ARTÍCULO DE INVESTIGACIÓN

Effective β-lactam antibiotics for *Agrobacterium tumefaciens* suppression in indica rice calli

Antibióticos beta-lactámicos efectivos para eliminar el *Agrobacterium tumefaciens* en callos de arroz índica

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Abstract

It was evaluated the antibacterial activity of seven β-lactam antibiotics against *A. tumefaciens* strain EHA105, living in indica rice calli. For detection of persistent *Agrobacterium* in callus tissues, homogenates of infected calli were spread over LB agar plates to count colony forming units per gram of calli. *Agrobacterium* growth was completely suppressed during plant regeneration at 250 mg/l of all of the antibiotics tested. Also it was appraised the effect of β-lactams on callus growth and plant regeneration. A similar tendency of increased calli fresh weight with 100 mg/l and 250 mg/l of all antibiotics was proven. But the use of 500 mg/l caused decreasing of callus growth. About 80% of calli formed shoots in a month when we used 100 mg/l or 250 mg/l of β-lactam during the regeneration stage. Two morphogenic responses were distinguished during regeneration stage: somatic embryogenesis and adventitious shoots organogenesis.

Key words: antibacterial activity, embryogenesis, shoot regeneration.

Resumen

Se evaluó la actividad antibacteriana de siete antibióticos β-lactámicos sobre la cepa EHA105 de *Agrobacterium tumefaciens* inoculada en callos de arroz. Para detectar el *Agrobacterium* persistente en los callos, se cultivó en medio LB sólido un extracto de callos infectados, y se contó el número de unidades formadoras de colonias por gramo de callo. La bacteria se eliminó totalmente en la fase de regeneración utilizando 250 mg/l de cualquiera de los antibióticos probados. Se evaluó además el efecto de los β-lactámicos sobre el crecimiento de los callos y la regeneración de plantas. El incremento del peso de los callos fue similar cuando se utilizó 100 y 250 mg/l de antibiótico, pero disminuyó significativamente cuando la concentración se elevó a 500 mg/l. La regeneración de plantas ocurrió a partir del 80% de los callos cultivados con 100 y 250 mg/l de antibiótico. En la regeneración se distinguieron dos respuestas morfogénicas: embriogénesis somática y organogénesis adventicia.

Palabras claves: actividad antibacteriana, embriogénesis, regeneración de brotes.

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Introduction

Establishment of high efficient *Agrobacterium tumefaciens*-mediated transformation protocols has greatly facilitated the widespread application of genetic transformation in rice (*Oryza sativa* L.), to introduce genes for varietal improvement (Lin and Zhang, 2005). For *Agrobacterium*-mediated transformation, elimination of *Agrobacterium* are essential to reduce the risk of damaging growth and regeneration of transformed tissues, and to minimize the hazard of releasing genetically engineered *Agrobacterium* into the environment (Ogawa and Mii, 2005).

β-Lactam antibiotics are frequently used for *Agrobacterium* eradication in plant tissues, they are penicillin, cephalosporin, cephamycin, oxacephem, monobactam and carbapenem, and are currently being developed to improve and enlarge antibacterial activity (Demain and Elander, 1999). These classes of antibiotics lyse and kill the bacteria by specifically interfering with the biosynthesis of the prokaryotic peptidoglycan component of the bacterial cell wall by binding to penicillin-binding proteins (Asbel and Levison, 2000). Carbenicillin and cefotaxime, which are penicillin and cephalosporin respectively, are regularly used for *Agrobacterium* elimination in transformed tissues. Other β-lactam antibiotics have also been employed, e.g., sulbenicillin (Zhang *et al*., 2000), augmentin (Ieamkhang and Chatchawankanphanich, 2005), meropenem and moxalactam (Ogawa and Mii, 2007). The ideal antibiotics should be stable, soluble, inexpensive, unaffected by pH and media components, lack side effects, as well as being non-toxic to plant cells.

The selection of the suitable antibiotics that do not inhibit growth, organogenesis or embryogenesis of plant tissues at effective concentrations for suppression of *Agrobacterium* is of paramount importance. Side effects of antibiotics may vary depending of plant species and explant sources (Nauerby *et al*., 1997).

Augmentin and timentin were used for suppressing *Agrobacterium tumefaciens* during tomato (*Lycopersicon esculentum*) genetic transformation (Ieamkhang and Chatchawankanphanich, 2005). The concentrations of augmentin and timentin used to eliminate *Agrobacterium* were 300 and 100 mg/l, respectively. Concentrations up to 500 mg/l of both antibiotics showed no significant toxicity to shoot regeneration. In 2005, Ogawa and Mii concluded that meropenem and moxalactam, novel and expensive β-lactams, are the most effective antibiotics for *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabacum*) leaves. Meropenem exhibited the highest antibacterial activity and it also offers a high shoot formation rate. Two years later, the same authors published the usefulness of both antibiotic in tomato and rice (Ogawa and Mii, 2007).

It has been reported that high concentrations of cefotaxime, carbenicillin and augmentin, may reduce the regenerability of rice calluses, as they resemble auxins in their structure, and in combination with 2,4-dichlorophenoxyacetic acid (2,4-D) may cause loss in regeneration potential (Okkels and Pederson, 1988). The reduction of culture of calli with the antibiotic led to a substantial increase in the regeneration potential of the transformed tissues (Tyagi *et al*., 2007).

In a previous study, was described a procedure for *Agrobacterium*-mediated transformation of calli of indica rice cultivar IACuba-28 (Pérez-Bernal *et al*., 2008) but, frequently, calli are affected by persistent *Agrobacterium* growth. The purpose of this work is to evaluate the effect of seven β-lactam antibiotics on the elimination of callus contamination with *Agrobacterium tumefaciens*, and to verify their repercussion on callus development and plant regeneration.

Materials and methods

Callus induction

Mature dehulled seeds of IACuba-28 rice variety, were surface sterilized with 70% ethanol for 1 min and with 2.5% active chlorine solution for 25 min. Seeds were rinsed exhaustively with distilled water and blot onto filter paper to dry them. The surface sterilized seeds were placed on callus induction medium (N6-2 medium), consisting of salts and vitamins from N6 (Chu *et al*., 1975), 30 g/l sucrose, 1 g/l casein hydrolysate, 2.5 mg/l 2,4-D and 2 g/l Phytagel™.

Bacterial strain and plasmid

Agrobacterium tumefaciens strain EHA105, harbouring the binary vector pCAMBIA1301, was used to transform rice calli. The vector contains a hygromycin-resistant gene (*hpt*) and μ*idA* reporter gene, interrupted by castor bean (*Ricinus communis*) catalase intron. Both genes are driven by the CaMV 35S promoter.

A. tumefaciens strain was grown in 50 ml of AB liquid media (Hiei *et al*., 1997) with 40 mg/l rifampicin and 50 mg/l kanamycin, at 28±1 °C, in the dark, shaking at 250 rpm. Cultures were centrifuged at 3000xg for 20 min, and *Agrobacterium* cells were resuspended in AAM medium (Hiei *et al*., 1997), to give OD_{600} of 1.0 (inoculum).

Inoculation and co-cultivation of calli with A. tumefaciens

Calli were submerged in a flask containing 20 ml of inoculum. Inoculation was carried out during 10 min at 26±1°C. Inoculum was then pippeted out and infected calli were blotted on sterile absorbent paper. Calli were then put on N6-2 medium modified with 10 g/l glucose, 100µM acetosyringone and pH 5.2. They were incubated at 20°C, in the dark, during 3 days.

Callus washing and antibiotic treatments

The β-lactam antibiotics used in the present study are listed in table 1. Following co-cultivation, calli were washed carefully with sterile distilled water, plus a final rinse during 10 min with 500 mg/l of one of the β-lactam antibiotic. Calli were blotted on sterile absorbent paper, and placed on N6-2 medium containing 100, 250 or 500 mg/l of the β-lactam antibiotic used in the final rinse of calli. Increase of calli fresh weight was determined after 21 days of callus culture on media with different concentrations of antibiotic tested.

Non-contaminated calli were transferred to regeneration medium, consisting of MS (Murashige and Skoog, 1962) salts and vitamins, 30 g/l maltose, 3 mg/l kinetin, 1 mg/l Naphthaleneacetic acid (NAA), 0.5 mg/l 6-Benzylaminopurine (6-BAP) and 4.5 g/l Phytagel™. There were proved three concentrations of β-lactams: 100, 250 and 500 mg/l, and a non-transformed control without antibiotics.

Calli forming shoots were quantified three weeks after the beginning of regeneration. Shoots were classified into bipolar (with stem and root), unipolar (without roots) and abnormal shoots (albinism and/or anomalous forms).

Regenerated shoots were transferred to flasks containing MS medium (Murashige and Skoog, 1962), 3 % sucrose and 3 g/l Phytagel™. This medium was free of beta lactams.

Detection of persistent Agrobacterium in plant tissues

For this assay 1.0 g of calli was weighed from each antibiotic treatment, two weeks after callus washing and 30 days after transferring calli to regeneration medium. Calli were homogenized in a mortar with 1.0 ml phosphate-buffered saline (PBS: 0.43 g/l KH_2PO_4, 1.48 g/l Na_2HPO_4, 7.2 g/l NaCl, pH 7.2). Homogenates were diluted with PBS and 200µM of them were spread over LB agar plates, with 40 mg/l rifampicin and 50 mg/l kanamycin. Plates were incubated at 28°C during 5 days for colony formation. The number of persistent *Agrobacterium* was expressed as colony forming units (cfu) per gram of calli.

Statistical analysis

One way analysis of variance was used to evaluate the increase of fresh weight growth of calli, the cfu per gram of calli, and the percentage of calli forming shoots, recorded in each antibiotic treatment. Three replications were made per treatment. Student-Newman-Keuls test were applied for multiple comparisons of means ($p \leq 0.05$). Analyses were carried out using version 11.5 of Statistical Package for the Social Sciences (SPSS).

Results and discussion

Suppression of Agrobacterium by β-lactams during callus culture and regeneration

Agrobacterium overgrowth was detected in all calli through co-cultivation. Three weeks after wash, bacterium was not visible over the calli, but its presence

Table 1. β-Lactam antibiotics used in the present study.

Antibiotic (assigned abbreviation)	Class	Manufacturer
Amoxicillin/sulbactam (AMX)	Penicillin	Suzhou Erye Pharmaceutical, Shanghai, China
Carbenicillin (CRB)	Penicillin	Sigma-Aldrich Chemie, Steinheim, Germany
Cefazoline (CFZ)	Cephalosporin	Quimefa, Havana, Cuba
Cefuroxime (CFR)	Cephalosporin	Quimefa, Havana, Cuba
Ceftriazone (CFTR)	Cephalosporin	Quimefa, Havana, Cuba
Ceftazidime (CFTZ)	Cephalosporin	Quimefa, Havana, Cuba
Cefotaxime (CFT)	Cephalosporin	Quimefa, Havana, Cuba

into the tissues was detected by means of assay on LB plates with rifampicin and kanamycin. Colony forming units (cfu) were counted in all antibiotic treatments in the period of callus culture (table 2). The high quantity of cfu was found when the culture medium had 100 mg/l of β-lactam, and the number of cfu was smallest when 500 mg/l of β-lactam was used. These results indicate that in the callus phase the *Agrobacterium* growth was not eliminated even using 500 mg/l of β-lactam. In the regeneration phase, cfu were detected only with 100 mg/l of β-lactams (table 2). At the end of the regeneration period, the concentration of 250 mg/l β-lactam completely suppressed the *Agrobacterium* growth.

There were no significant differences ($p \leq 0.05$) for *Agrobacterium* elimination between the β-lactam treatments. Concentrations of 500 mg/l for callus culture, and 250 mg/l for plant regeneration, were the most favourable to eliminate *Agrobacterium* contamination.

Several reports have described the most appropriate antibiotic treatment to effectively suppress *Agrobacterium* from target plant tissues of many species. Timentin at 100~250 mg/l and clavamox at 300 mg/l suppressed *Agrobacterium* growth in tomato (Ieamkhang and Chatchawankanphanich, 2005). Concentrations of 250~500 mg/l of cefotaxime and carbenicillin have been more widely used for many species. However, Ogawa and Mii (2007) described the usefulness of meropenem and moxalactam, two novel and expensive antibiotics, more active against *Agrobacterium* strains and less phytotoxic than carbenicillin and cefotaxime. Ogawa and Mii (2007) detected persistent *Agrobacterium* in regenerated shoots up to 16 weeks of culture on media containing 12,5 mg/l of meropenem, but without visible bacterium overgrowth.

Table 2. Colony forming units (cfu) expressing the persistent *Agrobacterium tumefaciens* during callus culture and plant regeneration with β-lactams.

Antibiotics	Concentration (mg/L)	cfu / g calli	
		Callus culture	Plant regeneration
AMX	100	2.3×10^7	2.1×10^3
	250	5.8×10^5	0
	500	1.6×10^5	0
CRB	100	2.8×10^7	3.4×10^3
	250	6.1×10^6	0
	500	6.2×10^5	0
CFTZ	100	7.0×10^8	1.2×10^3
	250	2.0×10^7	0
	500	2.2×10^5	0
CFR	100	1.8×10^7	2.4×10^3
	250	3.2×10^6	0
	500	7.1×10^5	0
CFTR	100	2.3×10^6	3.6×10^3
	250	3.3×10^5	0
	500	1.1×10^5	0
CFZ	100	4.4×10^6	2.0×10^3
	250	4.2×10^6	0
	500	2.5×10^5	0
CFT	100	3.9×10^8	4.2×10^3
	250	4.5×10^7	0
	500	1.4×10^6	0

Hiei and Komari (2006) obtained favourable results using carbenicillin and cefotaxime for rice immature embryos. They transformed the embryos with *A. tumefaciens* strain LBA4404 that harbored super-binary vector pTOK233 or pSB134. Bacterium was reduced in a first step with 250 mg/l cefotaxime and 100 mg/l carbenicillin. The elimination was completed in the later selection of the transformed embryos and regeneration of calluses using only 250 mg/l cefotaxime, which coincide with our results.

The β-lactams proposed in this work are efficient for *Agrobacterium* suppression and less expensive than meropenem and moxalactam. In addition, the elimination of viable *Agrobacterium* during plant regeneration, using 250 mg/l of assayed β-lactams, is as a guarantee to release *Agrobacterium*-free plants to the environment.

Side effects of β-lactams on callus growth and plant regeneration

In general, calli had a nodular appearance with yellow in colour and multiple globular structures. A similar tendency of increased calli fresh weight with 100 mg/l and 250 mg/l of all antibiotics was proven. But the use of 500 mg/l caused decreasing of callus growth (figure 1). These results indicated that callus growth is dependent on β-lactam concentration.

In the regeneration stage, the surface of the calli took a smooth nodular appearance, about a week after transfer to regeneration media. Calli turned green after the tenth day. About 80% calli formed shoots when 100 mg/l or 250 mg/l of β-lactams were used (figure 2). Some authors have explored the side effects of β-lactams on callus growth and plant regeneration. It has been suggested that certain β-lactams have auxin-like activities and these biological responses are derived from their chemical structure. These antibiotics contain 6-aminopenicillanic acid, phenylacetic acid and phenylmalonic acid in the β-lactam ring and the side chain, and may have important biological activities in plants (Ogawa and Mii, 2007).

High concentrations of cefotaxime, carbenicillin and augmentin may reduce the regenerability of rice (*Oryza sativa* L.) calluses as they resemble auxins in their structure, and in combination with other callus inducing hormones like 2,4-D, may cause loss in regeneration potential (Okkels and Pederson, 1988). The reduction of the period for which the calluses are subjected to antibiotic could increase the regeneration potential of the transformed calli (Tyagi *et al.,* 2007).

It is possible that some breakdown products of β-lactams may interfere with the physiological balance of *in vitro* tissues. But it perhaps depends on antibiotic concentration in culture media, because, in the present work, neither callus growth nor calli forming

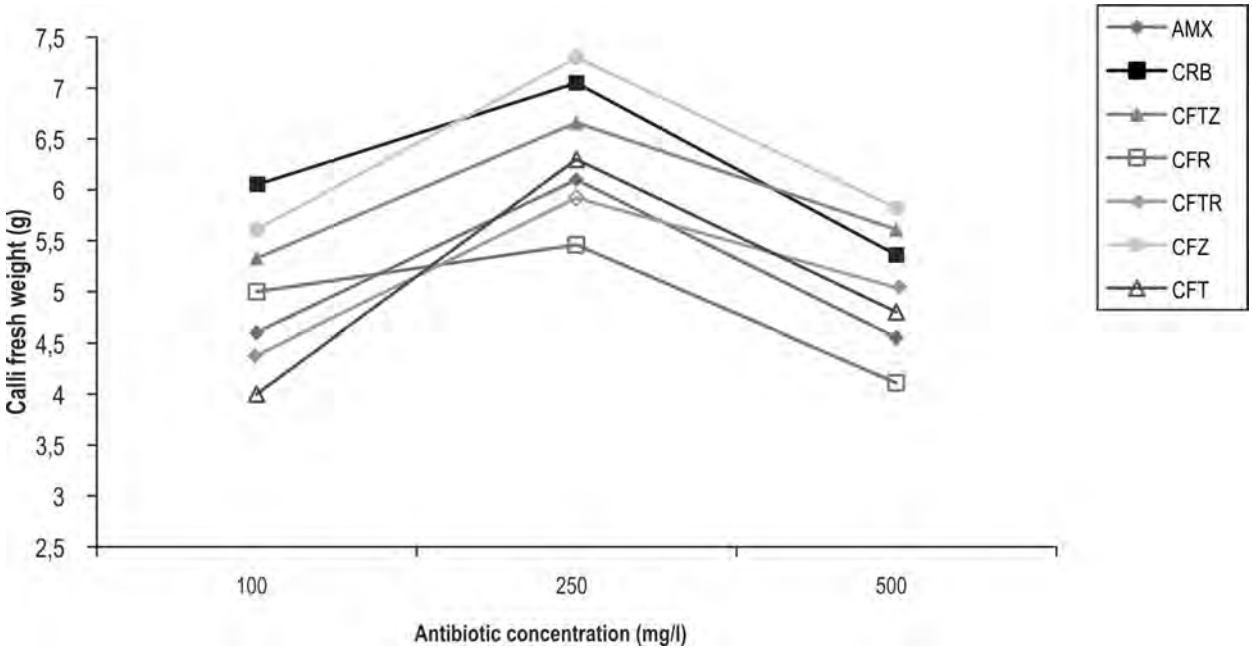

Figure 1. Effect of the type and concentration of β-lactam antibiotics on calli fresh weight.

shoots have been significantly affected ($p \leq 0.05$) when antibiotic concentration was 100 or 250 mg/l, compared with control without antibiotics (figure 2). However, the percentage of calli forming shoots decayed when 500 mg/l of β-lactams was applied. Excessive amount of β-lactams possibly inhibit growth of plant cells, without increasing antibacterial effects (Ogawa and Mii, 2004). For that reason, it is very important to use minimal antibiotic concentration to suppress *Agrobacterium* during regeneration phase.

Two morphogenic pathways in plant regeneration

In all antibiotic treatments and in control without antibiotics, it was verified that calli did not produce shoots in a synchronized way. Initially, globular proembryogenic masses were formed on the surface of the calli, and germination of bipolar shoots was observed 30 days after transfer to regeneration medium. Adventitious shoot organogenesis occurred later from the compact-inner portion of calli, and in more quantities than bipolar shoots. That coincides with previous results in plant regeneration of IACuba-28 rice cultivar, which occurred by two simultaneous morphogenic pathways: adventitious shoots organogenesis and somatic embryogenesis (Pérez Bernal, 2007).

In the present research we distinguished that the organogenesis prevailed over embryogenesis in all of β-lactams treatments. The percentages of abnormal shoots were insignificant. This was similar in all antibiotic treatments and in control without antibiotics (table 3).

Although eukaryotic plant cells are not targets for β-lactams, the chemicals can affect, positively or negatively, plant organogenesis, embryogenesis or callogenesis (Nauerby *et al*., 1997). Ieamkhang and Chatchawankanphanich (2005) reported that adventitious shoots were induced on media containing ti-

Table 3. Effect of different concentrations and types of β-lactams on embryogenic and organogenic rates, after three weeks of callus culture on regeneration medium.

Antibiotics	Conc. (mg/L)	Embryogenic rate		Organogenic rate		Abnormal shoots
		Bipolar shoots	%	Unipolar shoots	%	
AMX	100	12	40,00	18	60,00	-
	250	14	42,42	19	57,58	-
	500	5	31,25	11	68,75	-
CRB	100	11	34,37	21	65,63	-
	250	18	45,00	22	55,00	-
	500	9	40,90	13	59,10	-
CFTZ	100	9	37,50	15	62,50	-
	250	9	37,50	15	62,50	-
	500	4	33,33	7	58,33	1
CFR	100	9	31,03	20	68,97	-
	250	11	39,28	17	60,72	-
	500	3	42,85	4	57,15	-
CFTR	100	12	36,36	21	63,64	-
	250	14	42,42	19	57,58	-
	500	6	35,30	11	64,70	-
CFZ	100	10	34,03	17	62,97	-
	250	10	32,25	21	67,75	-
	500	3	30,00	6	60,00	1
CFT	100	14	37,83	23	62,17	-
	250	13	40,62	19	59,38	-
	500	4	25,00	10	62,50	2
Control without antibiotics		13	43,33	16	53,33	1

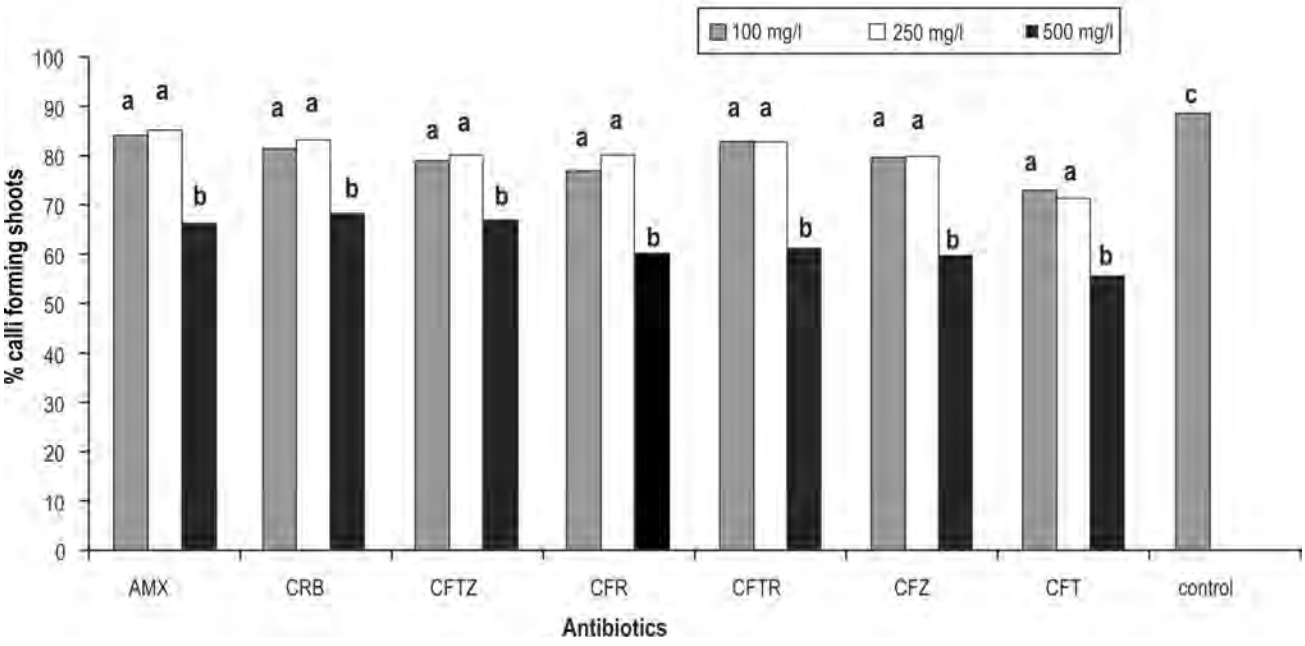

Figure 2. Effect of the type and concentration of β-lactam antibiotics on percentage of calli forming shoots. Means with different letters denote significant differences between antibiotic treatments (Student-Newman-Keuls test, $p \leq 0,05$).

mentin or clavamox with no significant differences. However, Han *et al*. (2006) showed that shoot formation rate was lower in clavamox and cefotaxime than in timentin. Timentin not only increased the regeneration rate, but also stimulated more adventitious shoots per callus.

On the other hand, a detrimental effect of some β-lactams, like carbenicillin, has been found in embryogenesis of some studied species. Decrease of embryo production in the presence of carbenicillin was observed for *Carica papaya* at 375 and 500 mg/l (Yu *et al*., 2001) and *Theobroma cacao* at 100-300 mg/l (Antúnez de Mayolo *et al*., 2003). The results of this work indicated that beta lactams evaluated may delay the maturation of somatic embryos of the calli, or the course from globular stage to bipolar shoots. For this reason they were found in less quantity than unipolar shoots.

The *Agrobacterium* suppression, obtained with the beta lactams, was definitively verified when regenerated shoots were cultured in MS medium without antibiotics. It was not observed visual signals of *Agrobacterium* in stems, leaves or roots. The plants grew healthy and vigorous, reaching an average height of 10.6 cm in seven days.

Conclusions

The beta-lactam antibiotics tested are effective for the total elimination of *Agrobacterium tumefaciens* in indica rice calli (variety J-104), and do not interfere negatively in callus growth and plant regeneration.

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