Effect of Antibiotics and Exposure to an Acidified Medium on the Elimination of *Agrobacterium tumefaciens* from Apple Leaf Explants and on Shoot Regeneration

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ABSTRACT. A range of antibiotics and short-term exposure to an acidified (pH 3.0) medium were evaluated for their effects on eliminating *Agrobacterium tumefaciens*, supervirulent strain EHA101 (pEHA101/pGT100), from leaf explants of 'Royal Gala' apple (*Malus $\times$ domestica* Borkh.) and on shoot regeneration. Exposure of leaf explants to regeneration and elongation media containing 100 $\mu\text{g}\cdot\text{mL}^{-1}$ concentrations of the antibiotics carbenicillin (crb), cefotaxime (cef), and cefoxitin [=mefoxin (mef)], singly or in combination for 52 days did not eliminate *A. tumefaciens* from the explants. The percentage of regeneration on crb, cef, and mef was 97%, 11%, and 50%, respectively, compared to 67% for the controls. Short-term (1- to 18-hour) vacuum infiltration with 500 $\mu\text{g}\cdot\text{mL}^{-1}$ of any of the above antibiotics did not inhibit regeneration and failed to eliminate *A. tumefaciens* from leaf explants. Cef (2000 $\mu\text{g}\cdot\text{mL}^{-1}$) did not inhibit the percentage of regeneration and was more effective than crb or mef in preventing growth of *A. tumefaciens* when vacuum infiltrated into apple leaf explants for 30 minutes. Further experiments demonstrated that the incidence of *A. tumefaciens* contamination could be reduced to 28% without negatively impacting shoot regeneration by using a 1-hour vacuum infiltration with an acidified medium, an 18-hour vacuum infiltration with cef (5000 $\mu\text{g}\cdot\text{mL}^{-1}$), and a 52-day incubation on regeneration and elongation media containing 100 $\mu\text{g}\cdot\text{mL}^{-1}$ each of mef and crb. Kan resistant, GUS (β -glucuronidase) positive, putative transformants without *A. tumefaciens* were generated by adding kan (10 $\mu\text{g}\cdot\text{mL}^{-1}$) to the regeneration and elongation media.

Successful transformation using *Agrobacterium tumefaciens* depends on the efficiency of the gene delivery and plant regeneration systems, and on the subsequent elimination of this bacterium from transformed cells. Transformation of apple following co-cultivation with *A. tumefaciens* has been reported previously (De Bondt et al., 1996; James et al., 1989; Maheswaran et al., 1992; Norelli et al., 1994; Yao et al., 1995); however, a major problem with this system has been the low efficiency of transformation (James and Dandekar, 1991). Although supervirulent *A. tumefaciens* strain A281 has been shown to be the most effective strain for apple transformation (De Bondt et al., 1994; Maheswaran et al., 1992; Martin et al., 1990), derivatives of this strain (e.g., EHA101, the disarmed A281, Hood et al., 1986) have not been used for transformation studies because bacterial growth can only be controlled with levels of cefotaxime that inhibit apple regeneration (Maheswaran et al., 1992; Yepes and Aldwinckle, 1994). Recently, De Bondt et al. (1996) reported on the regeneration of transgenic apple shoots following co-cultivation with *A. tumefaciens* strain EHA101; however, transformants could not be generated from several important apple cultivars (i.e., 'Gala') and

transformants were not screened for the presence or absence of this bacterium. Although Puite and Schaart (1996) generated transgenic 'Gala', 'Delicious', and 'Elstar' using supervirulent strains of *A. tumefaciens*, they did not test for the presence or absence of *A. tumefaciens* in their transformants. The importance of screening tissue cultures for this bacterium was demonstrated by Cooke et al. (1992), who reported that *A. tumefaciens* may persist in plant tissue cultures without growing out on the medium or producing symptoms on the plants. Eliminating *A. tumefaciens* from transformants is important because strong expression of β -glucuronidase (GUS) by *A. tumefaciens* can present a problem for identifying transformation (Martin et al., 1990) and, more importantly, because microbial contaminants in cultured plants can reduce multiplication and rooting rates or induce plant death (Cassells, 1991; Leifert et al., 1991a). The objective of this study was to develop a system that would eliminate supervirulent strain EHA101 from 'Royal Gala' apple explants following *A. tumefaciens* co-cultivation, without inhibiting apple shoot regeneration.

Materials and Methods

PLANT MATERIAL, EXPLANT PREPARATION, AND PLANT REGENERATION. Leaf material used in all experiments was taken from 15-d-old in vitro-cultured axillary shoots of 'Royal Gala' (a red-fruited sport of 'Gala') apple (Gercheva et al., 1994). All axillary shoots were derived from in vitro mother plants that were established as free of *A. tumefaciens* strain EHA101 by the absence of bacterial growth when basal stem sections were incubated on selective medium (see YEB medium below) for 14 d. The three to four youngest fully expanded leaves were excised and stored in petri dishes on sterile filter paper moistened with deionized distilled water. The basal one-third of each leaf (with petiole) was dissected

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and placed abaxial side up directly on apple regeneration (N6) medium or on N6 medium after *Agrobacterium* co-cultivation, antibiotic treatment, or both. Explants were subcultured every 14 d. After 42 d on N6 medium, regenerated shoots were transferred to elongation (J3-h) medium for 10 d, and then percentage of regeneration and shoot yield per explant were determined. The N6 medium consisted of N6 macrosalts (Chu et al., 1975), LS microsals (Linsmaier and Skoog, 1965), 0.56 mM myo-inositol, 3 μM thiamine HCl, 10 μM thidiazuron (TDZ), 87.6 mM sucrose, casein hydrolysate at 300 $\text{mg}\cdot\text{L}^{-1}$, and Difco-Bacto agar at 7 $\text{g}\cdot\text{L}^{-1}$ (Fasolo et al., 1989). The J3-h medium contained MS salts (Murashige and Skoog, 1962) supplemented with 0.56 mM myo-inositol, 1.2 μM thiamine HCl, 87.6 mM sucrose, and 7 $\text{g}\cdot\text{L}^{-1}$ Difco Bacto-agar. The N6 and J3-h media were adjusted to pH 5.4 and pH 5.6, respectively, before autoclaving. Unless noted otherwise, explants were incubated in the dark for 10 d at 26 °C and then transferred to a 16-h daily photoperiod provided by cool-white fluorescent tubes at 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

AGROBACTERIUM STRAIN AND CO-CULTIVATION. EHA101 carrying the virulence plasmid pEHA101 and the binary vector pGT100 (supplied by L.D. Owens, USDA-ARS, Beltsville, Md.) was used in all studies. The binary vector pGT100 is a tetracycline-selectable modification of pKYLX71 (Scharl et al., 1987) in which the 35S promoter and rbcS 3' fragments were replaced with the CaMV 35S promoter-GUS-nopaline synthase 3' from pBI121 (Clontech, Palo Alto, Calif.). This strain was maintained on YEB medium, pH 7 (Van Larebeke et al., 1977), and supplemented with kanamycin (kan, 50 $\mu\text{g}\cdot\text{L}^{-1}$) and tetracycline (tet, 10 $\mu\text{g}\cdot\text{L}^{-1}$). For co-cultivation, the bacteria were grown overnight at 28 °C (200 rpm), then were pelleted and resuspended to a density of 2×10^8 cfu/mL (by measuring absorbance at 550 nm) in N6 liquid medium containing 20 μM acetosyringone. Leaf explants were dipped in *A. tumefaciens* suspension, blotted on sterile filter paper and transferred (abaxial side up) to sterile filter paper saturated with N6 liquid medium containing 20 μM acetosyringone. After a 24- to 48-h co-cultivation, leaf explants were washed in N6-h liquid medium (N6 medium without TDZ), blotted, and transferred to media with or without antibiotics.

ANTIBIOTIC TREATMENTS AND SCREENING FOR THE PRESENCE OF *A. tumefaciens*. The antibiotics used in this study: carbenicillin (crb), cefotaxime (cef), and cefoxitin [=mefoxin (mef)] (Sigma, St. Louis) were all filter sterilized and added to autoclaved media. Antibiotics (100 to 5000 $\mu\text{g}\cdot\text{mL}^{-1}$) were tested alone or in combination to determine optimum levels to eliminate *A. tumefaciens* strain EHA101 without inhibiting regeneration. Following co-cultivation, leaf explants were either vacuum infiltrated with an antibiotic medium or placed on N6 and J3-h media with or without an antibiotic. For vacuum infiltration, explants were placed into individual wells of 12-well sterile tissue culture dishes, which were then placed in a vacuum desiccator at 68 kPa. Half of the vacuum-infiltrated explants were screened immediately by transferring them to YEB medium with kan and tet for 14 d. The other half of the explants were transferred to N6 and J3-h media with or without antibiotics for 52 d and then transferred to YEB medium with kan and tet. The percentage of contaminated explants was recorded during and immediately after the 14-d period in YEB medium. Except for our last study, all bacterial screening studies were replicated at least twice with three to ten explants per treatment per replication.

LONG-TERM EXPOSURE TO LOW LEVELS OF ANTIBIOTICS. Apple explants were transferred to N6 medium containing 100 or 200 $\mu\text{g}\cdot\text{mL}^{-1}$ of cef, crb, or mef, alone or in combination, and incubated at 26 °C for 10 d in the dark followed by incubation under a 16-h

photoperiod as described above. The low- and high-dose antibiotics were applied in separate experiments. Each experiment had an untreated control. The experiments were replicated three times with nine to ten explants per treatment per replication. For each replication, the relative frequency of explants with shoot counts in four categories (0, 1 to 10, 11 to 20, and >20) was recorded. The number of shoots per explant was estimated by assuming that the mean shoot counts for the four categories were 0, 5.5, 15.5, and 25.5, respectively. A linear means model was fitted to the approximate shoot counts using the SAS general linear model procedure (SAS Institute, Cary, N.C.). Pairwise comparisons were made using *t* tests. Apple explants were also co-cultivated with *A. tumefaciens* strain EHA101, transferred to N6 and J3-h media with cef, crb, or mef (100 to 200 $\mu\text{g}\cdot\text{mL}^{-1}$), alone or combined, and screened for the presence of *A. tumefaciens* in YEB liquid with kan and tet after 52 d.

SHORT-TERM EXPOSURE TO MODERATE OR HIGH LEVELS OF ANTIBIOTICS. Vacuum infiltration of cef, crb, or mef (500 or 2000 $\mu\text{g}\cdot\text{mL}^{-1}$) was studied to determine 1) if short-term exposure to moderate or high levels of antibiotics, either alone or combined with long-term exposure to low levels of antibiotics, would eliminate strain EHA101 from apple leaf explants without inhibiting shoot regeneration and 2) which antibiotic was most effective against strain EHA101. In the first experiment, leaf explants, with and without a 1-d co-cultivation with *A. tumefaciens*, were vacuum infiltrated with antibiotic (500 $\mu\text{g}\cdot\text{mL}^{-1}$) for either 1 or 18 h and then screened for the presence of *A. tumefaciens*. Three explants were screened per treatment, and this part of the experiment was repeated once. At the same time, leaf explants were also vacuum infiltrated with antibiotic and transferred sequentially to regeneration and elongation media without antibiotics and the percentage of regeneration and number of shoots per explant were recorded at 52 d. This part of the experiment was replicated three times with a minimum of eight explants per treatment per replication. In the second experiment, co-cultivated leaf explants were vacuum infiltrated with antibiotic (2000 $\mu\text{g}\cdot\text{mL}^{-1}$) for 30 min and either screened immediately or transferred to regeneration and elongation media supplemented with mef (100 $\mu\text{g}\cdot\text{mL}^{-1}$) and crb (100 $\mu\text{g}\cdot\text{mL}^{-1}$) and scored as above. This experiment was replicated three times with a minimum of eight explants per treatment per replication.

SHORT-TERM EXPOSURE TO HIGH LEVELS OF ANTIBIOTIC AND/OR TO ACIDIFIED MEDIUM. Vacuum infiltration of apple explants with cef (5000 $\mu\text{g}\cdot\text{mL}^{-1}$) for different amounts of time was studied to determine if increasing cef concentration and the exposure time or adding a 1-h exposure to N6-h at pH 3.0 would improve elimination of *A. tumefaciens* strain EHA101 from apple leaf explants without inhibiting regeneration. Following a 1- or 2-d co-cultivation with *A. tumefaciens*, leaf explants were vacuum infiltrated with a) cef (5000 $\mu\text{g}\cdot\text{mL}^{-1}$) for either 1 or 18 h; b) N6-h, pH 3.0, for 1 h; or c) N6-h, pH 3.0, for 1 h followed by cef (5000 $\mu\text{g}\cdot\text{mL}^{-1}$) for 18 h. Explants were either screened for *Agrobacterium* immediately after co-cultivation and vacuum infiltration or were transferred to N6 and J3-h media supplemented with crb and mef for 52 d and scored as above. The experiment was replicated at least three times with a minimum of eight explants per treatment per replication.

SHORT-TERM EXPOSURE TO AN ACIDIFIED MEDIUM AND TO HIGH LEVELS OF CEF, FOLLOWED BY LONG-TERM EXPOSURE TO LOW LEVELS OF CRB, MEF, AND KAN. To determine whether putative transformants could be produced under optimum conditions for elimination of *A. tumefaciens*, leaf explants were vacuum infiltrated with N6-h, pH 3.0, for 1 h followed by cef (5000 $\mu\text{g}\cdot\text{mL}^{-1}$) for 18 h following a 2-d co-cultivation and transferred to regeneration medium with crb

Table 1. Effect of long-term treatment of low levels of antibiotic on regeneration from apple leaf explants.^z

Treatment	Antibiotic ($\mu\text{g}\cdot\text{mL}^{-1}$)			
	100	200	100	200
	Regeneration (%) ^y		No. of shoots per explant ^x	
Control	67 $\pm$ 12	87 $\pm$ 7	12.0 b	15.8 c
Crb	97 $\pm$ 3	60 $\pm$ 12	23.3 c	6.0 bc
Cef	11 $\pm$ 6	47 $\pm$ 3	0.6 a	5.2 ab
Mef	50 $\pm$ 10	63 $\pm$ 14	8.4 b	10.5 cd
Crb/cef	63 $\pm$ 3	67 $\pm$ 8	8.3 b	9.0 bcd
Crb/mef	90 $\pm$ 6	63 $\pm$ 7	18.3 c	11.5 ed
Cef/mef	0 $\pm$ 0	7 $\pm$ 3	0.0 a	0.4 a

^zAfter 52 d on N6 regeneration and J3-h elongation media supplemented with the antibiotics carbenicillin (crb), cefotaxime (cef), or cefoxitin [=mefoxin (mef)]. Combined treatments contain 100 or 200 $\mu\text{g}\cdot\text{mL}^{-1}$ of each antibiotic as indicated.

^yEach value represents the mean $\pm$ SE of three replications with eight to ten explants per treatment per replication.

^xMean values within columns followed by the same letter are not significantly different, LSD 0.05.

and mef for 3 d, and then kan (10 $\mu\text{g}\cdot\text{mL}^{-1}$) was added to the regeneration and elongation media for the rest of the experiment. This experiment was replicated two times with 180 explants co-cultivated with *A. tumefaciens* per replication. After vacuum infiltration, 10 explants per replication were screened for *A. tumefaciens*. At the end of the experiment, 10 explants per replication and all putative transformants were screened. For each putative transformant, one upper expanded leaf and one 3-mm stem segment was removed, cut into several pieces, and screened. This screening was repeated two times. Histochemical localization of GUS in leaf tissue of putative transformants was performed as previously described (Jefferson et al., 1987).

Results

EFFECT OF LONG-TERM EXPOSURE TO LOW LEVELS OF ANTIBIOTICS ON ELIMINATION OF *A. tumefaciens* FROM APPLE EXPLANTS AND ON SHOOT REGENERATION. Adventitious shoot formation was highest on media with crb (100 $\mu\text{g}\cdot\text{mL}^{-1}$) or 100 $\mu\text{g}\cdot\text{mL}^{-1}$ each of crb and mef and was significantly greater than that for the no-antibiotics control (Table 1). Shoot formation on media containing cef alone or in combination with mef was significantly less than that for controls (Table 1). All of the explants plated on antibiotic medium after a 1-d co-cultivation with *A. tumefaciens* became infected with *A. tumefaciens* by the end of the experiment (data not shown), and many became overgrown making it impossible to monitor shoot regeneration.

EFFECT OF SHORT-TERM EXPOSURE TO MODERATE LEVELS OF ANTIBIOTICS ON ELIMINATION OF *A. tumefaciens* FROM APPLE EXPLANTS AND ON SHOOT REGENERATION. In the absence of co-cultivation, adventitious shoot formation following a 1-h vacuum infiltration with any of the antibiotics tested (at 500 $\mu\text{g}\cdot\text{mL}^{-1}$) was similar to that for controls that were vacuum infiltrated with medium alone (Table 2). Shoot formation was also high (more than nine shoots per explant) following an 18-h vacuum infiltration with any of the antibiotics (at 500 $\mu\text{g}\cdot\text{mL}^{-1}$), but somewhat less for cef and crb treatments (Table 2). All of the explants screened for the presence of *A. tumefaciens* immediately after co-cultivation were infected with this bacterium. All of the explants plated sequentially on regeneration and elongation media without antibiotics, after a 1-d co-cultivation with *A. tumefaciens*, were infected with agrobacteria by the end of the experiment (data not shown), and shoot formation could not be determined because of bacterial growth.

EFFECT OF SHORT-TERM EXPOSURE TO HIGH LEVELS OF ANTIBIOTIC AND/OR AN ACIDIFIED MEDIUM ON ELIMINATION OF *A. tumefaciens* FROM

APPLE EXPLANTS AND ON SHOOT REGENERATION. Short-term (30-min) vacuum infiltration with a high level of cef (2000 $\mu\text{g}\cdot\text{mL}^{-1}$) was more effective against *A. tumefaciens* than mef and crb as seen by the percentage of contamination after 2 and 5 d incubation in YEB medium (Table 3). However, none of the vacuum-infiltration treatments could completely kill this bacterium, and by 14 d all YEB tubes exhibited bacterial contamination. Explant contamination was reduced by 20% to 30% when vacuum infiltration was combined with plating explants on medium supplemented with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ each of crb and mef (Table 3). The percentage of explants regenerating shoots ranged from 73% to 93%, whether or not explants were co-cultivated with *A. tumefaciens* (Table 3).

Vacuum infiltration with a very high level of cef (5000 $\mu\text{g}\cdot\text{mL}^{-1}$) for 18 h was only slightly more effective for eliminating agrobacteria than vacuum infiltration for either 1 h with cef or 1 h with N6-h (pH 3.0) (Table 4). However, *A. tumefaciens* contamination following either a 1- or 2-d co-cultivation was reduced to 13% and 16%, respectively, when explants were screened immediately after a 1-h, pH 3.0, treatment plus an 18-h cef treatment, and 13% and 28%, respectively, when screened after the same treatment plus 52 d on regeneration and elongation media with crb and mef (Table 4). The percentage of explants regenerating shoots following most treatments was similar to that for controls (without co-cultivation, but

Table 2. Effect of short-term treatment of moderate levels of antibiotic on regeneration from apple leaf explants.

Antibiotic treatment ^z	Regeneration (%) ^y	No. of shoots per leaf explant ^y
1-h crb	87 $\pm$ 9	13.4 $\pm$ 2.3
1-h cef	87 $\pm$ 3	13.7 $\pm$ 1.0
1-h mef	72 $\pm$ 10	11.6 $\pm$ 1.8
1-h control ^x	89 $\pm$ 4	14.0 $\pm$ 3.3
18-h crb	73 $\pm$ 9	9.6 $\pm$ 0.6
18-h cef	69 $\pm$ 7	9.3 $\pm$ 0.8
18-h mef	90 $\pm$ 6	13.8 $\pm$ 1.3
18-h control ^x	83 $\pm$ 3	13.4 $\pm$ 2.1

^zAntibiotics carbenicillin (crb), cefotaxime (cef), or cefoxitin [=mefoxin (mef)] (500 $\mu\text{g}\cdot\text{mL}^{-1}$) in N6-h liquid medium (pH 5.4) were vacuum-infiltrated into leaf explants for either 1 or 18 h, after a 1-d co-cultivation of explants with *Agrobacterium tumefaciens*.

^yScored after 52 d on N6 regeneration and J3-h elongation media without antibiotics. Each value represents the mean $\pm$ SE of three replications with eight to ten explants per treatment per replication.

^xN6-h liquid medium, without antibiotics.

Table 3. Effect of short-term antibiotic treatments on elimination of *Agrobacterium tumefaciens* strain EHA101 from apple explants and on plant regeneration.

Antibiotic treatment ^a	Leaf explants with <i>Agrobacterium</i> (%)					No. of shoots per leaf explant ^x
	Placed in YEB medium immediately after treatment (d) ^z				Placed on N6 and J3-h media for 52 d then in YEB medium (d) ^y	
	2	5	7	14	14	
Carbenicillin	40	100	100	100	83 ± 17	10.4 ± 0.8
Cefotaxime	5	65	90	100	73 ± 3	9.6 ± 1.9
Mefoxin	20	100	100	100	80 ± 6	8.7 ± 1.8
Control ^w	---	---	---	0	0 ± 0	10.6 ± 0.9

^aEvaluated for the presence of *A. tumefaciens* after 2 to 14 d.

^yScored after 52 d on N6 regeneration and J3-h elongation media containing 100 µg·mL⁻¹ each of crb and mef. Each value represents the mean ± SE of three replications with eight to twelve explants per treatment per replication.

^xAntibiotics (2000 µg·mL⁻¹) in N6-h liquid medium (pH 5.4) were vacuum infiltrated into leaf explants for 30 min after a 1-d co-cultivation of explants with *A. tumefaciens*.

^wN6-h liquid medium, without antibiotics.

with antibiotic, pH 3.0, treatments) (Table 4).

EFFECT OF SHORT-TERM EXPOSURE TO HIGH LEVELS OF CEF AND TO AN ACIDIFIED MEDIUM, FOLLOWED BY LONG-TERM EXPOSURE TO LOW LEVELS OF MEF, CRB, AND KAN, ON THE ELIMINATION OF *A. tumefaciens* FROM APPLE EXPLANTS AND ON THE PRODUCTION OF PUTATIVE TRANSFORMANTS. Co-cultivation with *A. tumefaciens* for 2 d, followed by pH 3.0 and cef treatments and kan selection, generated 26 kan-resistant putative transformants, and *A. tumefaciens* contamination was reduced to 8% in explants (Table 4). None of the putative transformants was contaminated following repeated screenings for *A. tumefaciens* and one putative transformant was GUS positive.

Discussion

In this study, we describe a system that makes it possible to use *A. tumefaciens* supervirulent strain EHA101 in apple transformation experiments. A key feature is the reduction in explant contamination following co-cultivation while maintaining a high efficiency of regeneration. Strain selection is of particular importance with apple transformation studies, since the efficiency of transformation is influenced by the strain of *A. tumefaciens* (De Bondt et al., 1994; Maheswaran et al., 1992; Martin et al., 1990)

and apple explant genotype (De Bondt et al., 1994). Disarmed strain LBA4404 (derived from wild-type strain Ach5) (Hoekema et al., 1983) has been used in most apple transformation studies (James et al., 1989; Norelli et al., 1994; Yao et al., 1995); however, De Bondt et al. (1994) demonstrated that stable expression of the GUS gene was ≈10-fold greater following co-cultivation with strain EHA101 than with strain LBA4404. In addition, Maheswaran et al. (1992) and Martin et al. (1990) have demonstrated that the efficiency of transformation was greater with wild-type strain A281 (from which EHA101 is derived) than with strains C58, Ach5, and A348.

Recently, Yao et al. (1995) achieved a 1.8% frequency of transformation of 'Royal Gala' with strain LBA4404 and attributed the success of transformation to delayed selection.

Strain LBA4404 has been the strain of choice for apple transformation studies because, in contrast to strain EHA101, it could be effectively eliminated from apple explants with low levels of antibiotics following co-cultivation (James et al., 1989; Maheswaran et al., 1992). Because *A. tumefaciens* may stay latent (not producing visible growth on the medium or symptoms on the plant in vitro) for at least 12 months post-transformation in vitro (Matzk et al., 1996), attempts to eliminate this bacterium with antibiotics must be accompanied by appropriate screening procedures (Cooke

Table 4. Effect of short-term antibiotic treatment and low pH medium on elimination of *Agrobacterium tumefaciens* strain EHA101 from apple explants and on plant regeneration.^z

Treatment ^y	Leaf explants contaminated with <i>Agrobacterium</i> (%)			No. of shoots per leaf explant ^x
	Placed on YEB medium immediately after treatment (14 d)	Placed on N6 and J3-h media for 52 d then in YEB medium (14 d) ^x	Regeneration (%) ^x	
1-h cef (1-d c/c)	100 ± 0	77 ± 3	87 ± 7	14.1 ± 5.8
18-h cef (1-d c/c)	77 ± 7	67 ± 16	93 ± 3	15.1 ± 1.5
1-h (pH 3.0) (1-d c/c)	82 ± 4	82 ± 4	90 ± 10	17.0 ± 3.2
1-h (pH 3.0) + 18-h cef (1-d c/c)	13 ± 7	13 ± 7	83 ± 7	10.5 ± 2.3
1-h (pH 3.0) + 18-h cef (2-d c/c)	16 ± 9	28 ± 6	84 ± 5	12.2 ± 2.7
1-h (pH 3.0) + 18-h cef (control)	0 ± 0	0 ± 0	79 ± 6	9.7 ± 0.9
1-h (pH 3.0) + 18-h cef (2-d c/c)	25 ± 5.1	8 ± 3.8 ^w	7 ± 1 ^{w,v}	0.1 ± 0.0 ^{w,v}

^zUnless noted otherwise, each value represents the mean ± SE of two to three replications with a minimum of eight explants per treatment per replication.

^yCefotaxime (cef, 5000 µg·mL⁻¹) in N6-h liquid medium (pH 5.4) and N6-h (pH 3.0) were vacuum-infiltrated into leaf explants after either a 1- or 2-d co-cultivation (c/c) of explants with *A. tumefaciens*, or a 2-d treatment with N6-h (pH 5.4) treatment without co-cultivation (control).

^xScored after 52 d on N6 regeneration and J3-h elongation media with 100 µg·mL⁻¹ each of crb and mef.

^wRegeneration and elongation media also contain kan (10 µg·mL⁻¹).

^vEach replication contained 180 explants.

et al., 1992; Reed and Tanprasert, 1995). Cef and crb have been used extensively to eliminate *A. tumefaciens* from plant cultures following co-cultivation because they have a broad spectrum for bacterial inhibition (Wise et al., 1978) and low eukaryotic toxicity (Doerr et al., 1980; Pollack et al., 1983). These β -lactam antibiotics bind to peptidoglycan transpeptidases, which are involved in the final stages of cell wall synthesis, and induce cell lysis (Curtis et al., 1979). Our finding that cef is more active than crb and mef against *A. tumefaciens* strain EHA101 supports earlier studies that showed cef to be consistently and considerably more active against a wide range of pathogenic bacteria than either mef or crb (Wise et al., 1978). Other studies have also indicated that many species of bacteria are inhibited by plant media of pH 4.0 and below (Cassells, 1991) and, recently, Cooke et al. (1992) reported inhibition of *A. tumefaciens* and elimination of this bacterium from infected *Rosa* shoot cultures on media of pH 4.0 and below. Our results also demonstrate that, by combining a short-term exposure to acidic (pH 3.0) medium with a short-term exposure to high levels of cef, explant contamination could be reduced to a level acceptable for transformation studies. It is not clear why adding kan to the regeneration and elongation media further reduced contamination, since this bacterium can grow in the presence of kan. However, Leifert et al. (1991b) and Young et al. (1984) have reported eliminating bacteria from plant tissue cultures using combinations of antibiotics, and synergism between β -lactam and aminoglycoside antibiotics has been reported (Klastersky, 1975).

Because efficient regeneration is necessary for successful transformation and because some antibiotics have either a positive or a negative effect (often depending on antibiotic concentration) on cell growth or plant regeneration (Barrett and Cassells, 1994; Mathias and Mukasa, 1987; Pollack et al., 1983), phytotoxicity studies should be conducted in conjunction with studies to eliminate *A. tumefaciens* from plant explants as a prerequisite to transformation research. Extensive studies by Yepes and Aldwinckle (1994) on the effects of cef, crb, and kan on regeneration from leaf explants of seven apple cultivars indicated that cef at $250 \mu\text{g}\cdot\text{mL}^{-1}$ stimulated, whereas crb at $500 \mu\text{g}\cdot\text{mL}^{-1}$ inhibited regeneration. However, the effect of different concentrations of these antibiotics on eliminating *A. tumefaciens* strain EHA101 from apple leaf explants was not investigated. In our studies with 'Royal Gala' apple, long-term exposure of leaf explants to cef at $100 \mu\text{g}\cdot\text{mL}^{-1}$ inhibited regeneration, whereas, crb or crb plus mef at $100 \mu\text{g}\cdot\text{mL}^{-1}$ stimulated regeneration. This may explain the inability by De Bondt et al. (1994) to generate kan-resistant plants of several cultivars including 'Gala', since they used $200 \mu\text{g}\cdot\text{mL}^{-1}$ cef and $200 \mu\text{g}\cdot\text{mL}^{-1}$ triacillin to prevent *A. tumefaciens* overgrowth on the medium. Only short-term exposure of explants to a pH 3.0 medium followed by short-term exposure to high levels of cef and then long-term exposure to crb plus mef (each at $100 \mu\text{g}\cdot\text{mL}^{-1}$) effectively controlled strain EHA101 while allowing efficient regeneration. When combined with kan selection, this system has proven useful for generating kan-resistant, GUS-positive apple plants. Although it has been demonstrated that the expression of GUS by agrobacteria that have not been eliminated from transformed tissues can cause problems with the early detection of transformation in apple (Martin et al., 1990), repeated testing of our putative transformants suggests that they did not harbor *A. tumefaciens* and that our system for elimination of *A. tumefaciens* may prove useful as an alternative to the De Bondt et al. (1994) system, for apple cultivars whose regeneration is inhibited by low levels of cef.

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