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Evaluation of the Effectiveness of Selected Antibiotics in the Suppression of *Agrobacterium* from Cowpea (*Vigna Unguiculata* L. Walp.) Embryo Explants and as Potential Selective Agents in *Agrobacterium*-Mediated Transformation

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Abstract

The purpose of the study was to provide baseline information on *Agrobacterium* growth control and suitable selective agent(s) for use in *in vitro* cowpea genetic transformation studies. Ampicillin was identified as an effective alternative to cefotaxime, in suppressing *Agrobacterium tumefaciens*. It shows no toxicity to cowpea tissues at a concentration of up to 500 mgl^{-1}. Cefotaxime did not inhibit shoot regeneration or growth but ampicillin is more economical than cefotaxime. This study also examined the effect of four different aminoglycoside antibiotics; geneticin, paromomycin, kanamycin and neomycin, on the regeneration of cowpea decapitated embryos, in an attempt to develop a selection system for *in vitro* cowpea transformation and regeneration. Plant regeneration was completely inhibited by geneticin (50-500 mgl^{-1}), kanamycin (200-500 mgl^{-1}), paromomycin (400-500 mgl^{-1}) and neomycin at (300-500 mgl^{-1}). Kanamycin (200 mgl^{-1}) and geneticin (10 mgl^{-1}) are suggested as potential agents for selection of transformed cowpea tissues.

Keywords: ***Agrobacterium tumefaciens,*** **aminoglycoside antibiotics, genetic transformation and regeneration,** ***Vigna unguiculata.***

Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is grown throughout the tropics and subtropics as a pulse, a vegetable, for fodder and as a cover crop (Singh *et. al*., 1992). However, its production is constrained mainly by insect pests. Genetic transformation has been suggested to be the recourse for transfer of post-flowering insect resistance traits to cowpea (Machuka, 2000). *Agrobacterium*-mediated transformation is probably the most effective and widely used approach to introduce foreign DNA into crops (Ling *et. al*., 1998). Although legumes generally were previously not considered to be susceptible to *Agrobacterium* (DeCleene and Delay, 1976), it has since been determined that leguminous species can be suitable hosts for *A. tumefaciens* (Mauro *et al.* 1995; Cheng *et al;* 1996; Zhang *et. al.* 1997). Effective elimination of bacteria, after co-culturing with infected tissues, is necessary for successful transformation. Cefotaxime is one of the two most extensively used antibiotics for this purpose. However, this antibiotic is expensive and has been observed to inhibit regeneration in some plants (Sarma *et. al*., 1995). Cheng *et. al*. (1998) presented timentin as an alternative antibiotic, for the suppression of *Agrobacterium* from tobacco and siberian elm tissues.

Effective selection, using suitable selectable marker genes, can lead to a substantial reduction in the number of untransformed regenerants. The neomycin phosphotransferase gene (*npt II*) has been used widely as a selectable marker in plant transformation vectors (Fraley *et. al*., 1986). Due to its specificity, neomycin phosphotransferase is active against a limited group of aminoglycoside antibiotics that include kanamycin, geneticin (G418), neomycin and paromomycin (Yoshikura, 1989). A general approach in transformation studies is to establish a kill curve for the selective agent and use the lowest level of selective agent which inhibit 100 % of the control growth (Park *et. al*., 1998). Plant regeneration from cowpea decapitated embryos was previously described (Pellegrineschi, 1997; Machuka *et al.,* 2000). For effective coupling of regeneration with transformation, it is necessary first to establish the level of antibiotic(s) which can effectively control *Agrobacterium* growth in culture. Secondly, it is necessary to establish a reliable selection system for cowpea transformation. The main objective of this work was to determine the effective selective agent(s) for use in *in vitro* cowpea transformation and regeneration. The other objective was to evaluate the effectiveness of ampicillin as an alternative antibiotic to cefotaxime, for the elimination of *Agrobacterium* from cowpea explants *in vitro* and the effect on regeneration.

Materials and Methods

Plant materials and chemicals

Murashige and Skoog [(MS), 1962] medium was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, USA). All other chemicals were obtained from Sigma

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Chemical Co. (St. Louis, USA). Seeds of an improved cowpea cultivar (IT 86D 1010) were obtained from the gene bank of the International Institute of Tropical Agriculture (IITA), Ibadan. Surface sterilization was done by soaking mature seeds overnight in freshly prepared solution of 0.6 % (w/v) calcium hypochlorite. A drop of Tween 20 per 100 ml of distilled water was added, to act as a surfactant. Seeds were rinsed thoroughly three times with autoclaved water, prior to sowing.

Plant tissue culture

Embryo axes were excised from the seeds and decapitated. Explants were cultured on shoot induction medium (SIM) which is based on MS formulations, with the following additions: 3 % sucrose, 0.8 % agar and 0.5 mgl^{-1} BAP. The pH was adjusted to 5.8 prior to autoclaving. All cultures were incubated at $26 \pm 2^{o}C$ under 16 h photoperiod.

Determination of *Agrobacterium* growth inhibition levels of ampicillin and cefotaxime

Agrobacterium strains, LBA 4404, PGV 3850 and AGL1 were grown in Luria Bertani (LB) broth (10 gl^{-1} tryptone, 10 gl^{-1} yeast extract and 5 gl^{-1} NaCl) for 24 h. The strains were streaked onto Petri-plates containing MS medium supplemented with various concentrations of either ampicillin or cefotaxime at concentrations of 0, 100, 200, 300, 400, and 500 mgl^{-1}. Each treatment consisted of three Petri-plates, which were placed under fluorescent light with a 16 h photoperiod. *A. tumefaciens* growth was evaluated after 3 weeks.

Effect of antibiotics on cowpea shoot regeneration

Decapitated cowpea embryos were cultured on the MS basal medium supplemented with antibiotics at 0, 100, 200, 300, 400, and 500 mgl^{-1}. Each experiment was replicated three times, with ten explants/plate. Shoot regeneration was evaluated at the end of 3 weeks.

Effect of antibiotics on the suppression of *A. tumefaciens* from cowpea infected tissues

Decapitated embryos were vacuum infiltrated in cell suspension of *A. tumefaciens* strain, LBA 4404, at 28 in. Hg vacuum for 20 seconds. Explants were blotted dry on sterile paper towel and cultured on co-cultivation medium (MS basal medium). After 3 days of co-culturing, explants were transferred to SIM medium with either ampicillin or cefotaxime at concentrations of 0, 100, 200, 300, 400, 500 mgl^{-1}. Each treatment had 3 petri-plates, with 10 decapitated embryos per plate. After 4 weeks of culture, regenerating explants, which showed no growth of *Agrobacterium*, were excised and transferred to antibiotic-free medium for 10 days, to determine whether the bacterium was suppressed or killed.

Effect of selective antibiotics on cowpea shoot regeneration

Four different aminoglycoside antibiotics were tested: paromomycin, kanamycin, neomycin and geneticin. Each of the antibiotics was filter sterilized and separately added to SIM at 0, 50, 100, 200, 300, 400 and 500 mgl^{-1}. Decapitated embryos were placed onto each of these selection media (ten explants/plate). Each treatment was replicated three times. Shoot regeneration was evaluated after 3 weeks.

Effect of geneticin on root induction from excised cowpea shoots

Decapitated embryos were sown on antibiotic-free medium for a week. The root system of the germinating embryos was removed. The excised shoots were cultured on root induction medium (RIM) which contained MS basal medium supplemented with NAA (0.05 mgl^{-1}) and geneticin at 0, 2.5, 5, 10, 20, 25, and 50 mgl^{-1}. The root formation index (RI) was measured qualitatively by comparing root development in all treatments to that of the control cultured in the absence of antibiotics.

Statistical analysis

All experiments were repeated three times. Data were statistically analyzed by the SAS software using a completely randomized design and means were compared at the $p = 0.05$ level of significance using Duncan's multiple range test (SAS GLM, $P<0.05$; SAS Institute, 1989).

Results

Effect of antibiotics on *Agrobacterium* growth

Growth of *Agrobacterium* strains LBA4404 and PGV 3850 was strongly inhibited following streaking on medium containing ampicillin or cefotaxime at 300 mgl^{-1}. Strain AGL1 grew at all concentrations in media containing ampicillin. However, growth of this strain was inhibited on medium containing 500 mgl^{-1} cefotaxime. No significant effect was observed with the antibiotic treatments on shoot regeneration (Table 1). The two antibiotics did not adversely affect shoot regeneration of decapitated embryos. Although there was no significant difference in the number of shoots regenerated per explant in all treatments, shoot growth was slightly enhanced by ampicillin (data not shown). This may likely suggest a stimulatory role of ampicillin in cowpea shoot growth.

In the experiment to test for the effectiveness of ampicillin and cefotaxime on the suppression of

Agrobacterium, growth of the bacterial strain was observed from the infected explants on the medium with 200 mgl^{-1} ampicillin and cefotaxime and in the control without the antibiotics. However, when *A. tumefaciens*-infected tissues which had been sub-cultured twice at two weeks intervals were transferred to antibiotic-free medium, *Agrobacterium* growth was detected in all the treatments (Table 2).

Effect of aminoglycoside antibiotics on cowpea regeneration

Plant regeneration from cowpea decapitated embryos was completely inhibited at all the concentrations of geneticin after 3 weeks of cultivation on regeneration medium (Table 3). Although significant shoot regeneration was observed at 50 mgl^{-1} geneticin, root formation was completely inhibited. Kanamycin (200-500 mgl^{-1}) and paromomycin (300-500 mgl^{-1}) completely inhibited plant regeneration. Lower concentrations of kanamycin and paromomycin (50-150 mgl^{-1}) allow shoot regeneration and secondary root formation. However, cowpea explants were more tolerant to neomycin than other aminoglycoside antibiotics. Although, plant regeneration was inhibited at 400 mgl^{-1} neomycin, the percentage of explant death was below 50 %.

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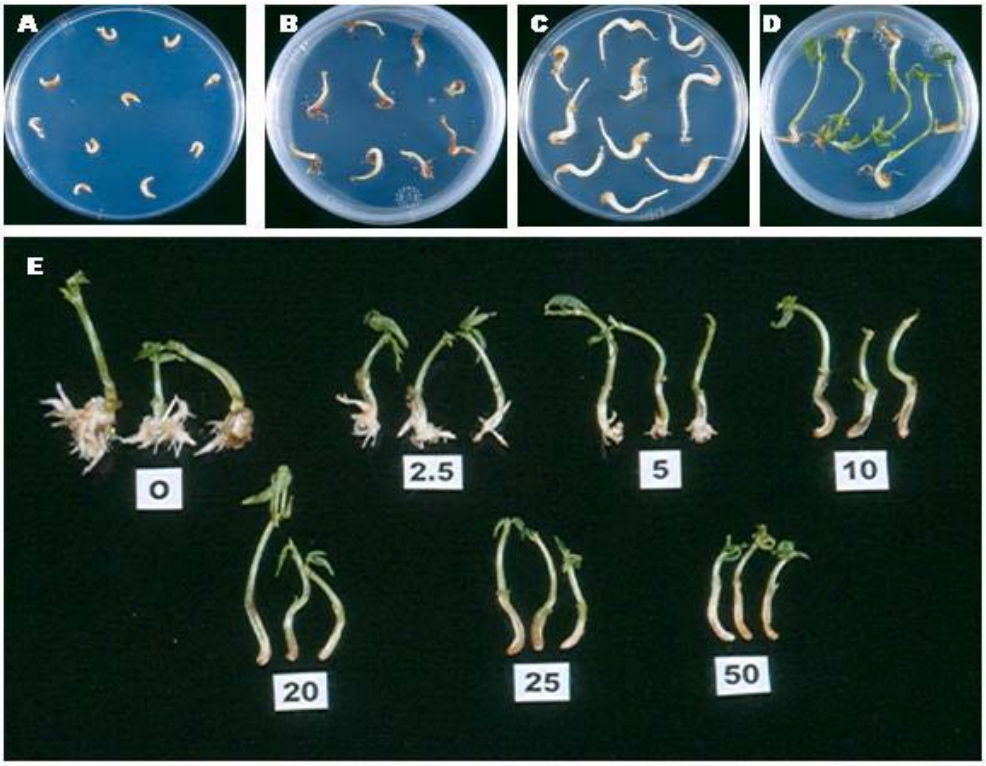

Figure 1. Effect of aminoglycoside antibiotics on cowpea regeneration (A-D) and root induction (E). A) Geneticin (100 mgl^{-1}); B) Neomycin (400 mgl^{-1}); C) Kanamycin (400 mgl^{-1}); D) No antibiotic –control; E) Effect of different concentrations of geneticin on root induction from excised cowpea shoots

Table 1. Effect of ampicillin and cefotaxime on cowpea shoot regeneration

Antibiotic (mgl^{-1})	Percentage explants regenerated*	Number of shoots/explant*
Ampicillin		
0	90.0 ± 5.8^a	1.7 ± 0.0^a
200	86.7 ± 3.3^a	1.7 ± 0.1^a
300	83.3 ± 3.3^a	1.6 ± 0.0^a
400	83.3 ± 6.7^a	1.7 ± 0.0^a
500	80.0 ± 5.8^a	1.7 ± 0.1^a
Cefotaxime		
200	83.3 ± 3.3^a	1.6 ± 0.1^a
300	80.0 ± 0.0^a	1.7 ± 0.1^a
400	80.0 ± 5.8^a	1.6 ± 0.0^a
500	83.3 ± 6.7^a	1.7 ± 0.1^a

*Mean ± SE. Means have the same letter and are therefore not significantly different (p=0.05) according to Duncan's multiple range test.

Table 2: Effect of antibiotics on the elimination of *A. tumefaciens* from cowpea tissues

Agrobacterium-infection	Antibiotic (mgl^{-1})	Percentage of segments showing *Agrobacterium* growth after twosubcultures *
	Ampicillin	
No	200	00.0 ± 0.0^b
Yes	300	40.0 ± 5.8^a
Yes	400	36.7 ± 6.7^a
Yes	500	40.0 ± 5.8^a
	Cefotaxime	
Yes	300	43.3 ± 3.3^a
Yes	400	40.0 ± 5.8^a
Yes	500	46.7 ± 3.3^a

*Mean ± SE. Means having the same letter are not significantly different (p=0.05) according to Duncan's multiple range test.

The root formation index (RI) was measured qualitatively by comparing root development in all treatments to that of the control cultured in the absence of antibiotics (Fig.1A-D). The RI decreases with increasing concentration of the antibiotics. Geneticin adversely inhibited root development, probably making nutrient uptake impossible. High doses of kanamycin caused cowpea explants to turn pale yellow whereas high doses of geneticin, paromomycin and neomycin resulted in necrosis. Inspite of the inhibitory effect of high levels of kanamycin on cowpea regeneration, development of root hairs and enlargement of explants were observed in all cultures (Fig. 1c).

Although 100 % explant response was observed with cultures on 0, 2.5 and 5 mgl^{-1} of geneticin (Table 4), there was marked difference in root proliferation from the control shoots as compared to others (Fig.1 E). Geneticin at 10 mgl^{-1} inhibited prolific root formation, which was observed at lower levels. It only allowed the formation of few root initials

Table 3. Establishment of lethal doses of four aminoglycoside antibiotics on cowpea regeneration

Antibiotic (mgl^{-1})	% explants forming shoots/buds*	% explants forming lateral roots*	% dead explants after 21 days of culture*	root index (max. 5)
Kanamycin				
0	100.0 ± 0.0^{a}	50.0 ± 0.0^{a}	00.0 ± 0.0^{i}	5
50	100.0 ± 0.0^{a}	43.3 ± 3.3^{de}	00.0 ± 0.0^{i}	4
100	100.0 ± 0.0^{a}	23.3 ± 8.8^{gh}	50.0 ± 5.8^{ef}	3
150	93.3 ± 3.3^{ab}	16.7 ± 8.8^{h}	56.7 ± 3.3^{cde}	1
200	86.7 ± 3.3^{b}	00.0 ± 0.0^{i}	63.3 ± 3.3^{cd}	0
300	50.0 ± 5.8^{e}	00.0 ± 0.0^{i}	76.7 ± 3.3^{b}	0
400	00.0 ± 5.8^{g}	00.0 ± 0.0^{i}	86.7 ± 3.3^{b}	0
500	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0
Geneticin				
50	63.3 ± 0.0^{d}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0
100	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0
150	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0
200	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0
300	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0
400	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0
500	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0
Neomycin				
50	100.0 ± 0.0^{a}	73.3 ± 6.7^{b}	26.7 ± 3.3^{h}	4
100	100.0 ± 0.0^{a}	53.3 ± 3.3^{cd}	30.0 ± 5.8^{h}	4
150	100.0 ± 0.0^{a}	50.0 ± 0.0^{cde}	30.0 ± 0.0^{h}	3
200	90.0 ± 5.8^{b}	40.0 ± 5.8^{ef}	36.7 ± 6.7^{gh}	2
300	76.7 ± 3.3^{c}	20.0 ± 5.8^{gh}	43.3 ± 3.3^{fg}	2
400	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	46.7 ± 3.3^{efg}	0
500	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	66.7 ± 3.3^{c}	0
Paromomycin				
50	93.3 ± 3.3^{ab}	56.7 ± 3.3^{c}	36.7 ± 3.3^{gh}	3
100	93.3 ± 3.3^{ab}	50.0 ± 5.8^{cde}	53.3 ± 8.8^{def}	2
150	$90.0 + 5.8^{b}$	43.3 ± 3.3^{de}	53.3 ± 3.3^{def}	2
200	86.7 ± 3.3^{b}	30.0 ± 5.8^{fg}	56.7 ± 3.3^{cde}	1
300	36.7 ± 3.3^{f}	00.0 ± 0.0^{i}	63.3 ± 3.3^{cd}	1
400	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	86.7 ± 3.3^{b}	0
500	00.0 ± 0.0^{g}	00.0 ± 0.0^{i}	100.0 ± 0.0^{a}	0

*Mean ± SE. Means having the same letter are not significantly different (p=0.05) according to Duncan's multiple range test.

Discussion

Whereas growth of *Agrobacterium* strains LBA4404 and PGV 3850 was inhibited by ampicillin and cefotaxime, the hypervirulent strain AGL1 was only inhibited by a high (500 mgl^{-1}) cefotaxime concentration. Ampicillin, a derivative of penicillin G, and cefotaxime, are β-lactam antibiotics, which inhibit bacterial cell wall synthesis (Ling *et. al*., 1998). They inhibit the cross-linking of peptidoglycans by binding and inactivation of transpeptidases leading to nicks in the cell walls by which the cell membrane protrudes into the hypotonic environment and finally ruptures as a result of osmotic shock (Ling *et. al.,* 1998).

Table 4. Effect of geneticin on root induction from excised cowpea shoots.

Geneticin (mgl^{-1})	% explants forming roots/ roots initials	root index (max. 5)
0	100	5
2.5	100	3
5	100	2
10	50	0.5
20	0	0
25	0	0
50	0	0

Furthermore, ampicillin slightly enhanced growth of cowpea embryo explants in culture (data not shown). Stimulatory effects on callus growth and organogenesis *in vitro* have been reported with antibiotics in several plants (Eapen and George, 1990; Yepes and Aldwinckle; 1994; Lin *et. al*., 1995). Penicillin G possesses auxin-like structural features (Robert *et. al*; 1998) which break down in culture medium, to physiologically active levels of the auxin phenylacetic acid (Holford and Newbury, 1992).

Following antibiotic suppression of *Agrobacterium*, subsequent transfer of clean cowpea cultures to antibiotic-free medium and further sub-culture led to re-emergence of *Agrobacterium*. This suggests that both antibiotics were effective as bacterio-static but not bactericidal agents. This is as expected, since suppression of bacterial growth is what is usually achieved in most *Agrobacterium*-mediated transformations. It is often very difficult to completely eliminate *Agrobacterium* from the tissues of some species (Hammerschlag *et. al*., 1995; Shackelford and Chlan, 1996). The results of these experiments have demonstrated that ampicillin may be an effective, cheaper alternative compared to cefotaxime, vancomycin and timentin, in suppressing *A. tumefaciens*. This cost effectiveness is most desirable when developing an optimized transformation system for recalcitrant species (De Bondt *et. al*., 1994) like cowpea. For biosafety and food safety reasons, the *nptII* gene encoding neomycin phosphotransferase may be more acceptable than the *bar* gene encoding phosphinothricin acetyl transferase in genetic transformations designed for public or commercial release (IFT Report, 2000).

Although, plant regeneration was inhibited at 400 mgl^{-1} neomycin, the percentage of explant death was below 50 %. A similar result was reported for apple tissues (Norelli and Aldwinkle, 1993). The RI decreases with increasing concentration of the antibiotics. Geneticin inhibited root development, probably making nutrient uptake impossible. This suggests phytotoxicity of the antibiotic to cowpea tissues within the concentration gradient tested in this work. Pena *et. al.* (1997) also reported that geneticin was too toxic to lime tissues. High doses of kanamycin caused cowpea explants to turn pale yellow whereas high doses of geneticin, paromomycin and neomycin resulted in necrosis (Fig. 1). The mild inhibitory effect of high levels of kanamycin on cowpea regeneration may imply that kanamycin would be the preferred selective agent in future work on cowpea transformation and regeneration. The data obtained in this work also indicate that geneticin (at $\geq$ 10 mgl^{-1}) can be considered as a candidate selective agent for screening for both regenerated transformed shoots and putative transformed tissues of T_1 plants. The possible doses of the other three antibiotics that may be applied for selection of transformants are as follows: neomycin (300 mgl^{-1}), paromomycin (250 mgl^{-1}) and kanamycin (200 mgl^{-1}). These recommended levels will need to be tested and verified in the course of future efforts to develop reliable cowpea transformation protocols. From the data presented here, it may be concluded that growth of *Agrobacterium* strains LBA4404 and PGV 3850 (and not strain AGL1) can be controlled with both ampicillin and cefotaxime at levels that are not inhibitory to cowpea tissue culture and regeneration. The effective levels of suitable selective agents for selection and screening for cowpea transformed tissues were also established. The results provide a basis for further work on *in vitro Agrobacterium* transformation and regeneration of cowpea.

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