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Moxalactam as a counter-selection antibiotic for *Agrobacterium*-mediated transformation and its positive effects on *Theobroma cacao* somatic embryogenesis

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Abstract

Previous work in a number of laboratories has led to the development of a highly efficient cacao somatic embryogenesis (SE) protocol, establishing the basis for an *Agrobacterium tumefaciens*-mediated genetic transformation system. Such a system will be valuable as a basic research tool, and possibly in the future as a means to introduce specific disease resistance or other genes into cacao. However, during our initial attempts at transformation, cacao tissue was frequently destroyed due to *Agrobacterium* overgrowth following co-cultivation. Furthermore, the addition of the antibiotic cefotaxime, commonly applied to tissue culture media to eliminate *Agrobacterium* post infection, decreased cacao somatic embryo production by 86%. To define antibiotic conditions sufficient to suppress *Agrobacterium* while not interfering with cacao SE, we evaluated the efficacy of four antibiotics. Two cephalosporins, cefotaxime and moxalactam; and two penicillins, amoxicillin and carbenicillin. Moxalactam, a β-Lactam antibiotic, was proven to effectively suppress *Agrobacterium* growth. Furthermore, at certain concentrations, it also significantly enhanced the efficiency of cacao SE. A possible mechanism for this enhancement is proposed.

Keywords: *Theobroma cacao*; Cacao; Cefotaxime; Antibiotics; Plant tissue culture; *Agrobacterium* selection

1. Introduction

Theobroma cacao, a perennial tree belonging to the Malvaceae family, is native to the American tropics [1,2]. Production of cacao is mainly the labor of small crop farmers, who cultivate close to 5 million hectares worldwide. Cacao seeds, fermented and dried, are a major cash crop for a number of developing countries with an annual value estimated at $2.9 billion US [3]. Additionally, cacao farming provides ecological benefits such as habitat and watershed conservation, enhancement of the connectivity between ecosystem fragments, soil stabilization and carbon sequestration [4].

Due to increasing disease pressures, cacao farmers are in urgent need of pest and pathogen resistant varieties. For example, over the past decade, Brazil lost 68% of its cacao production to 'witches broom', a fungal disease caused by infection of *Crinipellis pernicosa*. Ghana has experienced 29% crop loss due to capsids insects, cacao swollen shoot virus and the fungal disease pod rot [3]. Future predictions for crop loss are equally dim. The rapid spread of the lepidopteron pest cocoa pod borer, if left uncontrolled is predicted to reduce Indonesia's production by half, while Nigeria is predicted to incur production losses as much as 70% over the next few years if black pod continues to spread at the same rates [3]. Recent efforts in plant breeding, disease management, and agro-forestry have begun to make a positive impact on these problems.

Research in plant biology has developed an array of new techniques for crop improvement including: tissue

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culture, quantitative trait loci mapping, marker assisted selection, genetic engineering and the use of molecular techniques to investigate basic physiological mechanisms. Our group, along with colleagues worldwide, is committed to applying these technologies to cacao tree improvement with the ultimate goal of enhancing the income of smallholder farmers. One of our objectives has been the development of a genetic transformation system for cacao for use as a basic research tool, and to enable the method for potential future use in crop improvement. Following on prior work [5–7], we first developed an efficient in vitro primary somatic embryogenesis (SE) system for cacao using floral parts [8], and an even more efficient secondary somatic embryogenesis (2-SE) protocol using cotyledon slices from mature primary somatic embryos [9]. These systems have established the regenerative basis for the development of a cacao genetic transformation protocol via *Agrobacterium tumefaciens* co-cultivation [10]. However, developing a transformation system requires not only a reliable regeneration system but also an efficient cell transformation procedure, a mechanism for selection of the transformed cells, and antibiotic conditions for the inhibition of *Agrobacterium* growth in the in vitro culture post-infection [11]. It is essential that the conditions used for each of these steps do not interfere with tissue regeneration.

Our initial attempts at developing an *Agrobacterium*-mediated transformation system were hampered by bacterial overgrowth, which often destroyed the infected tissue weeks after infection. In our laboratory, *Agrobacterium* overgrowth during tobacco, *Arabidopsis* and apple transformations has been routinely controlled utilizing cefotaxime antibiotic at a concentration 400 mg l^{-1} [12,13]. However, using these conditions during cacao transformation resulted in *Agrobacterium* overgrowth, requiring frequent transfers of explants to media with fresh antibiotic and often, tissue necrosis. In addition, preliminary studies indicated that these conditions decreased somatic embryo regeneration by as much as 86% [14].

In this study, we evaluated the effects of different antibiotics on SE and their potential as *Agrobacterium* counter selection agents during cacao genetic transformation. A dilution series of two major antibiotic classes: cephalosporins (cefotaxime and moxalactam) and penicillin (amoxicillin and carbenicillin) were investigated. Using an in vitro disk diffusion assay, [15], we first established the efficacy of the antibiotics in controlling *Agrobacterium* growth. Each of the antibiotics were also added to embryo regeneration media at four different concentrations to evaluate their effects on callus and embryo formation during cacao secondary embryogenesis and to assess their efficacy in controlling *Agrobacterium* growth after co-cultivation with cacao somatic embryo cotyledon explants. Our results indicated that moxalactam, a β-Lactam antibiotic, efficiently suppressed the growth of *Agrobacterium* strain AGL1 during the in vitro disk assays and tissue transformation. Surprisingly, the results also indicated that moxalactam significantly enhances the efficiency of somatic embryo formation. The results demonstrate that in addition to controlling *Agrobacterium* overgrowth, moxalactam enhanced embryo production by approximately 2.5-fold. Using these conditions, the successful production of transgenic cacao embryos and plants has been achieved [10].

2. Materials and methods

2.1. Antibiotics

All antibiotics were purchased from Sigma (St. Louis, MO, USA). Stock solutions of cefotaxime (Sigma #C7039), carbenicillin (Sigma #C3416) and moxalactam (Sigma #M8158) were dissolved in water (100 mg l^{-1}), and filter sterilized. Amoxicillin (Sigma #A8523) was dissolved to the same concentration in 95% ethanol. Stock antibiotics were stored at −20 °C for up to 2 months. Antibiotics were added to the tissue culture media after media was autoclaved and cooled to 55 °C, before plates were poured.

2.2. Bacterial cultures

Twenty five ml of 523 media [16] containing 20 mg l^{-1} rifampicin (Sigma #R7382) and 50 mg l^{-1} kanamycin (Sigma #K1377) were inoculated with *A. tumefaciens* strain AGL1 [17], containing plasmid pCP108. Binary vector pCP108 was constructed by the ligation of EGFP gene (Clontech) expression cassette as a *Hind*III-*Hind*III fragment into the *Hind*III cloning site of binary transformation vector pBI121 Clontech (Palo Alto, CA, USA) [19]. An EGFP gene was driven by a CaMV 35s derivative, the E12Ω5′ promoter [18]. Additionally, pBI121 contains a neomycin phosphotransferase (*nptII*) plant selectable marker gene and a *β-glucuronidase* (GUS) reporter gene. *Agrobacterium* cultures were grown overnight on a rotary shaker at 200 rpm, at 25 °C. The optical density of the cultures was adjusted to 0.5 OD at 420 nm by dilution with fresh 523 bacteria media just before use.

2.3. Disk diffusion assay

Filter paper discs, 5 mm in diameter, (Whatman 3 mm chromatography paper), were autoclaved and saturated with 15 μl of antibiotic solutions, each with one of four different concentrations: 100, 150, 200, and 300 mg l^{-1}. Bacterial solution (50 μl, 0.5 OD at 420 nm) was evenly spread on the surface of solid 523 media, and then the

four discs were placed on top of two replicate 523 agar plates. Plates were wrapped in parafilm and incubated for 48 h at 25 °C. The zone of bacterial inhibition was defined as the distance in mm from the edge of the paper disc to the edge of the bacterial growth area.

2.4. Cacao tissue culture

Immature flowers were collected from Scavina-6 cacao plants grown in a greenhouse at The Pennsylvania State University. Primary somatic embryos were produced as described by Li et al. [8] by culturing staminode explants on primary callus growth (PCG) medium, followed by transfers to secondary callus growth (SCG) medium and were maintained on plant growth regulator free embryo development (ED) medium [8]. Secondary somatic embryos were initiated on SCG medium, followed by transfer to ED [9]. Cotyledon pieces, 4×4 mm in size, were excised from mature primary somatic embryos at different times after primary culture initiation depending on the experimental objectives (see below). All moxalactam treatments contained three replicate plates with 25 explants each and the experiments were repeated three times, except for the initial experiment containing all four antibiotics, in which case only 25 explants per plate per treatment were initiated and recorded. Primary and secondary cultures were maintained in the dark at 25 °C. In all experiments, embryo production and morphology was observed and recorded for the duration of the experiments. The total numbers of embryos present in the cultures were counted every other week for 22 weeks after culture initiation. Mature embryos were also classified as either normal (containing an axis, healthy hypocotyl and two cotyledons) or abnormal (multiple embryos fused at the axis, embryos lacking cotyledons or those with multiple cotyledons) as previously described [9].

3. Results

3.1. Effect of various antibiotics on the growth of A. tumefaciens strain AGL1

To determine the most effective antibiotic for controlling the growth of *A. tumefaciens*, a disk diffusion bioassay was performed using four antibiotics at concentrations of 100, 150, 200 and 300 mg l^{-1}. From the antibiotics tested, moxalactam, at all concentrations demonstrated effective control over bacterial growth and produced the largest bacterial inhibition areas, up to 27 mm at 200 mg l^{-1} (Fig. 1). The second largest inhibition area observed was for cefotaxime (18 mm) at the concentration of 200 mg l^{-1}, same inhibition zone as that observed for the lowest moxalactam concentration.

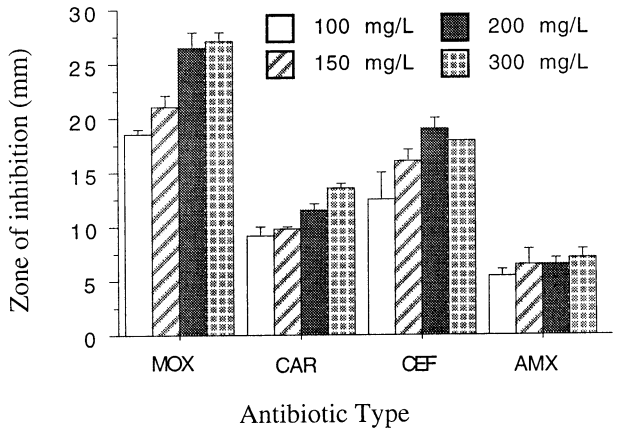

Fig. 1. Antibiotic disk diffusion assay. *Agrobacterium* strain AGL1 was challenged with four antibiotics in a disk diffusion assay. The effects of the antibiotics on bacterial growth is represented as the zone of inhibition in millimeters (mm) from the paper disk to the edge of bacteria growth after 48 h of co-cultivation at 25 °C. Abbreviations are CEF-cefotaxime, CAR-carbenicillin, AMX-amoxicillin, and MOX-moxalactam.

Carbenicillin and amoxicillin were somewhat effective in controlling bacterial growth in this assay, but the zones of inhibition were smaller than those observed for the other two antibiotics. Similar results were also seen with the *Agrobacterium* strain EHA104 (data not shown).

3.2. A. tumefaciens infection and counter selection

Cotyledon explants from 20-weeks-old primary somatic embryo cultures were inoculated and co-cultivated with *A. tumefaciens* (AGL1::pCP108) as described for apple [14], with the addition of a 30 s sonication treatment in a Bransonic water bath (Model 1510, VWR, Pittsburgh, PA), at 25 °C. After 48 h, co-cultivation in the dark at 25 °C, the explants were transferred to SCG medium containing various antibiotics. The types and the concentrations of the antibiotics used for *Agrobacterium* counter selection were as described in the disk diffusion assay. The treatments included *Agrobacterium* infection and co-cultivation followed by counter selection with all four antibiotics at four different concentrations.

Moxalactam, at all concentrations tested, effectively controlled *Agrobacterium* growth with no explants lost in any of the treatments (Table 1). Explants grown on media with the other antibiotics needed more frequent transfer due to bacterial overgrowth, especially during the first 4 weeks following co-cultivation. Unlike the results from the disk assay, carbenicillin was more effective than cefotaxime at suppressing *Agrobacterium* growth after co-cultivation. This was demonstrated by the fewer explants lost on the carbenicillin medium compared with those on cefotaxime (Table 1), especially at the lower concentrations (100, 150 mg l^{-1}) where

Table 1
Effect of antibiotics on the percent of explants lost to *Agrobacterium*-AGLl overgrowth 10 weeks after cocultivation

Concentration (μg ml^{-1})	CEF	CAR	AMX	MOX
100	15	5	30	0
150	14	2	24	0
200	10	0	20	0
300	8	0	15	0

The percentages were calculated from the total number of explants lost per treatment plate. One plate containing 25 total number of explants was initiated per concentration and antibiotic treatment. Abbreviations are CEF, cefotaxime, CAR, carbenicillin, AMX, amoxicillin, and MOX, moxalactam.

considerably fewer explants were lost on the carbenicillin plates than on cefotaxime. Amoxicillin provided little control over *Agrobacterium*, resulting in a high percentage of explants lost at all four concentrations tested.

To investigate the effects of the antibiotics on SE, cotyledon explants were cultured with the various antibiotics at varying concentrations. Tissues were transferred to fresh media every other week. Twenty-five explants were cultured per treatment. Ten weeks after culture initiation, the numbers of explants lost to *Agrobacterium* overgrowth were recorded. Additionally, the total numbers of SEs produced per treatment and the numbers of explants producing SEs were recorded.

At the concentrations tested, all antibiotics had some effect on the regeneration potential of cacao secondary somatic embryogenesis (2-SE) compared with the control cultures without antibiotics (Fig. 2). Cotyledons exposed to 200 mg l^{-1} moxalactam responded positively, with the highest increase in the total number of embryos produced per cotyledon explant. Moxalactam at concentrations above 100 mg l^{-1} showed a strong positive effect on embryo regeneration. The highest production was achieved at 200 mg l^{-1} with an average number of embryos per explant 3-fold higher than the control plates without antibiotic. At 300 mg l^{-1}, the effect of moxalactam on embryogenesis was reduced to an average of 11 embryos per explant compared with 200 mg l^{-1}, with an average of 17 embryos per explant. At 300 mg l^{-1}, we observed an average increase in the total number of embryos compared with the control regeneration plates, which only produced seven embryos per explant. Cefotaxime enhanced regeneration only at 150 mg l^{-1}. Negative effects were observed at all of other cefotaxime concentrations tested, including 300 mg l^{-1}, the minimum concentration required for moderate control of the *Agrobacterium* during transformation. Carbenicillin and amoxicillin had negative effects at all concentrations tested, decreasing cacao 2-SE.

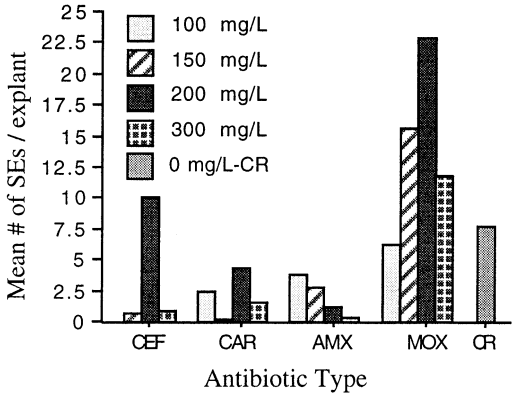

Fig. 2. Effects of antibiotic type and concentration on the average number of embryos produced per cotyledon explant in the absence of *Agrobacterium*. Twenty five explants per treatment were plated on media containing various antibiotics. The total number of embryos produced and the total numbers of explants initiated per treatment were recorded. The average number of embryos per total explant was calculated. Abbreviations: CEF-cefotaxime, CAR-carbenicillin, AMX-amoxicillin, and MOX-moxalactam.

3.3. Detailed analysis of the effects of moxalactam on cacao primary and secondary embryogenesis

To determine the optimal moxalactam concentration for primary embryogenesis, and the moxalactam concentration in combination with explant age for secondary embryogenesis, primary embryogenesis was initiated immediately after flower harvest, and cotyledon explants were excised from mature SEs at 20, 21 and 24 weeks after primary culture initiation from primary SE cultures. Moxalactam concentrations applied were 100, 150, 200 and 300 mg l^{-1}. Due to tissue limitations, 150 mg l^{-1} concentration was applied only to secondary embryogenesis treatments initiated at 20 and 21 weeks.

3.4. Effect of moxalactam on primary SE

Staminode cultures were monitored for a 22-week period. The total number of embryos produced and the number of explants producing embryos were recorded. As seen in the previously described experiment, we observed that moxalactam had a significant positive effect on regeneration, increasing primary embryo production by 42% at 100 mg l^{-1}. No significant effect was observed for explants plated on 200 mg l^{-1} compared with the control, while at 300 mg l^{-1}, moxalactam had negative effects on the tissue regeneration (Table 2), producing white hard calli, forming numerous roots and developing very few embryos.

In previous work, we have observed the appearance of both normal and abnormal embryos in culture [8,9]. Thus, we counted the numbers of normal and abnormal embryos in these experiments to determine possible antibiotic effect on the embryo quality (Fig. 3B). Although moxalactam at concentrations higher than

Table 2
Average number of normal embryos produced per treatment after 20 weeks culture initiation

Explant	Age of tissue (weeks)	Control	MOX 100	MOX 150	Mox 200	Mox 300
Staminode	0	11.0±2.1	35.0±2.8		35.5±3.7	22.0±3.0
Cotyledon	20	141.0±3.6	153.0±7.3	163.0±6.4	218.0±4.0	158.0±3.4
Cotyledon	21	85.3±5.9	135.6±3.0	146.7±6.2	249.0±6.2	147.0±5.3
Cotyledon	24	97.3±4.3	140.3±2.4		361.3±4.7	124.6±3.9

Data represents the average number of normal embryo per explant±standard error. Averages were generated from three replicate plates containing 25 explants each. Abbreviations: MOX, moxalaetam. Concentrations are expressed in mg l^{-1}.

100 mg l^{-1} had a negative effect on the number of embryos produced, the higher concentrations did not affect the percentage of normal and abnormal embryos produced, 25 and 75%, respectively. A one-way ANOVA analysis at the 95% confidence interval showed no significant difference ($P = 0.772$) in embryo quality among all antibiotic treatments.

Another important parameter associated with the efficiency of cacao SE is the percentage of explants which regenerate and produce at least one embryo (Fig. 3C). Moxalactam at 100 mg l^{-1} had a positive effect on the percentage of staminodes producing embryos. However, as reported in Table 2, antibiotic concentrations higher than 100 mg l^{-1} had negative effects on the number of SEs produced from staminodes, an effect that became accentuated with increasing antibiotic concentrations.

3.5. Effect of moxalactam on secondary SE

An increase in embryo production was also observed for 2-SE cultures at all concentrations of moxalactam tested. The highest numbers observed were at the antibiotic concentration of 200 mg l^{-1} (Table 2). The age of the primary culture at the time of 2-SE initiation was also important, even in the presence of moxalactam. Moxalactam treatment resulted in increases of 36, 91 and 264 % embryo production for the cultures initiated at age 20, 21 and 24 weeks, respectively, after primary culture initiation. The effects of moxalactam on 2-SE (Fig. 4) were similar to those observed with primary embryogenesis, (Fig. 3). At all concentrations tested, moxalactam increased embryo production compared with the control treatments. Similar to the result with primary embryogenesis, a significant increase in the number of embryos was recorded at all treatments, with optimal moxalactam concentration at 200 mg l^{-1} (Fig. 4A and C).

In our experiments, the control treatments exhibited the lowest average number of embryos per explant, followed by 100, and 150 mg l^{-1} moxalactam. At moxalactam concentrations of 200 mg l^{-1}, we observed the best response from the tissue, and at 300 mg l^{-1} embryo production started to decline compared with 200 mg l^{-1} (Fig. 4A). At the lower three concentrations, no difference in the percentage of normal versus abnormal embryos was recorded ($P = 0.954$). However, at 300 mg l^{-1} a statistically significant decrease ($P < 0.001$) in the production of normal embryos was observed (Fig. 4B) when compared with all other treatments (two-sample t-tests at the 95 percent confidence interval).

3.6. Comparison of cefotaxime and moxalactam effects on cacao secondary embryogenesis

In an effort to further investigate the effects of cefotaxime and moxalactam on cacao SE, an experiment was designed to evaluate the antibiotics effects on embryo production and quality. For this experiment cotyledons from 24-week-old primary culture were exposed to cefotaxime and moxalactam at the concentrations of 0, 100, 200 and 300 mg l^{-1} ($2 \times 4 = 8$ treatments). At the concentrations tested, both antibiotics altered secondary embryo production (Fig. 5A and B). The highest numbers of embryos produced by the cefotaxime treatment was at 100 mg l^{-1}, with the average number of embryos decreasing at the higher concentrations of 200 and 300 mg l^{-1}. In contrast, moxalactam averages peaked at 200 mg l^{-1}, decreasing to 300 mg l^{-1} with averages well above the control regeneration plates. When comparing the numbers of embryos produced at 300 mg l^{-1} cefotaxime, the values are not significantly different from those produced on control regeneration plates ($P = 0.412$). Nonetheless, at 300 mg l^{-1} cefotaxime, we observed a slight increase ($P = 0.044$) in embryo abnormality compared with the control (Fig. 5B). Similar increases in embryo abnormality were also observed at 300 mg l^{-1} moxalactam (Fig. 4B). In addition, we compared the effects of cefotaxime on normality rates using a two-sample t-test. In this analysis, we observed no significant difference between abnormal embryo production in the controls 47% ($P = 0.412$), the 100 mg l^{-1} treatments 34% ($P = 0.44$), or in the 200 mg l^{-1} treatment 45% ($P = 0.135$) assuming equal variance. A significant difference was identified at the 300 mg l^{-1} concentration when the treatment produced a total of 53% abnormal embryos ($P = 0.002$) compared with all the other treatments.

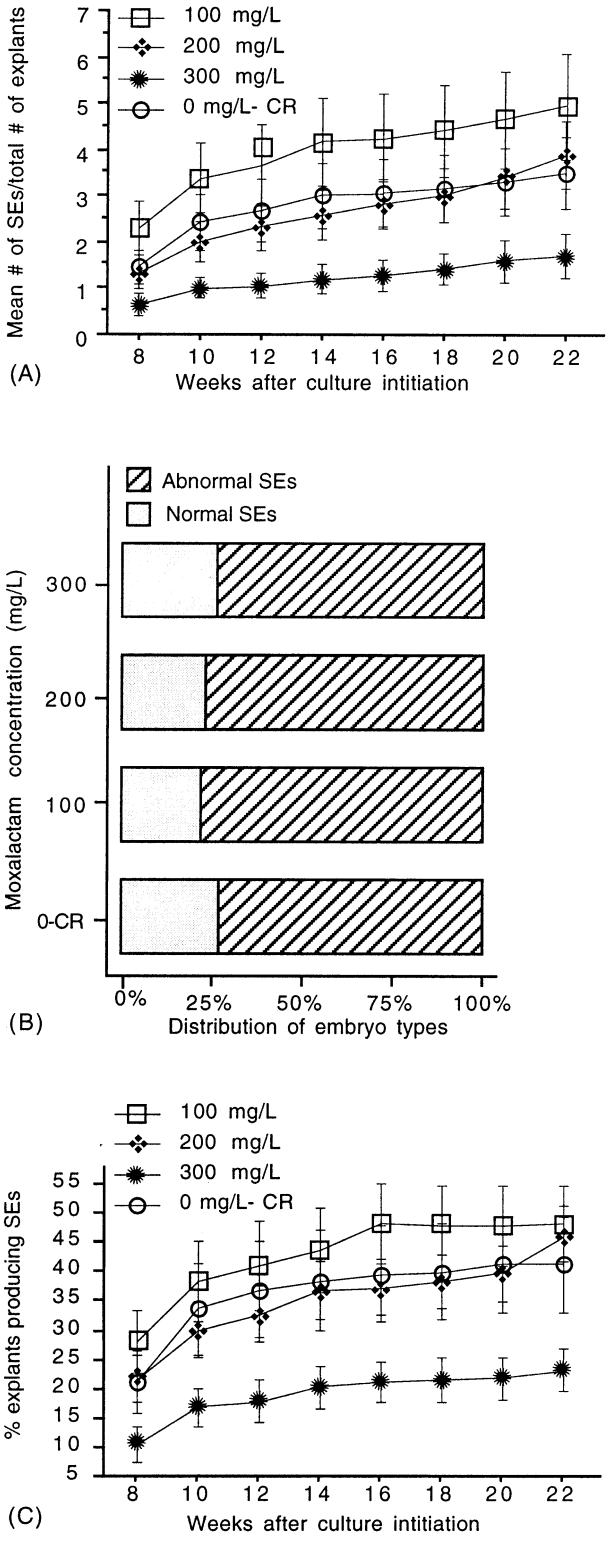

Fig. 3

3.7. Effects of moxalactam time exposure on cacao secondary embryogenesis

In the previous experiments, the explants were cultured continuously on antibiotics; therefore, we also investigated the effect of moxalactam time exposure on secondary embryogenesis. Twenty-four week-old primary cotyledons were placed on SCG for secondary embryogenesis and exposed to 200 mg l^{-1} moxalactam for 0, 4, 6, or 20 weeks, followed by growth on antibiotic free media. Starting 8 weeks after culture initiation, the number of explants producing embryos and the total number of embryos produced per treatment were recorded every other week for 20 weeks.

Our data indicated that 4 weeks of exposure to moxalactam had a positive effect on embryogenesis compared with untreated controls (Fig. 6). The number of embryos produced increased further after 6 weeks exposure to moxalactam and were slightly higher for those explants that remained in the 200 mg l^{-1} antibiotic concentration throughout the experiment. Thus, we can conclude that for the first 20 weeks after culture initiation, moxalactam had no negative effect on the regeneration potential of cacao cotyledon tissue; conversely, extended exposure to the antibiotic results in increased regeneration.

4. Discussion

Development of an effective transformation system for cacao depends on the availability of tissue culture techniques that permit efficient DNA delivery, selection of transformed cells and the regeneration and recovery of whole transgenic plants. *Agrobacterium* mediated transformation is a method of choice for the introduction of foreign genes into plants. During the development of a transformation protocol for cacao, it was noted at an early stage that controlling *A. tumefaciens* growth after gene delivery and increasing the efficiency of the SE system would be critical for success. The research described here contributed to the development of a working transformation system [10], which is very useful as a basic research tool for cacao. Using moxalactam antibiotic in the transformation protocol, we have generated 17 independent transgenic 2-SE lines from ten *Agrobacterium*-mediated transformation ex-

Fig. 3. Effects of moxalactam antibiotic and concentration on primary embryogenesis. Staminode explants were cultured on media containing various concentrations of moxalactam. Quantitative and qualitative data of the embryo production were collected at 2 week intervals for the entire culture period. (A) Average number of somatic embryos produced per cotyledon explant. Data points represent the means of three replicate plates. (B) Percentages of normal vs. abnormal embryos produced on moxalactam compared with control regeneration without antibiotic. The columns represent the cumulative mean percentages at 20 weeks from three replicate plates. Standard errors vary from 1.2–2.5% for all data sets collected. (C) Percentage of explants producing somatic embryos over time. Data points represent mean percentages of three replicate plates. Mean separations were performed using Fisher's PLSD test at 5% level of significance.

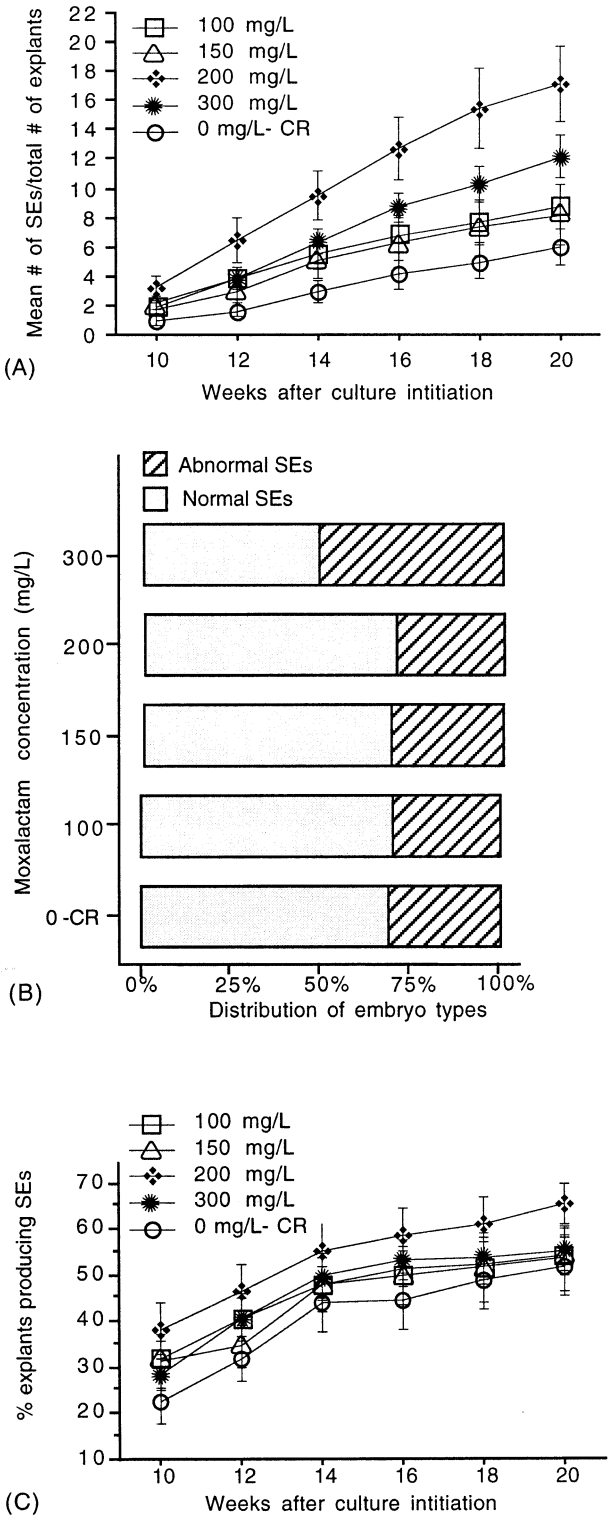

Fig. 4

periments [10]. While the efficiency is low, and work is ongoing to improve it, transgenic lines of plants have been generated using various transformation vectors, including a vector containing a *T. cacao* class I chitinase

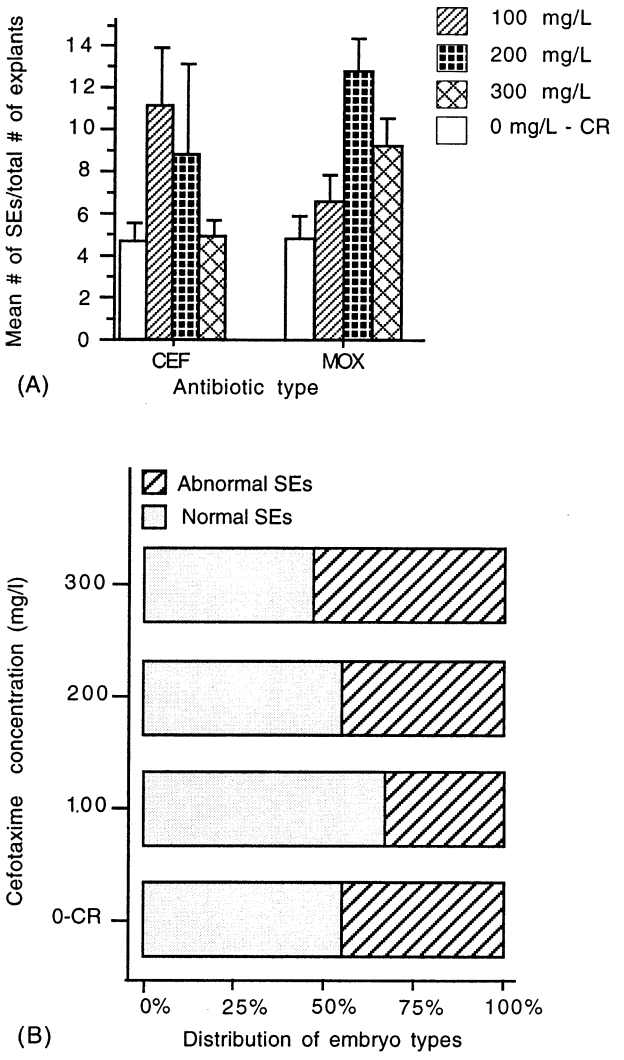

Fig. 5. Effects of cefotaxime antibiotic and concentration on secondary embryogenesis. (A) Direct comparison between the effects of cefotaxime and moxalactam antibiotic after a 20 week cultivation period of tissue on media containing antibiotics at the indicated concentrations. (B) Percentages of normal vs. abnormal embryos produced on moxalactam compared with control regeneration without antibiotic. Stack columns represent cumulative data at 20 weeks of mean percentages from three replicate plates. Standard errors vary from 2.37 to 3.83% for all data sets collected.

Fig. 4. Effects of moxalactam on secondary embryogenesis. Cotyledon explants were cultured for 20 weeks on media containing various concentrations of moxalactam. Quantitative and qualitative data of embryo production were collected every 2 weeks for the entire culture period. (A) Average numbers of somatic embryos produced per cotyledon explant. Data points represent the means of three replicate plates. (B) Percentages of normal vs. abnormal embryos produced on moxalactam compared with control regeneration without antibiotic. The columns represent cumulative mean percentages at 20 weeks from three replicate plates. Standard errors vary from 1.3 to 2.29% for all data sets collected. C. Percentages of explants producing somatic embryos over time. Data points represent mean percentages of three replicate plates. Mean separations were performed using Fisher's PLSD test at 5% level of significance.

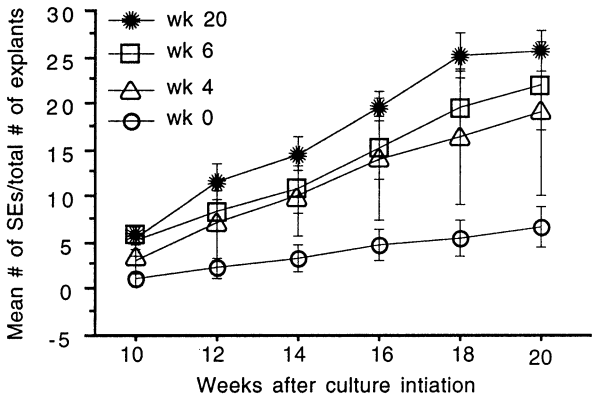

Fig. 6. Effect of time exposure of the antibiotic moxalactam on secondary embryogenesis. Twenty five cotyledon explants per plate in triplicate were plated on media containing 200 mg l^{-1} moxalactam antibiotic for the four specific time treatments of 0, 4, 6 or 20 weeks. After treatments, explants were grown on antibiotic free media for a total of 20 weeks. The total number of embryos produced, and the total numbers of explants initiated per treatment were recorded. The average numbers of embryos produced per explant were calculated. Abbreviations: CEF-cefotaxime, CAR-carbenicillin, AMX-amoxicillin, and MOX-moxalactam.

and several other marker genes. Further analysis of these lines awaits embryo development into plants with sufficient tissue for molecular examination.

The antibiotics cefotaxime and carbenicillin are commonly used for counter-selection of *Agrobacterium* [11,15]. It is generally assumed that these antibiotics, at concentrations required for bacterial control, are not detrimental to the plant tissue in vitro. The effect of these antibiotics on morphogenesis of plants in highly regenerative microculture systems like tobacco leaf discs may be negligible [11]. However, that is not the case for the *T. cacao* SE system, which in the past has proven relatively recalcitrant to tissue culture practices.

The antibiotics tested in this study belong to two major classes, cephalosporins and penicillins. These drugs are known as β-Lactams, which despite their different structural compositions inhibit the synthesis of bacterial cell walls [20]. Both classes of antibiotics become covalently linked to the cell's penicillin-binding proteins, enzymes responsible for constructing or modifying the bacterial cell wall. Antibiotic binding prevents cell wall synthesis, causing leakage and/or triggering of active enzymatic processes, leading to the degradation of the cell wall [20]. The cephalosporin antibiotics tested in this study were moxalactam and cefotaxime, and we observed that both cephalosporin type antibiotics showed good control of *Agrobacterium*, while also increasing embryo regeneration, compared with control regeneration.

In an effort to determine the optimal counter-selection conditions for *Agrobacterium*-mediated transformation of cacao, we tested various antibiotics and their individual effects on *A. tumefaciens* growth and on the SE regeneration system. The results from the disk diffusion assay demonstrated that moxalactam is most effective for growth inhibition of strain AGL1. Similar results were observed when testing the same four antibiotics with *Agrobacterium* strains EHA 101 and LBA4404, where moxalactam proved to be the antibiotic with the largest bacterial zone of inhibition, even at the low concentration of 100 mg l^{-1} [15]. Similarly, for strain AGL1::pCP108, we observed that the application of moxalactam at 100 mg l^{-1} resulted in an inhibition zone of 18 mm, equivalent to that of the next most effective antibiotic, cefotaxime at 200 mg l^{-1}. The addition of 200 mg l^{-1} moxalactam to the regeneration media not only effectively eliminated bacterial growth, but also enhanced secondary embryo production even in the absence of *Agrobacterium*. Moxalactam also contributed to an increased number of cotyledon explants capable of embryo regeneration.

The effects of moxalactam antibiotic were evaluated in combination with the other factors required for a successful transformation protocol, including explant type, tissue age, and *Agrobacterium* strain. Here, we demonstrated that the use of moxalactam in cacao primary and 2-SE not only controlled *Agrobacterium*, but also enhanced embryogenesis frequencies. Consistent with previous observations [9], our results showed that secondary embryogenesis is more efficient than primary embryogenesis (3.5-fold higher) in terms of average numbers of embryos produced per explant cultured. Similarly, secondary embryogenesis produced a higher percentage of normal embryos per explant. This indicates the distinct advantages of utilizing secondary embryogenesis for development of a transformation protocol.

A clue to a possible mechanism of action explaining the enhancement of cacao SE by moxalactam is found in the structural composition of this antibiotic. After its breakdown, moxalactam is thought to degenerate into several key functional groups [21], one of which resembles phenylacetic acid, a naturally occurring auxin. This suggests that increased auxin concentration during SE may promote higher regeneration frequencies. However, for our transformation system, moxalactam function is doubled by not only serving potentially as an additional auxin source, but also by exhibiting β-Lactam activity against gram negative bacteria, including *Agrobacterium* [20,22], thus enhancing embryogenesis, preventing bacterial overgrowth and tissue loss.

It is important to note that significant genotypic effects on cacao SE efficiencies has been observed [9]. The results presented here were obtained with the genotype *Scavina* 6 and it is likely that the effects of antibiotics on other genotypes will vary significantly. Additionally there is a need to test the seasonal effects on cacao SE, since our observations over time indicate a difference in the frequency of somatic embryos pro-

duced from tissue initiated at different times of the year [8,9]. Under optimal somatic embryo formation frequencies, the moxalactam effect may be limited or negligible. Long-term experiments to test this effect are ongoing at the present time.

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