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Short communication

Phytotoxic effects of different antibiotics on leaf explants and their potential as *Agrobacterium* counter selection agents during gene transfer in apple

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ABSTRACT

Different antibiotics were evaluated for their effects on *in vitro* callusing of leaf explants and suppressing the growth of *Agrobacterium tumefaciens* strain LBA 4404 in suspension cultures as well as during co-cultivation in apple rootstock MM 111. Maximum callusing on leaf explant (85-93%) was observed in 300 mg/l carbenicillin, 200 mg/l augmentin and 100-200 mg/l timentin. Low conc. of timentin (100-200 mg/l) effectively inhibited the bacterial growth in suspensions and completely controlled the bacterial overgrowth during co-cultivation after 1st and 2nd blot without leaving any deleterious effect on the leaf explants of MM 111.

Key words: Antibiotics, apple, gene transfer, co-cultivation, phytotoxicity.

Successful transformation using *Agrobacterium tumefaciens* depends on the efficiency of the gene delivery, plant regeneration systems and on the subsequent elimination of this bacterium from the transformed cells as soon as it is no longer needed (Tang *et al*., 8). The bacterial growth needs to be suppressed so as not to interfere with the growth and development of the transformed plant cells. Therefore, *Agrobacterium*-mediated gene transfer requires the use of efficient antibiotic(s) in the selection and regeneration media (leamkhang and Chatchawankanphanich, 2) in order to eliminate the bacterium and to check their potential on the explant growth. Majority of times, ampicillin, carbenicillin and cefotaxime have been used as effective antibiotics for the elimination or suppression *of Agrobacterium* cells (Tang *et al*., 8). Tran and Mishra (9) reported that the nature and concentration of antibiotics used can extensively affect the regeneration potential of explants. During the gene transfer studies in apple, leaf explants were frequently destroyed due to agrobacterial overgrowth in cefotaxime supplemented medium following the co-cultivation. Therefore, in order to seek an effective alternative antibiotic, we evaluated the effect of different antibiotics on growth of leaf explants and their potential as *Agrobacterium* counter selection agents during apple genetic transformation.

Agrobacterium tumefaciens strain LBA 4404 harbouring the plasmid pCAMBIA 1300-bar-ubi-*chill* (plasmid kindly provided by Dr S. Muthukrishanan, Deptt. of Biochemistry, Kansas State University, Manhattan, USA) carrying *hpt* (hygromycin phosphotransferase) and *bar* (phosphinothricin acertyltransferase) as selectable marker genes under the control of CaMV35S and *chitinase* gene of rice under the control of ubiquitin promoter was used. Leaf explants were excised from four-week-old *in vitro* shoots, wounded and cultured for shoot induction on MS (Murashige and Skoog, 6) medium (SIM) supplemented with BA (4.0 mg/l), IAA (1.0 mg/l), sucrose (30 g/l) and Difco Bacto-agar (7.0 g/l). Preliminary antibiotics tests were performed to know the resistance threshold of non-transformed leaf explant against the selection agents. For this, the antibiotics cefotaxime, carbenecillin, timentin and rifampicin (Hi-media) and augmentin (Glaxo Smith Kline) after filter sterilization were incorporated at a concentration of 100-500 mg/l to the shoot induction medium (SIM). For co-cultivation, the bacteria were grown on YMB (Yeast Mannitol Broth) medium overnight at 28°C (200 rpm), then were pelleted and resuspended in half-strength MS liquid medium to a density of 3×10^8 cells/ ml by measuring absorbance at 540 nm. Leaves were dipped in this bacterial suspension, blotted on sterile filter paper and transferred to SIM containing antibiotics. After 48 h co-cultivation, these were washed in autoclaved double-distilled water, blotted and transferred to SIM with antibiotics. All the explants were incubated at 26 ± 2°C for 16/8 h light/dark periods (36 µmol m^{-2} light radiation). Similar antibiotics were added to YMB and suspension was prepared as mentioned above. After 28 h, bacterial cell numbers as colony forming units per ml (cfu/ml) was estimated by using the serial dilution method followed by spread plate technique. Each experiment was performed twice by taking three replications each time and each replication consisted of 6 flasks. In case of regeneration experiments, each

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flask contained five leaf explants and performance was evaluated after 45 days of incubation. The data recorded for the different parameters were subjected to CRD. Per cent data were subjected to Arc sine transformation prior to statistical analysis. The statistical analysis based on mean values per treatment was made by using analysis of variance (ANOVA) of CRD.

It was observed that browning of leaf explants was directly proportional to the concentration of antibiotics added to the medium. However, callusing enhanced only at some moderate concentrations of the antibiotics and declined at their further increase in the concentration (Table 1). Out of different types and concentrations of antibiotics tested for inhibiting the growth of cultured leaf explants, a gradual but consistent browning of non-transformed leaf explants was observed in the SIM supplemented with 100-400 mg/l of cefotaxime and very less number of explants (4.40, 7.73, 16.63 & 18.83%) induced callus. SIM supplemented with 300 mg/l of carbenicillin showed maximum callusing, *i.e*., 93.30% followed by 84.43% with 200 mg/l, while 500 mg/l was able to kill 73.3% of leaf explants. The enhancement in the growth and callus induction of leaf explants may be a result of the possible release of auxin-like compounds from the carbenicillin disodium salt, as reported earlier (Lin *et al.*, 4). In our study, carbenicillin was found to be non-toxic up to 300 mg/l. Similar observations were reported in chrysanthemum (Chen *et al*., 1). In case of 500 mg/l augmentin, around 75.6% explants turned pale yellow, whereas at 200 mg/l, 85.53% explants induced callus, while all other concentrations revealed 4-10% callusing (Table 1). Browning of the leaf explants did not occur when SIM was supplemented with 100-300 mg/l of timentin. Moreover, callus induction was increased to 88.67 and 98.87% at 100-200 mg/l and was reduced at higher concentrations. Both augmentin and timentin had no adverse effect on the leaf tissues of apple rootstock MM 111 even at higher concentration of 400 mg/l as seen with other antibiotics used in this study. This could be due to the reason that both antibiotics contain β-Lactam antibiotic in the penicillin G group that is ticarcillin and amoxicillin, respectively and the breakdown product of this group is auxin-like compound that might interact with auxin added to the culture medium. Maneekard *et al.* (5) compared the effect of carbenicillin and cefotaxime with timentin and reported the non adverse effects of augmentin and timentin in various crops. In tomato and *Artemisia annua,* non-deleterious effects of these two antibiotics were reported (leamkhang *et al*., 3; leamkhang and Chatchawankanphanich, 2) even at higher concentration of 500 mg/l, which support our findings. Rifampicin, with every 100

Table 1. Effect of antibiotics on leaf explants before and after co-cultivation with *A. tumefaciens* strain LBA 4404 in shoot induction medium.

	% explant browning (EB) and % callus induction (CI) before co-cultivation										% explants showing agrobaterial growth after co-cultivation									
Conc. (mg/l)	Cefotaxime		Carbenicillin		Augmentin		Timentin		Rifampicin		Cefotaxime		Carbenicillin		Augmentin		Timentin		Rifampicin	
	EB	CI	EB	CI	EB	CI	EB	CI	EB	CI	1st blot	2nd blot	1st blot	2nd blot	1st blot	2nd blot	1st blot	2nd blot	1st blot	2nd blot
0	0.00	20.00	0.00	20.00	0.00	20.00	0.00	20.00	0.00	20.00	100	100	100	100	100	100	100	100	100	100
	(0.00)	(26.55)	(0.00)	(26.55)	(0.00)	(26.55)	(0.00)	(26.55)	(0.00)	(26.55)	(90)	(90)	(90)	(90)	(90)	(90)	(90)	(90)	(90)	(90)
100	4.40	4.40	5.50	4.4	0.00	4.40	0.00	88.67	5.54	4.40	100	93.30	100	100	100	100	99.00	0.00	99.33	98.77
	(11.94)	(2.30)	(13.41)	(11.94)	(0.00)	(11.90)	(0.00)	(70.63)	(13.46)	(2.30)	(90)	(75.31)	(90)	(90)	(90)	(90)	(85.38)	(0.00)	(86.26)	(85.08)
200	6.63	7.73	15.50	84.43	6.63	85.53	0.00	98.87	25.3	6.63	100	82.77	92.73	67.63	87.63	34.30	0.00	0.00	99.10	91.67
	(14.60)	(2.94)	(23.16)	(66.96)	(14.6)	(68.11)	(0.00)	(86.46)	(30.19)	(2.71)	(90)	(65.49)	(74.45)	(55.33)	(69.43)	(35.85)	(0.00)	(0.00)	(85.69)	(73.27)
300	11.10	16.63	28.63	93.30	29.97	9.96	0.00	14.40	54.4	6.96	68.97	64.87	66.30	33.63	0.00	0.00	0.00	0.00	81.63	77.03
	(19.42)	(4.18)	(32.32)	(75.31)	(33.17)	(18.24)	(0.00)	(22.27)	(47.58)	(2.65)	(56.15)	(53.65)	(54.52)	(35.45)	(0.00)	(0.00)	(0.00)	(0.00)	(64.64)	(61.38)
400	15.30	18.83	43.3	9.96	48.20	7.73	6.65	6.63	85.43	5.50	59.30	32.40	29.00	16.13	0.00	0.00	0.00	0.00	72.60	66.80
	(23.00)	(4.41)	(41.10)	(18.24)	(43.97)	(16.07)	(14.62)	(14.60)	(68.03)	(2.52)	(50.36)	(34.69)	(32.58)	(23.67)	(0.00)	(0.00)	(0.00)	(0.00)	(58.45)	(54.86)
500	56.53	4.40	73.3	1.10	75.57	4.40	73.2	1.10	97.77	0.00	25.07	15.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	(48.81)	(2.30)	(59.12)	(3.48)	(60.54)	(11.94)	(59.05)	(3.48)	(85.00)	(1.00)	(30.04)	(22.93)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)
$CD_{0.05}$	5.34	0.9	6.38	8.05	4.80	5.85	5.12	8.90	9.17	1.14	1.01	3.22	1.62	1.07	0.78	0.39	3.03	2.90	4.13	4.05

% shoot induction in control medium (MS medium + 1 mg/l NAA and 3 mg/l BA) = 34
Values in parentheses are transformed values

mg/l increase in its concentration, per cent mortality increased two times. Presently, rifampicin showed adverse effects on leaf explants even at 300 mg/l, as a result the callus induction was found very poor at all the concentrations used in the study.

Present results revealed that OD values were inversely proportional to the concentration of all the antibiotics (Fig. 1). OD values remained approximately the same at 100 and 200 mg/l of carbenecillin, *i.e.*, 1.00 and 0.90, respectively, whereas a consistent decrease was clearly observed at 300-500 mg/l. In case of cefotaxime, 100 mg/l showed small decline up to 1.14 as compared to control and 200-400 mg/l showed more or less the same values. Timentin and augmentin showed similar trend of decrease in OD values with the increase in the concentration of antibiotics. Estimation of Colony Forming Units (cfu/ ml) are demonstrated in 3D bar diagram (Fig. 1). All antibiotics showed decline in cfu/ml count with the increase in the concentration of antibiotics from 100 to 500 mg/l. Timentin at a low concentration of 100 mg/l showed only 2×10^1 cfu/ml. No colony was observed at 200-500 mg/l timentin and at 500 mg/l carbenicillin and augmentin. After studying OD values of the bacterial samples as well as counting cfu/ml from the antibiotic supplemented bacterial suspensions it was found that 200-500 mg/l timentin was the most effective antibiotic for the suppression of *Agrobacterium* growth in suspension followed by 500 mg/l augmentin. Rifampicin was proved to be the least effective. These findings hold true with the work done by leamkhang and Chatchawankanphanich (3) on tomato. While evaluating leaf explants co-cultivated with *Agrobacterium*, it has been observed that cefotaxime at 500 mg/l showed the bacterial growth in 25.07% of explants after first blot, which was reduced to 15.2% after 2nd blot (Table 1). On the other hand, carbenicillin and rifampicin were able to suppress bacterial growth at 500 mg/l, whereas, 200 & 300 mg/l timentin and augmentin were sufficient to inhibit the growth of *A. tumefaciens* strain LBA 4404. In addition, a high concentration of timentin upto 400 mg/l showed non-significant toxicity to leaf explants. Augmentin and timentin at 300 mg/l completely controlled the bacterial overgrowth during co-cultivation even after 1st blot. It has also been

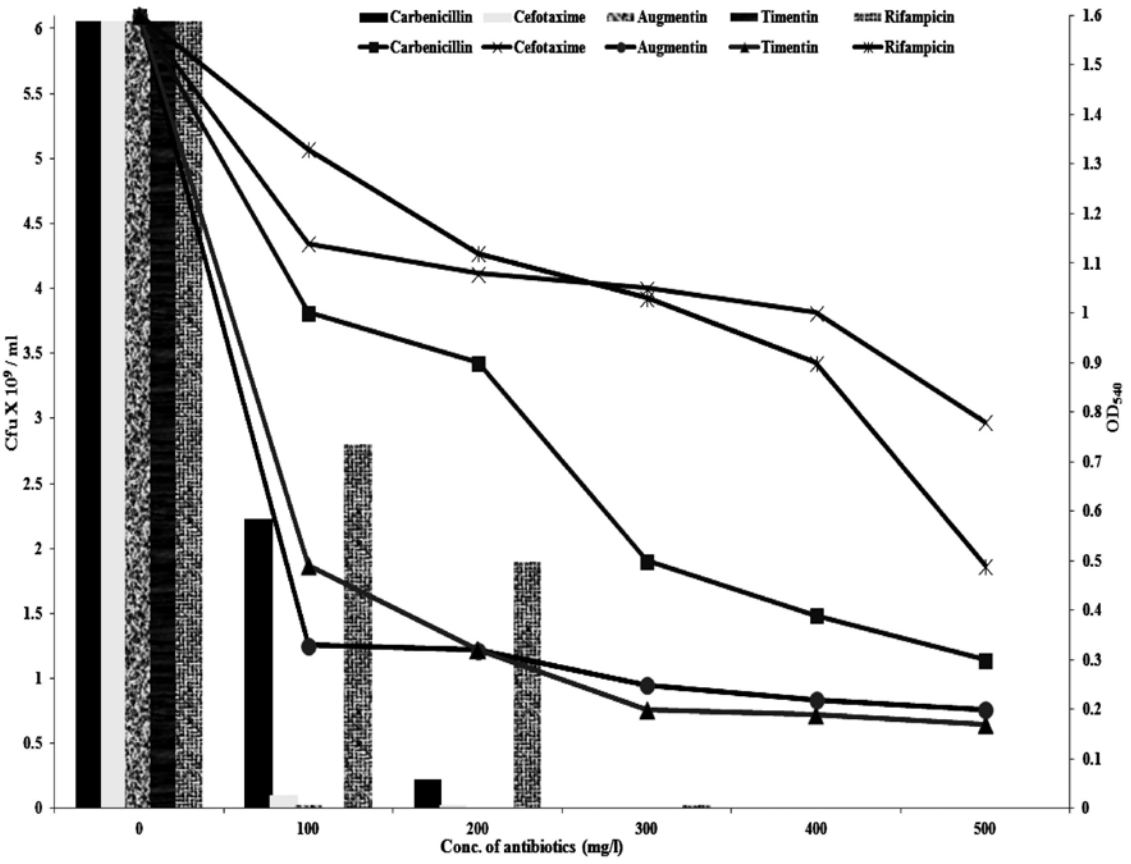

Fig. 1. Effect of different antibiotics on growth of *Agrobacterium* in suspension cultures in terms of colony forming units (cfu/ ml x 10^9) and OD values.

observed that all the antibiotics at 100 mg/l could not control the overgrowth of *Agrobacterium* because percent explants showing bacterial growth after 1st and 2nd blot remained the same (approx. 100%), except for timentin, which showed no overgrowth on explants after 2nd blot. It has already been suggested that timentin is an effective antibiotic in suppressing the *Agrobacterium* growth and it has little negative effect on the tissue growth and regeneration on tomato (Ieamkhang *et al*., 3). Similar observation has been reported in *indica* rice by Priya *et al*. (7). Augmentin has successfully been used as an alternative antibiotic for suppressing *Agrobacterium* growth during tomato transformation (Ieamkhang and Chatchawankanphanich, 2). When the efficiency of augmentin and timentin was compared in our studies, it was found that augmentin was effective at 300 mg/l, while timentin at 100 mg/l and both had no detrimental effect on leaf explant. Augmentin is an antibacterial combination consisting of a penicillin derivative amoxicillin and clavulanic acid. Since, ticarcilin is more potent then amoxicillin, thus lower concentration of timentin suppressed *Agrobacterium* growth. These reasons can be attributed to their effectiveness over the rest of the antibiotics. Thus, it has been shown that timentin and augmentin at low doses can be used effectively to control *Agrobacterium* overgrowth in apple rootstock MM 111. However, we observed that shoot induction is greatly affected / blocked by the presence of antibiotics which suggests the need to change the type and concentration of phyto-hormones in shoot induction medium in the presence of antibiotics.

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