meropenem trihydrate (Mer; ACS Dobfar, Brazil), and timentin (Tim; SmithKline Beecham Pharmaceuticals, USA), were used in the present study to evaluate their effects on somatic embryogenesis of untransformed cotyledon explants. Either 250–500 mg l^{-1} cefotaxime (Cef), 6.25–12.5 mg l^{-1} meropenem (Mer), or 150–300 mg l^{-1} timentin (Tim) were applied to standard SCG-2 and ED media, devoid of polyamines, for regeneration of secondary somatic embryos. These concentrations were based on the *in planta* antiagrobacterial activities and phytotoxicities (Ogawa and Mii 2005). The antibiotics were dissolved in water, filter-sterilized, and added to the medium after autoclaving and cooling.

Hygromycin as a selective agent

For the experiments testing hygromycin as a selective agent, untransformed cotyledon explants were cultured on standard SCG-2 and ED media containing 0, 5, 7.5, 10, 15, or 20 mg l^{-1} hygromycin, aiming to identify the most suitable concentration for selecting transformed secondary somatic embryos.

Factors affecting uidA gene transfer

For improvement of the *Agrobacterium*-mediated transformation protocol, four different factors were evaluated for their effects on *uidA* gene transfer. Different treatments were compared for each factor, as follows: (1) the explants were sonicated for 0, 50, 100, 150, 200, 250, or 300 s in *Agrobacterium* solution (Ultrasonic processor, 20 kHz, Sigma, USA); (2) the explants were incubated for 0, 10, 20 or 30 min in *Agrobacterium* solution; (3) incubation was done in OD_{600} at 0.4 ($\approx 2 \times 10^8$ cfu ml^{-1}), 0.6 ($\approx 3 \times 10^8$ cfu ml^{-1}), 0.8 ($\approx 4 \times 10^8$ cfu ml^{-1}), or 1.0 ($\approx 5 \times 10^8$ cfu ml^{-1}) *Agrobacterium* concentration, and; (4) 2-days cocultivation was performed on cocultivation medium supplemented with 0, 25, 50, 100, or 200 μM acetosyringone (AS) or tobacco feeder layers (Horsch et al. 1985). Following the order of the experiments described above, the best treatment identified in the previous experiment was used as parameter to set up the next one.

Experimental design and statistical analysis

Experiments of polyamines, hygromycin, and β-lactam antibiotics were performed with 25 explants per Petri dish, five Petri dishes per treatment, and repeated at least twice. The percentage of embryogenic calli over the total number of cultured explants, representing the frequency of embryogenic calli and average number of embryos per explant were determined 2 months after culture initiation. The transformation experiments were performed with 100 explants per treatment and repeated at least twice. Transient expression was evaluated after 7 days on selective SCG-2 medium based on percentage of GUS positive (GUS+) explants under a dissecting microscope. The data were subjected to polynomial regression (linear, quadratic, and cubic levels). Statistical analysis was performed with the software BIOESTAT (Universidade Federal do Pará, Brazil), which tested the experiments as a completely randomized design. Data of frequency (%) were $\arcsin\sqrt{x}$ transformed prior to statistical analysis. Analysis of variance (ANOVA) was applied and for means comparison Bonferroni's test was employed, with a critical value of $P = 0.05$.

Results and discussion

Establishment of primary SE

Petal explants from the elite cacao clone 'TSH 565' enlarged two to three times their original size within 1 week of cultivation on PCG medium. Callus formation over the entire explant was apparent by the end of the culture period on SCG-2 medium. About 2 weeks after transfer the explants to ED medium, the presence of two morphologically distinct types of clustered cells was evident. The first type consisted of elongated cells and whitish appearance (Fig. 1a). Since roots instead embryos usually developed from this type of cell cluster, it was called

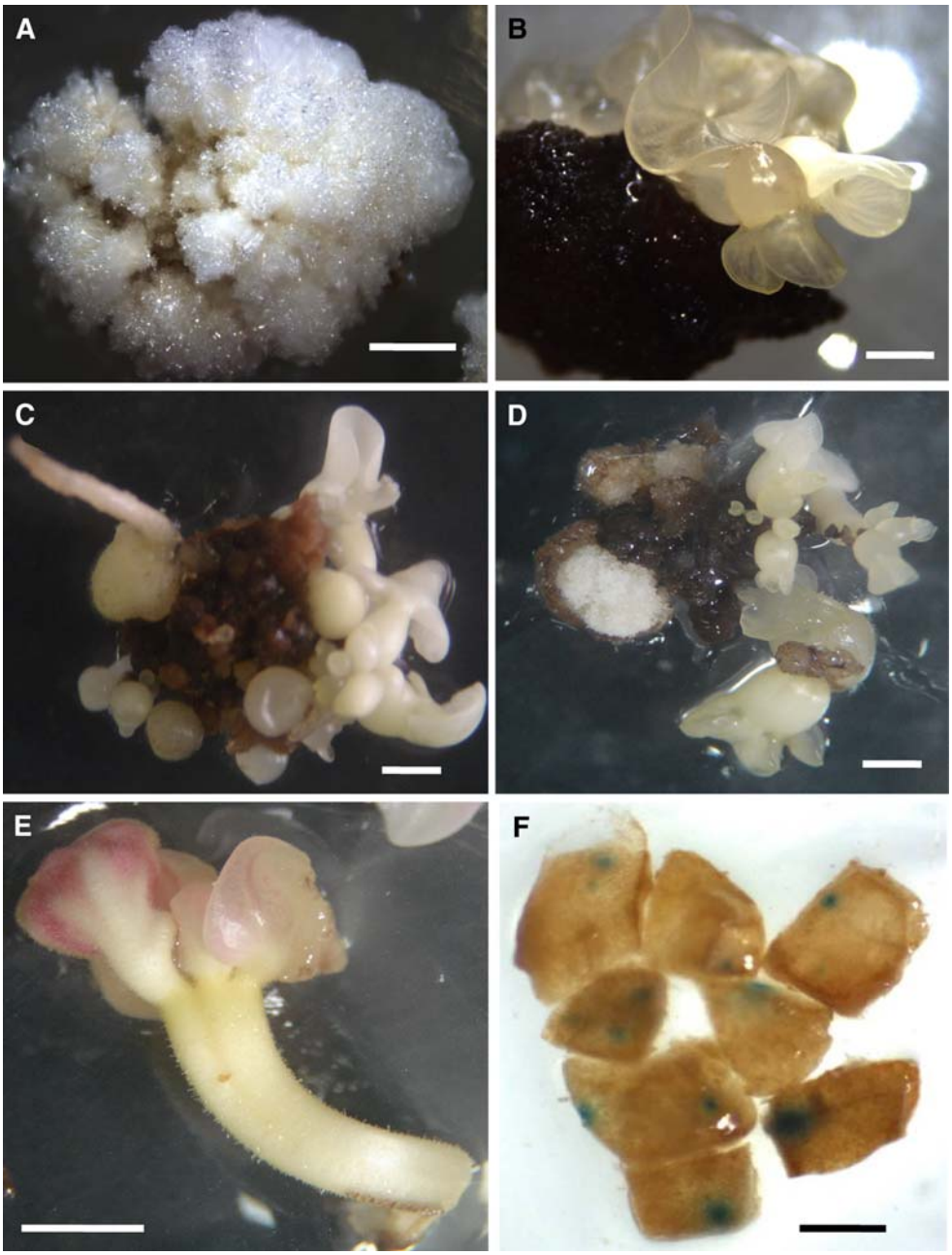

Fig. 1 Primary somatic embryogenesis and expression of the *uidA* gene in cotyledon explants from primary somatic embryos of the cacao elite clone 'TSH 565'. **a** Rhyzogenic callus. **b** Embryogenic callus with somatic embryos. **c** Embryogenic callus with embryos at different developmental stages. **d** Embryogenic callus with milky and translucent embryos. **e** Mature somatic embryo. **f** GUS assay performed 7 days after *Agrobacterium*-mediated transformation. *Bars* = 2 mm

rhyzogenic callus. The second type consisted of round cells and brownish appearance (Fig. 1b). These cell clusters were often associated with somatic embryos.

Pre-embryonic protuberances started to emerge from the surface of embryogenic calli, often in clusters, by the end of 2 weeks cultivation on ED medium. Four weeks after culture on ED medium, or about 2 months after culture initiation, the surfaces of some calli were covered with somatic embryos of distinct morphology and developmental stage (Fig. 1c, d). Cotyledons from mature primary embryos (Fig. 1e) were used as explant source for the experiments performed in the present study.

Effects of polyamines on secondary SE

Polyamines significantly affected secondary SE in cacao (Fig. 2). All polyamines and their tested concentrations, with exception of Put at 1 μM, increased both the percentage of embryogenic callus (Fig. 2a) and the average number of embryos per embryogenic callus (Fig. 2b), as compared to the control treatment (absence of polyamines). The general trend observed was a higher morphogenic response as the concentration of each polyamine increased in the culture medium. However, the highest percentage of embryogenic callus and numbers of embryos per

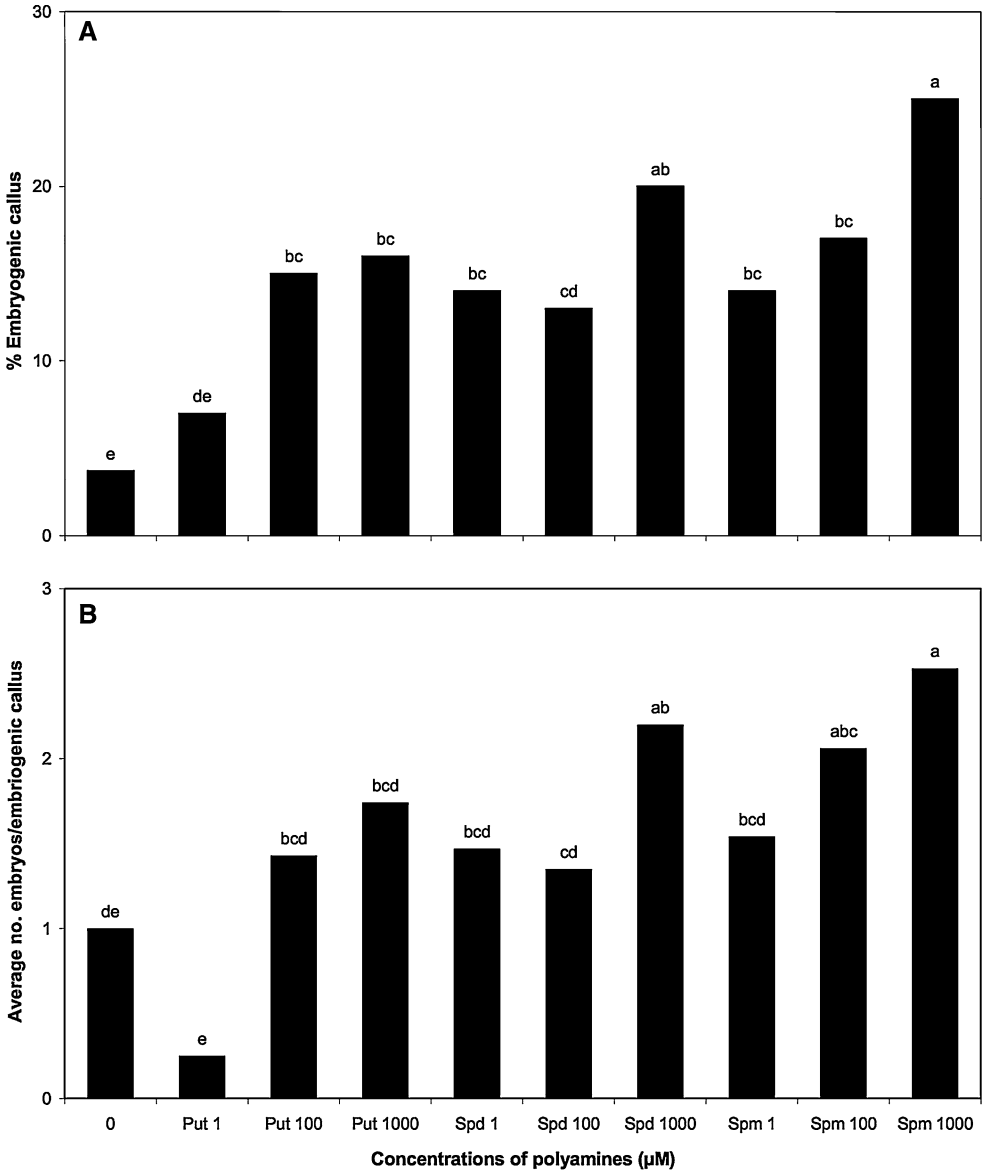

Fig. 2 Effects of the exogenous polyamines putrescine (Put), spermidine (Spd) and spermine (Spm) on the secondary somatic embryogenesis of cacao. **a** Percentage of embryogenic callus. **b** Average number of embryos per embryogenic callus. Data are from two independent experiments. Means followed by the *same letter* are not statistically significant by Bonferroni's test (at 5% of probability)

embryogenic callus were obtained in culture medium supplemented with Spm at 1,000 μM.

Polyamines, mainly Put, Spd and Spm, are an important group of naturally occurring low molecular weight, polycationic, aliphatic amines present in all plant cells, which have been implicated in several important cellular processes, including cell division, DNA replication and protein synthesis (Bais and Ravishankar 2002). A number of studies have demonstrated that increased polyamine biosynthesis proceeds or accompanies SE in several plant species and that inhibition of its biosynthesis leaded to delayed or reduced SE (Montague et al. 1979; Fobert and Webb 1988; Robie and Minocha 1989; Minocha et al. 1991, 2004; Garrido et al. 1995; Helleboid et al. 1995;Paul et al. 2009; Wu et al. 2009). Moreover, induction of SE via application of exogenous polyamines or overexpression of polyamine biosynthesis genes provides strong evidence about the involvement of polyamines in SE (Bastola and Minocha 1995; Kevers et al. 2000; Niemi et al. 2002; Silveira et al. 2006; Steiner et al. 2007; Paul et al. 2009; Wu et al. 2009). However, the exact mechanism whereby polyamines exert their effect on SE still is not clear. Our data show that exogenous application of polyamines is a simple method to improve regeneration in *Theobroma cacao* and, therefore, increase the efficiency of the genetic transformation protocol, as it has already been shown in other plant species. The beneficial effect of Spm on somatic embryo formation has been imputed to the enhancement of DNA amplification, increased lag time of somatic embryo formation, and/or suppression of the protein secretion from cells (Takeda et al. 2002).

Effects of β-lactam antibiotics on secondary SE

An efficient *Agrobacterium*-mediated transformation method requires not only the development of a reliable regeneration system, but also the use of antibiotics to control bacterial growth that have negligible effects on regeneration potential of the transformed cells. In the present work, we have evaluated the effects of the novel β-lactams timentin and meropenem on secondary SE of cacao in comparison to cefotaxime, a β-lactam antibiotic commonly used to suppress and eliminate *Agrobacterium* after cocultivation. In general, it was observed a non-detrimental effect of the antibiotics timentin and meropenem on SE, depending on their concentration, whereas cefotaxime clearly inhibited SE in cacao, mainly at 500 mg l^{-1} (Fig. 3). The percentage of embryogenic callus on culture medium containing 150 mg l^{-1} timentin or 6.25–12.5 mg l^{-1} meropenem did not significantly differ from that obtained on the control treatment (culture medium devoid of antibiotics) (Fig. 3a). However, cefotaxime significantly decreased the percentage of embryogenic callus, irrespective of the tested concentration. A similar result was obtained for the average number of embryos per embryogenic callus, whose treatments containing meropenem at 6.25 mg l^{-1} and timentin at 150 mg l^{-1} did not statistically differ from that without antibiotics (Fig. 3b). Cefotaxime at 500 mg l^{-1} gave the worst responses, decreasing cacao somatic embryo production by 72%.

β-lactam antibiotics have commonly been used to suppress and eliminate *Agrobacterium*, since they specifically inhibit prokaryotic cell wall synthesis and kill bacteria, with little or no detrimental effect on eukaryotic plant cells (Pollock et al. 1983; Asbel and Levison 2000). Unfortunately, noticeable inhibitory effects of these antibiotics, mainly carbenicillin and cefotaxime, on plant cell growth, organogenesis and embryogenesis have been well documented (Nauerby et al. 1997; Ogawa and Mii 2005; Wiebke et al. 2006; Mendes et al. 2009). Recently, many β-lactams have been developed to enhance and expand antibacterial activity (Demain and Elander 1999). These include timentin, a penicillin derivative (ticarcillin) coupled with the β-lactamase inhibitor clavulanic acid, and meropenem, a carbapenem antibiotic highly resistant to degradation by β-lactamases and cephalosporinases. The effects of these novel β-lactams on cacao SE have not been evaluated yet. It has been previously shown that carbenicillin, cefotaxime and amoxicillin had negative effects on cacao SE, whereas moxalactam had a positive effect on embryo regeneration (de Mayolo et al. 2003). Our data demonstrate that timentin and meropenem can be also antibiotics of choice to control *Agrobacterium* growth in genetic transformation experiments of cacao.

Hygromycin as a selective agent in cacao

Kanamycin has been the only selective agent used in genetic transformation of cacao to date (Maximova et al. 2003, 2006). However, the ineffectiveness of this antibiotic as a selective agent in experiments of genetic transformation has been well known (Gutiérrez-E et al. 1997; Seabra and Pais 1998; Humara and Ordas 1999; Niu et al. 2000; da Silva and Fukai 2003; Velcheva et al. 2005; Shin et al. 2007; Tian et al. 2009). Therefore, we decided for the first time to evaluate the antibiotic hygromycin as an alternative selective agent in cacao transformation, aiming not only to identify a more efficient antibiotic for recovering transformed cacao somatic embryos, but also to test another selective agent that could be used for re-transformation of cacao plants already containing the *nptII* selective marker gene. Cotyledon explants were cultured on media containing several concentrations of the antibiotic, in order to identify that most suitable for selection of transformed somatic embryos. As shown in Fig. 4, hygromycin significantly and quadratically decreased the average number of

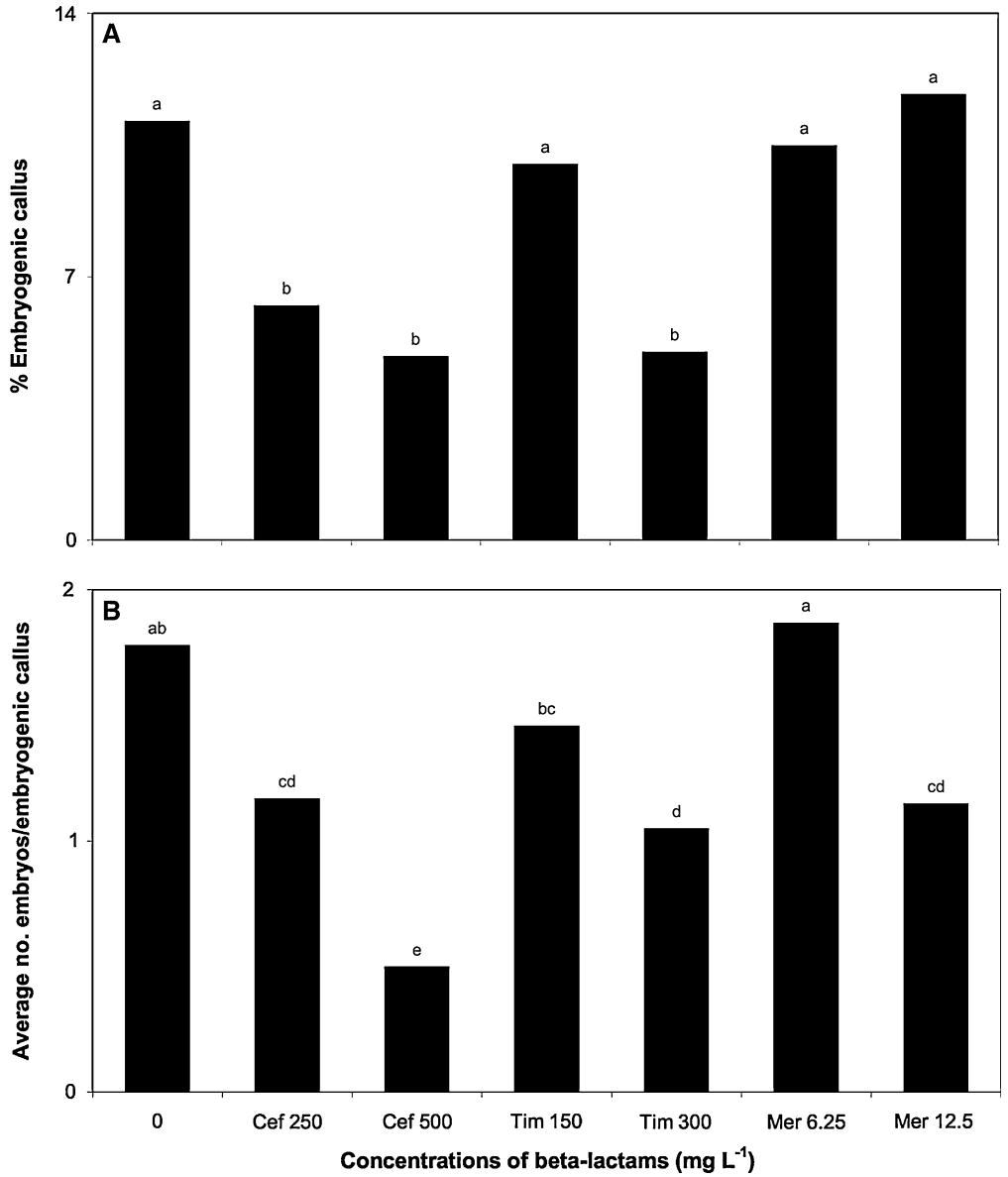

Fig. 3 Effects of the β-lactam antibiotics cefotaxime (Cef), timentin (Tim) and meropenem (Mer) on the secondary somatic embryogenesis of cacao. **a** Percentage of embryogenic callus. **b** Average number of embryos per embryogenic callus. Data are from two independent experiments. Means followed by the *same letter* are not statistically significant by Bonferroni's test (at 5% of probability)

embryos per embryogenic callus ($F = 15.8$, $P < 0.05$). Based on these data, 20 mg l^{-1} hygromycin is the most suitable concentration for use in genetic transformation experiments of cacao.

Factors affecting the *uidA* gene transfer

Developing of a reliable *Agrobacterium*-mediated transformation method requires also the development of an efficient procedure of T-DNA delivery into plant cells. Besides the establishment of a protocol for genetic transformation in the past few years (Maximova et al. 2003), a systematic study of factors affecting the *uidA* gene transfer in cacao has not been reported yet. In the present work, we have evaluated the effects of SAAT, incubation period in *Agrobacterium* solution, *Agrobacterium* concentration, and composition of the cocultivation medium on the efficiency of *uidA* gene transfer (Fig. 1f).

SAAT

SAAT affected T-DNA delivery to cells from cacao cotyledons. The transient GUS expression sharply increased from 0 to 100 s SAAT and then decreased after longer times of SAAT (Fig. 5a). Such a profile fitted better to the cubic polynomial trend. It is important to note that no GUS

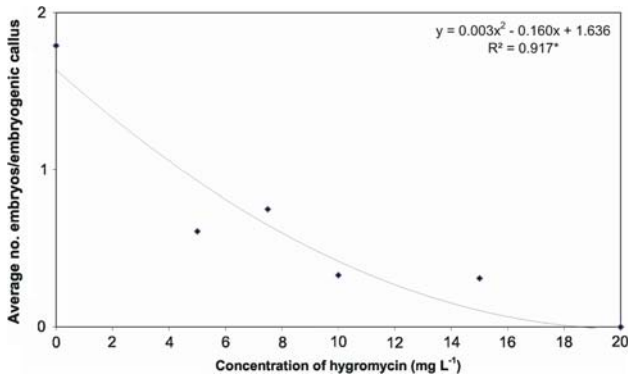

Fig. 4 Effect of the antibiotic hygromycin on the secondary somatic embryogenesis of cacao. The average number of somatic embryos per embryogenic callus was evaluated 2 months after culture initiation. R^2* Significant at $P_{0.05}$ level

expression was detected in the absence of SAAT, using the ordinary "dipping" method, demonstrating the relative recalcitrance of cacao to the practices of *Agrobacterium*-mediated transformation.

SAAT has been proved to be an efficient system of T-DNA delivery to cells in a number of plants (Santarém et al. 1998; Tang et al. 2001; Zaragozá et al. 2004; Beranová et al. 2008; de Oliveira et al. 2009), especially those that are typically more recalcitrant to *Agrobacterium*-mediated transformation (Trick and Finer 1997). This method involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*. It creates microwounding released from the cavitation of microbubbles causing minute visible wounds within and on the tissue (Gaba et al. 2006). Such phenomena allow better access and infection of plant cells by *Agrobacterium* (Beranová et al. 2008). In addition, the wounded tissue often produces inducers of the T-DNA transfer process, caused by the secretion of more phenolic compounds, enhancing the accessibility of putative cell wall binding factor to the *Agrobacterium* during transformation (Stachel et al. 1985). Our results show that these mechanisms are essential for successful *Agrobacterium*-mediated transformation of cacao.

Incubation period

The transient GUS expression quadratically increased as the incubation period of the explants in *Agrobacterium* solution increased (Fig. 5b). The highest levels of GUS expression were obtained when the explants were incubated for 20-min in *Agrobacterium* solution. Shorter or longer incubation times in *Agrobacterium* yielded lower levels of GUS expression.

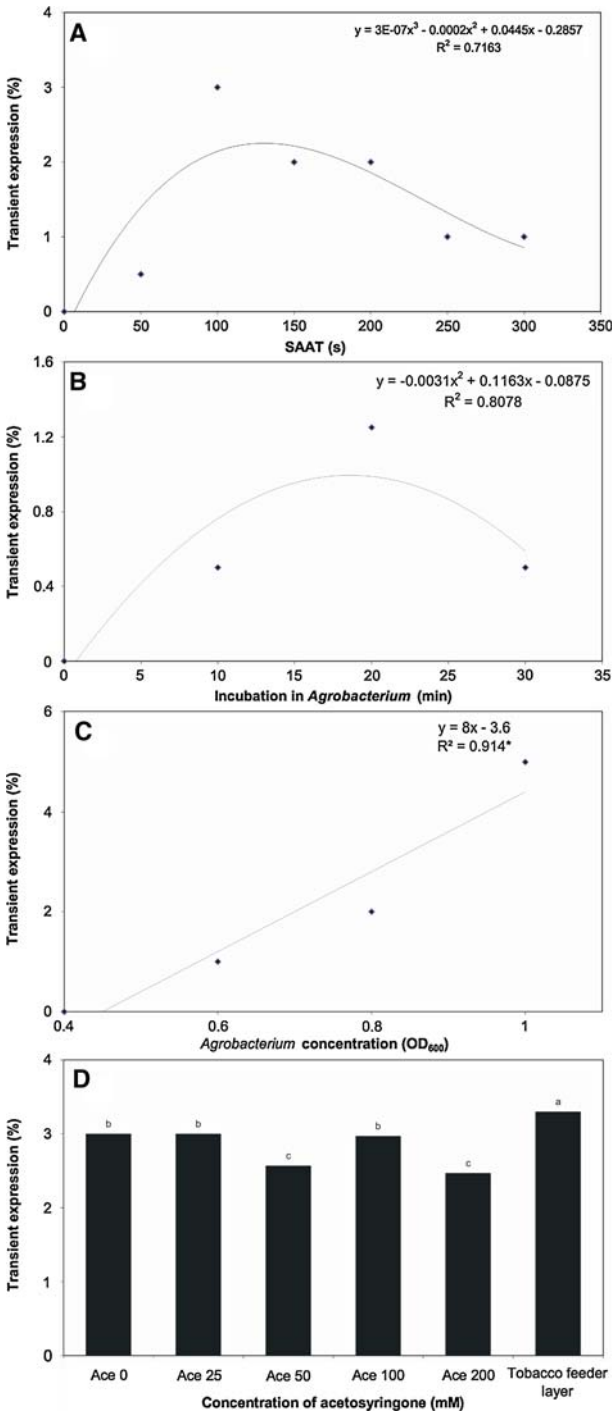

Fig. 5 Frequency of transient GUS expression in cotyledon explants from primary somatic embryos of cacao. **a** Effect of duration of sonication. **b** Effect of the incubation period in *Agrobacterium* solution. **c** Effect of the concentration of *Agrobacterium* ($OD_{600\ nm}$). **d** Effect of acetosyringone (Ace) at different concentrations and tobacco feeder layer during the cocultivation period. Data are from at least three independent experiments. R^2* Significant at $P_{0.05}$ level. Means followed by the *same letter* are not statistically significant by Bonferroni's test (at 5% of probability)

In cacao, a 10-min incubation period of the explants in *Agrobacterium* resuspension has been typically employed (Maximova et al. 2003, 2006). However, experiments comparing different incubation periods side-by-side have not been reported in cacao. We demonstrate that higher transformation rates can be obtained increasing the incubation period of the explants in *Agrobacterium* for 20-min.

Agrobacterium concentration

The concentration of *Agrobacterium* increased linearly the levels of GUS expression (Fig. 5c). The highest levels of expression were observed at $OD_{600} = 1.0$ ($\approx 5 \times 10^8$ cfu ml^{-1} *Agrobacterium* concentration). Lower levels of GUS expression were obtained in lower *Agrobacterium* concentrations.

The number of *Agrobacterium* cells in the inoculum is considered a critical factor in the efficiency of transformation, since an excessive number of bacteria can stress plant cells and affect their regeneration potential, whereas low concentrations can reduce the frequency of T-DNA transfer (Michelmore et al. 1987; Curtis et al. 1999; Humara et al. 1999). In cacao, an OD_{420} of 0.5 ($\approx 2.5 \times 10^8$ cfu ml^{-1}) has been used in genetic transformation experiments (Maximova et al. 2003). Our data show that higher transformation rates can be obtained by increasing the *Agrobacterium* concentration, although further investigations need to establish if the increased concentration of *Agrobacterium* could negatively affect the regeneration potential of the cells.

Cocultivation medium

The use of acetosyringone in the cocultivation medium has been shown to increase *Agrobacterium*-mediated transformation frequencies (Birch 1997). Therefore, we performed experiments aiming to investigate the effect of acetosyringone in the cocultivation medium of cacao. Surprisingly, the addition of this compound to the cocultivation medium at different concentrations did not increase the transient GUS expression (Fig. 5d). Acetosyringone is a phenolic compound produced during wounding of plant cells that induces the transcription of the virulence genes of *A. tumefaciens*. Its positive role has been demonstrated in the genetic transformation of several woody fruit species, including apple (James et al. 1993), kiwifruit (Janssen and Gardner 1993), citrus (Cervera et al. 1998), blueberry (Song and Sink 2004), and almond (Costa et al. 2006). In cacao, it has not been usually added to the cocultivation medium (Maximova et al. 2003), but its role as transformation enhancer was not investigated. We have shown here that acetosyringone does not affect the transformation frequencies in cacao explants. A possible explanation for this finding could be the naturally high production of hydroxycinnamic amides which has been reported in cacao explants during tissue culture phase (Alemanno et al. 2003). Such compounds are considered potent inducers of the virulence (*vir*) genes mediating T-DNA transfer (Berthelot et al. 1998).

It has been reported that the cocultivation of the explants onto feeder layers, such as tobacco feeder layers, also increased transformation frequencies in several species (Horsch et al. 1985; McCormick et al. 1986; Fillatti et al. 1987a, b). Figure 5d shows that cocultivation of cacao cotyledon explants on tobacco feeder layers increased the transient GUS expression in comparison with explants cocultivated on cocultivation medium without or with different concentrations of acetosyringone. The feeder layers also act as suppliers of *vir*-inducing compounds but, in addition, certain auxins (mainly 2,4-D) present in the feeder layers have been demonstrated to be beneficial for the transformation frequencies when added to the cocultivation and/or pre-cultivation medium in plants such as tomato (Fillatti et al. 1987a), carnation (Lu et al. 1991), arabidopsis (Sangwan et al. 1992), petunia (Villemont et al. 1997), and citrus (Cervera et al. 1998; Costa et al. 2002). Our results suggest that phytohormones, especially auxins, in the solid tobacco feeder layer may play an important role in increasing the transformation frequencies in cacao.

Optimized genetic transformation protocol

Overall, the results from this study indicated that all factors analyzed affected secondary SE as well as the transformation frequencies in cacao. These experiments suggested that a suitable transformation protocol for cacao should include sonication of the explants for 100 s in *A. tumefaciens* solution at $OD_{600} = 1.0$, followed by 20-min of bacterial incubation at 22°C and gentle agitation, and then transfer of the explants to tobacco feeder layers for 48 h of cocultivation, at 25°C in the dark. Following cocultivation, the explants must be transferred to fresh media, according to the somatic embryogenesis protocol as previously reported (Maximova et al. 2003), containing 1,000 μM spermine, 150 mg l^{-1} timentin or 6.25 mg l^{-1} meropenem for *A. tumefaciens* counter-selection and 20 mg l^{-1} hygromycin for *hpt* selection.

In summary, efforts have been made to improve the transformation protocol for cacao. It has been now applied in functional analysis of candidate resistance genes recently obtained from a *T. cacao–Moniliophthora perniciosa* interaction EST (expressed sequence tags) library (Gesteira et al. 2007). The hemibiotrophic fungus *M. perniciosa* is the causal agent of the cocoa's witches' broom, one of the most important diseases of cacao in the western hemisphere.

Acknowledgments To International Foundation for Science (IFS) for financial support; to The State of Bahia Research Foundation (FAPESB), for financial and fellowship support; to National Council for Scientific and Technological Development (CNPq) and Coordination of Higher Education and Graduate Training (CAPES), for fellowships support.

References

Alemanno L, Ramos T, Gargadenec A, Andary C, Ferriere N (2003) Localization and identification of phenolic compounds in *Theobroma cacao* L. somatic embryogenesis. Ann Bot 92:613–623. doi:10.1093/aob/mcg177

Asbel LE, Levison ME (2000) Cephalosporins, carbapenems, and monobactams. Infect Dis Clin North Am 14:435–447

Bais HP, Ravishankar GA (2002) Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue Organ Cult 69:1–34. doi:10.1023/A:1015064227278

Bastola DR, Minocha SC (1995) Increased putrescine biosynthesis through transfer of mouse ornithine decarboxylase cDNA in carrot promotes somatic embryogenesis. Plant Physiol 109:63–71

Beranová M, Rakouský S, Vávrová Z, Skalický T (2008) Sonication assisted *Agrobacterium*-mediated transformation enhances the transformation efficiency in flax (*Linux usitatissimum* L.). Plant Cell Tissue Organ Cult 94:253–259. doi:10.1007/s11240-007-9335-z

Berthelot K, Buret D, Guérin B, Delay D, Negrel J, Delmotte FM (1998) Vir-gene-inducing activities of hydroxycinnamic acid amides in *Agrobacterium tumefaciens*. Phytochemistry 49:1537–1548. doi:10.1016/S0031-9422(98)00209-X

Birch RG (1997) Plant transformation: problems and strategies for practical applications. Annu Rev Plant Physiol Plant Mol Biol 48:297–326. doi:10.1146/annurev.arplant.48.1.297

Cervera M, Pina JA, Juarez J, Navarro L, Peña L (1998) *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. Plant Cell Rep 18:271–278. doi:10.1007/s002990050570

Costa MGC, Otoni WC, Moore GA (2002) An evaluation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradisi* (Macf.) and the production of transgenic plants containing carotenoid biosynthetic genes. Plant Cell Rep 21:365–373. doi:10.1007/s00299-002-0533-1

Costa MS, Miguel C, Oliveira MM (2006) An improved selection strategy and the use of acetosyringone in shoot induction medium increase almond transformation efficiency by 100-fold. Plant Cell Tissue Organ Cult 85:205–209. doi:10.1007/s11240-005-9073-z

Curtis IS, Power JB, Hedden P, Ward DA, Phillips A, Lowe KC, Davey MR (1999) A stable transformation system for the ornamental plant, *Datura meteloids* D.C. Plant Cell Rep 18:554–560. doi:10.1007/s002990050621

da Silva JAT, Fukai S (2003) Effect of aminoglycoside antibiotics on in vitro morphogenesis from cultured cells of chrysanthemum and tobacco. J Plant Biol 46:71–82. doi:10.1007/BF03030434

de Mayolo GA, Maximova SN, Pishak S, Guiltinan MJ (2003) Moxalactam as a counter-selection antibiotic for *Agrobacterium*-mediated transformation and its positive effects on *Theobroma cacao* somatic embryogenesis. Plant Sci 164:607–615. doi:10.1016/S0168-9452(03)00012-8

de Oliveira MLP, Febres VJ, Costa MGC, Moore GA, Otoni WC (2009) High-efficiency *Agrobacterium*-mediated transformation of citrus via sonication and vacuum infiltration. Plant Cell Rep 28:387–395. doi:10.1007/s00299-008-0646-2

Demain AL, Elander RP (1999) The β-lactam antibiotics: past, present, and future. Antonie Van Leeuwenhoek 75:5–19

Driver JA, Kuniyuki AH (1984) In vitro propagation of *Paradox walnut* rootstock. HortScience 19:507–509

Fillatti JJ, Kiser J, Rose R, Comai L (1987a) Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Biotechnology 5:726–730. doi:10.1038/nbt0787-726

Fillatti JJ, Sellmer J, McCown B, Haissig B, Comai L (1987b) *Agrobacterium*-mediated transformation and regeneration of Populus. Mol Gen Genet 206:192–199. doi:10.1007/BF00333574

Fobert PR, Webb DT (1988) Effects of polyamines, polyamine precursors, and polyamine biosynthetic inhibitors on somatic embryogenesis from eggplant (*Solanum melongena*) cotyledons. Can J Bot 66:1734–1742. doi:10.1139/b88-238

Fulton RH (1989) The cacao disease trilogy: black pod, monilia pod rot, and witches' broom. Plant Dis 73:601–603

Gaba V, Kathiravan K, Amutha S, Singer S, Xiaodi X, Anantha-krishnan G (2006) The use of ultrasound in plant tissue culture. In: Gupta SD, Ybaraki Y (eds) Plant tissue culture engineering. Springer, The Netherlands, pp 417–426

Garrido D, Chibi F, Matilla A (1995) Polyamines in the induction of *Nicotiana tabacum* pollen embryogenesis by starvation. J Plant Physiol 145:731–735

Gesteira AS, Micheli F, Carels N, da Silva AC, Gramacho KP, Schuster I, Macedo JN, Pereira GAG, Cascardo JCM (2007) Comparative analysis of expressed genes from cacao meristems infected by *Moniliophthora perniciosa*. Ann Bot 100:129–140. doi:10.1093/aob/mcm092

Gutiérrez-E MA, Luth D, Moore GA (1997) Factors affecting Agrobacterium-mediated transformation in citrus and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of *Citrus tristeza* virus. Plant Cell Rep 16:745–753. doi:10.1007/s002990050313

Helleboid S, Couillerot JP, Hilbert JL, Vasseur J (1995) Inhibition of direct somatic embryogenesis by α-difluoromethylarginine in Cichorium hybrid: effects on polyamine content and protein patterns. Planta 196:571–576. doi:10.1007/BF00203658

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218. doi:10.1007/BF01977351

Horsch RB, Fry JE, Hoffmann NL, Eighholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231. doi:10.1126/science.227.4691.1229

Humara JM, Ordas RJ (1999) The toxicity of antibiotics and herbicides on in vitro adventitious shoot formation on *Pinus pinea* L. cotyledons. In Vitro Cell Dev Biol Plant 35:339–343. doi:10.1007/s11627-999-0045-6

Humara JM, López M, Ordás RJ (1999) *Agrobacterium tumefaciens*-mediated transformation of *Pinus pinea* L. cotyledons: an assessment of factors influencing the efficiency of uidA gene transfer. Plant Cell Rep 19:51–58. doi:10.1007/s002990050709

Hurst WJ, Tarka SM, Powis TG, Valdez F, Hester TR (2002) Cacao usage by the earliest Maya civilization. Nature 418:289–290. doi:10.1038/418289a

James DJ, Uratsu S, Cheng J, Negri P, Viss P, Dandekar AM (1993) Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. Plant Cell Rep 12:559–563. doi:10.1007/BF00233060

Janssen B-J, Gardner RC (1993) The use of transient GUS expression to develop an *Agrobacterium*-mediated gene transfer system to kiwifruit. Plant Cell Rep 13:28–31. doi:10.1007/BF00232310

Jefferson RA, Kanavagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3001–3907

Kennedy AJ, Lockwood G, Mossu G, Simmonds NW, Tan GY (1987) Cocoa breeding: past, present and future. Cocoa Grower's Bull 38:5–22

Kevers C, Nathalie LG, Monteiro M, Dommes J, Gasper T (2000) Somatic embryogenesis of *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathways. Plant Growth Regul 31:209–214. doi:10.1023/A:1006344316683

Li ZJ, Traore A, Maximova S, Guiltinan MJ (1998) Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In Vitro Cell Dev Biol Plant 34:293–299. doi:10.1007/BF02822737

Lobão DEVP (2007) The cocoa tree agroecosystem of Bahia: cocoa-cabruca and forest fragments on the conservation of tree species. Thesis (Ph.D), Universidade Estadual Paulista "Júlio de Mesquita Filho", Jaboticabal, 98 pp

Lu C-Y, Nugent G, Wardley-Richardson T, Chandler SF, Young R, Dalling MJ (1991) *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus* L.). Biotechnology 9:864–868. doi:10.1038/nbt0991-864

Maximova SN, Alemanno L, Young A, Ferriere N, Traore A, Guiltinan MJ (2002) Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. In Vitro Cell Dev Biol Plant 38:252–259. doi:10.1079/IVP2001257

Maximova S, Miller C, de Mayolo GA, Pishak S, Young A, Guiltinan MJ (2003) Stable transformation of *Theobroma cacao* L. and influence of matrix attachment regions on GFP expression. Plant Cell Rep 21:872–883. doi:10.1007/s00299-003-0596-7

Maximova SN, Marelli J-P, Young A, Pishak S, Verica JA, Guiltinan MJ (2006) Over-expression of a cacao class I chitinase gene in *Theobroma cacao* L. enhances resistance against the pathogen, *Colletotrichum gloeosporioides*. Planta 224:740–749. doi:10.1007/s00425-005-0188-6

McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep 5:81–84. doi:10.1007/BF00269239

Mendes AFS, Cidade LC, de Oliveira MLP, Otoni WC, Soares-Filho WS, Costa MGC (2009) Evaluation of novel beta-lactam antibiotics in comparison to cefotaxime on plant regeneration of *Citrus sinensis* L. Osb. Plant Cell Tissue Organ Cult 97:331–336. doi:10.1007/s11240-009-9518-x

Michelmore RW, Marsh E, Seely S, Landry B (1987) Transformation of lettuce (*Lactuca sativa*) mediated by *Agrobacterium tumefaciens*. Plant Cell Rep 6:439–442. doi:10.1007/BF00272777

Minocha SC, Papa NS, Khan AJ, Samuelsen AI (1991) Polyamines and somatic embryogenesis in carrot III. Effects of methylglyoxal bis(guanylhydrazone). Plant Cell Physiol 32:395–402

Minocha R, Minocha SC, Long S (2004) Polyamines and their biosynthetic enzymes during somatic embryo development in red spruce (*Picea rubens* Sarg.). In Vitro Cell Dev Biol Plant 40:572–580. doi:10.1079/IVP2004569

Montague MJ, Armstrong TA, Jaworski EG (1979) Polyamine metabolism in embryogenic cells of *Daucus carota* II. Changes in arginine decarboxylase activity. Plant Physiol 63:341–345. doi:10.1104/pp.63.2.341

Nauerby B, Billing K, Wyndaele R (1997) Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. Plant Sci 123:169–177. doi:10.1016/S0168-9452(96)04569-4

Niemi K, Sarjala T, Chen XW, Haggman H (2002) Spermidine and methylglyoxal bis(guanylhydrazone) affect maturation and endogenous polyamine content of scots pine embryogenic cultures. J Plant Physiol 159:1155–1158. doi:10.1078/0176-1617-00634

Niu X, Li X, Veronese P, Bressan RA, Weller SC, Hasegawa PM (2000) Factors affecting *Agrobacterium tumefaciens*-mediated transformation of peppermint. Plant Cell Rep 19:304–310. doi:10.1007/s002990050017

Ogawa Y, Mii M (2005) Evaluation of 12 β-lactam antibiotics for *Agrobacterium*-mediated transformation through in planta antibacterial activities and phytotoxicities. Plant Cell Rep 23:736–743. doi:10.1007/s00299-004-0885-9

Paul A, Mitter K, Raychaudhuri SS (2009) Effect of polyamines on in vitro somatic embryogenesis in *Momordica charantia* L. Plant Cell Tissue Organ Cult 97:303–311. doi:10.1007/s11240-009-9529-7

Pollock K, Barfield DG, Shields R (1983) The toxicity of antibiotics to plant cell culture. Plant Cell Rep 2:36–39

Robie CA, Minocha SC (1989) Polyamines and somatic embryogenesis in carrot I. The effects of difluoromethylornithine and difluoromethylarginine. Plant Sci 65:45–54. doi:10.1016/0168-9452(89)90206-9

Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel B (1992) Characterization of competent cells and early events of Agrobacterium-mediated genetic transformation in *Arabidopsis thaliana*. Planta 188:439–456. doi:10.1007/BF00192812

Santarém ER, Trick HN, Essing JS, Finer JJ (1998) Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. Plant Cell Rep 17:752–759. doi:10.1007/s002990050478

Seabra RC, Pais MS (1998) Genetic transformation of European chestnut. Plant Cell Rep 17:177–182. doi:10.1007/s002990050374

Shin YM, Choe G, Shin B, Yi G, Yun PY, Yang K, Lee JS, Kwak SS, Kim KM (2007) Selection of nptII transgenic sweetpotato plants using G(418) and paromornycin. J Plant Biol 50:206–212. doi:10.1007/BF03030631

Silveira V, Santa-Catarina C, Tun NN, Scherer GFE, Handro W, Guerra MP, Floh EIS (2006) Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert) O Ktze. Plant Sci 171:91–98. doi:10.1016/j.plantsci.2006.02.015

Song G-Q, Sink KC (2004) *Agrobacterium tumefaciens*-mediated transformation of blueberry (*Vaccinium corymbosum* L.). Plant Cell Rep 23:475–484. doi:10.1007/s00299-004-0842-7

Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells which activate the T-DNA transfer process in *Agrobacterium tumefaciens*. Nature 318:624–629. doi:10.1038/318624a0

Steiner N, Santa-Catarina C, Silveira V, Floh EIS, Guerra MP (2007) Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. Plant Cell Tissue Organ Cult 89:55–62. doi:10.1007/s11240-007-9216-5

Takeda T, Hayakawa F, Oe K, Matsuoka H (2002) Effects of exogenous polyamine on embryogenic carrot cells. Biochem Eng J 12:21–28. doi:10.1016/S1369-703X(02)00037-2

Tang W, Sederoff R, Whetten R (2001) Regeneration of transgenic loblolly pine (*Pinus taeda* L.) from zygotic embryos transformed with *Agrobacterium tumefaciens*. Planta 213:981–989. doi:10.1007/s004250100566

Tian LN, Canli FA, Wang X, Sibbald S (2009) Genetic transformation of *Prunus domestica* L. using the hpt gene coding for hygromycin resistance as the selectable marker. Sci Hortic 119:339–343. doi:10.1016/j.scienta.2008.08.024

Trick H, Finer JJ (1997) SAAT: sonication-assisted *Agrobacterium*-mediated transformation. Transgenic Res 6:329–336. doi:10.1023/A:1018470930944

Velcheva M, Faltin Z, Flaishman M, Eshdat Y, Perl A (2005) A liquid culture system for *Agrobacterium*-mediated transformation of

tomato (*Lycopersicon esculentum* L. Mill.). Plant Sci 168:121–130. doi:10.1016/j.plantsci.2004.07.037

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Sangwan-Norreel BS (1997) Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of Petunia: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201:160–172. doi:10.1007/BF01007700

Wiebke B, Ferreira F, Pasquali G, Bodanese-Zanettini MH, Droste A (2006) Influence of antibiotics on embryogenic tissue and *Agrobacterium tumefaciens* suppression in soybean genetic transformation. Bragantia 65:543–551

Wood GAR, Lass RA (1985) Cocoa. Longman, London 620 p

Wu XB, Wang J, Liu JH, Deng XX (2009) Involvement of polyamine biosynthesis in somatic embryogenesis of Valencia sweet orange (*Citrus sinensis*) induced by glycerol. J Plant Physiol 166:52–62. doi:10.1016/j.jplph.2008.02.005

Zaragozá C, Muñoz-Bertomeu J, Arrilaga I (2004) Regeneration of herbicide-tolerant black locust transgenic plants by SAAT. Plant Cell Rep 22:832–838. doi:10.1007/s00299-004-0766-2