AAB BIOFLUX
Advances in Agriculture & Botanics-
International Journal of the Bioflux Society

Delivery of an antisense ACC oxidase gene into *Citrus reticulata* Blanco. mediated by *Agrobacterium tumefaciens*

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Abstract. This study was carried out to optimize the conditions for plantlet regeneration and genetic transformation of *Citrus reticulata* Blanco. with an antisense ACC oxidase gene mediated by *Agrobacterium tumefaciens*. The highest percentage of plantlet regeneration of 100 was successfully achieved on the MS medium supplemented with 3 µM BA, while the half-strength MS medium amended with 8 µM NAA was suitable for root induction. Explants that had high potency of regeneration were shoots, nodes and internodes, respectively. The delivery of an *antisense ACC oxidase* gene into the species *C. reticulata* mediated by *Agrobacterium tumefaciens* strain EHA 105 was successful by co-cultivating explants with the strain EHA105 for 15 min, following that by eliminating the bacterium with 200 mg/l cefotaxime, and subsequently selecting transformed embryoid with 20 mg/l hygromycin. Verified histochemically by GUS assay, putative transformants showed the percentage of gus gene expression of 100. Molecular analysis using PCR confirmed the integration of the antisense ACC oxidase gene into plant genome.
Key Words: *Citrus reticulata*, *Agrobacterium tumefaciens*, antisense ACC oxidase, cefotaxime, hygromycin.

Introduction. Citrus is one of the most economically important fruit crops in the world. Harvested fruits are usually stored before they reach the market for fresh consumption (González-Candelas et al 2010). During this postharvest period fruits are subjected to both biotic and abiotic stress conditions. Excess ethylene production during the degreening process of citrus fruits (Biale et al 1954; Cooper et al 1969a; Eaks 1960; Ben-Yehoshua & Eaks 1970; Hyodo 1977) resulting in citrus postharvest decay is particularly among factors that reduce citrus fruit quality. Although citrus fruit is considered to be non-climacteric and produces very low amounts of ethylene at mature green stage (Aharoni 1968), low temperature treatment (5ºC) of attached and mature-green grapefruit (*Citrus paradisa* Macf.) as well as harvested tangerine (*C. reticulata* Blanco.) fruits were found to enhance both ethylene production and the yellowing of the citrus peel (Cooper et al 1969b), thus shortening the postharvest storage time and resulting in low quality of fruits. To improve postharvest quality of fruits, ethylene production should be effectively controlled, implying that it cost growers extra money to cope with ethylene-induced decay of their citrus fruits. Transgenic citrus with a superior trait of enhanced resistance to environmental stresses, especially ethylene, and improved postharvest quality could overcome the ethylene-induced decay. Success in blocking ethylene biosynthesis using either antisense ACC synthase or ACC oxidase gene (Hamilton et al 1990; Oeller et al 1991) has clearly elucidated that antisense RNA technology is a powerful and feasible means to prevent endogenous ethylene production in a specific plant organ at a specific developmental stage.

This study was therefore aimed to optimize the conditions for plantlet regeneration and genetic transformation of *Citrus reticulata* Blanco. with an antisense ACC oxidase gene by microparticle bombardment.

Material and Method

Seed germination and shoot formation from plantlet parts. Mature seeds of *C. reticulata* were initially washed with mild detergent and rinsed with tap water for 3 times. They were subsequently surface-sterilized in 70% (v/v) ethyl alcohol for 5 minutes and 20% (v/v) sodium hypochlorite with 2 drops of Tween-20 for 10 minutes. After being rinsed 3 times with sterile distilled water, seeds were cultured on Murashige & Skoog (MS) medium (Murashige & Skoog 1962) supplemented with 30 g/l sucrose and 8 g/l agar, pH 5.8. The cultures were kept at 25±2ºC under a long photoperiod (16 h light: 8 h dark) with light intensity of 40 µmol m^{-2} s^{-1} for 5 weeks.

Shoots, nodes and internodes from the upper part of a plantlet's cotyledons were used as explants for shoot formation. Briefly, explants were cultured on the MS medium added with 30 g/l, 8 g/l and BA concentrations of 0, 1, 2, 3, 4 and 5 µM. The cultured were maintained for 4 weeks at 25±2ºC under a long photoperiod (16 h light: 8 h dark) with light intensity of 40 µmol m^{-2} s^{-1}.

For root initiation, shoots obtained from the previous experiment were cultured on the half-strength MS medium supplemented with 30 g/l, 8 g/l agar and NAA concentrations of 0, 3, 5, 8 and 11 µM and kept for 4 weeks at 25±2ºC under a long photoperiod (16 h light: 8 h dark) with light intensity of 40 µmol m^{-2} s^{-1}.

Effect of antibiotics on shoot regeneration. To determine the effect of antibiotics on shoot regeneration, shoots, 0.5 cm in height, were cultured on the MS medium supplemented with 3 µM BA and cefotaxime concentrations of 0, 100, 200, 300, 400, 500, 600 and 700 mg/l. The effective concentrations of hygromycin were also determined. The concentrations tested were 0, 10, 15, 20 and 25 mg/l. All kinds of antibiotics were added to the medium after autoclaving. The cultures were maintained at 25±2ºC under a long photoperiod (16 h light: 8 h dark) with light intensity of 40 µmol m^{-2} s^{-1} for 5 weeks.

Agrobacterium-mediated transformation. *Agrobacterium tumefaciens* strain EHA105 (pCAMBIA1305.1) were used for the establishment of the transformation. The plasmid pCAMBIA1305.1 carried GUS gene and hygromycin-resistant (*hpt*II) gene, each expressed under the CaMV35S promoter. The bacterial strain was cultured in Luria Broth (LB) liquid medium supplemented with 100 mg/l kanamycin and maintained on a reciprocal shaker at 28ºC for 48 hours until OD_{600}=1.5-1.8.

Shoots were used as explants for transformation in this experiment. The explants were soaked in *Agrobacterium* suspension for 0, 5, 10, 15, 20 and 25 minutes. Then they were co cultivated on the MS medium for 3 days. After co cultivation, the explants were washed thoroughly in sterile distilled water containing 300 mg/l cefotaxime for 15 minutes. Explants were subsequently transferred to the MS medium supplemented with 3 µM BA, 300 mg/l cefotaxime and 15 mg/l hygromycin for 3 weeks.

Histochemical GUS assay. The histochemical assay for GUS gene expression was performed according to the method of Jefferson (1987), using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as a substrate. Briefly, putative transformants were transferred to a 1.5 ml microtube containing X-gluc and subsequently incubated overnight at a temperature of 37ºC.

PCR analysis. Total genomic DNA was extracted from shoots of transformed plantlets and non-transformed control plantlets by the CTAB method (Doyle & Doyle 1987). The primer sequences for PCR were as follows: NOS forward sequence (F)5'-GAATCCTGTTGCCGGTCTTG-3', reverse sequence (R)5'-TTATCCTAGTTTGCGCGCTA-3' to yield a 180 bp fragment. The DNA was denatured at 94 °C for 4 min, followed by 35 cycles of amplification (1 min at 92 °C; 1 min at 55 °C; 2 min at 72ºC). The final incubation at 72 °C was extended to 4 min, and the reaction material was cooled and kept at 4 °C. The PCR products were visualized by running the completed reaction on a 2% agarose gel containing ethidium bromide.

Results

Seed germination and shoot formation from plantlet parts. Seeds successfully developed into whole plantlets and grew up to 5 cm in height after 5 weeks of seed sowing in the MS medium without plant growth regulators added. It was evident that different plant parts showed different capacities in producing new shoots (Figure 1).

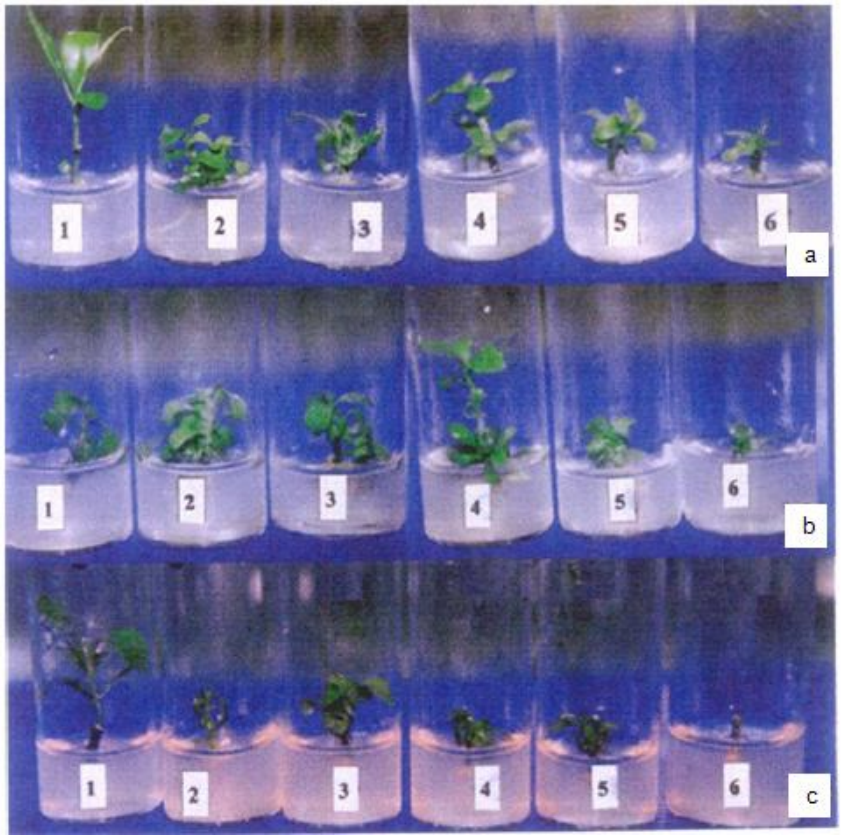


Figure 1. Newly developed shoots derived from (a) shoots, (b) nodes and (c) internodes cultured on the MS medium supplemented with varied concentrations of BA for 5 weeks: 1 = MS; 2 = MS + 1 µM BA; 3 = MS + 2 µM BA; 4 = MS + 3 µM BA; 5 = MS + 4 µM BA; 6 = MS + 5 µM BA.

Shoots cultured in the presence of 3 µM BA performed the best in the capacity for producing new shoots (100%) with the highest number of shoots per explant (3.33), followed by nodes cultured in the presence of 3 µM BA (100%) with the number of shoots per explant of 2.73. Internodes from the upper part of a plantlet's cotyledons cultured in the presence of 4 µM BA showed 40% of new shoot induction with the number of shoots per explants of 1.27 while those cultured in the presence of 5 µM did not produce new shoots but died within 4 weeks of culture (Figures 2 & 3).

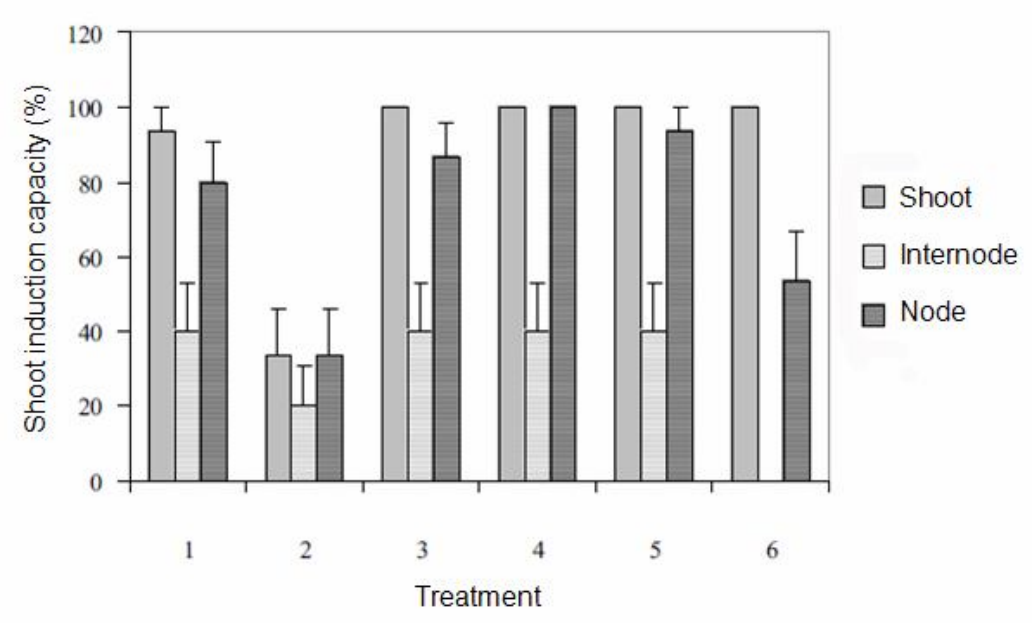


Figure 2. New shoot induction percentage obtained from different plant parts cultured under different conditions for 5 weeks: 1 = MS; 2 = MS + 1 µM BA; 3 = MS + 2 µM BA; 4 = MS + 3 µM BA; 5 = MS + 4 µM BA; 6 = MS + 5 µM BA.

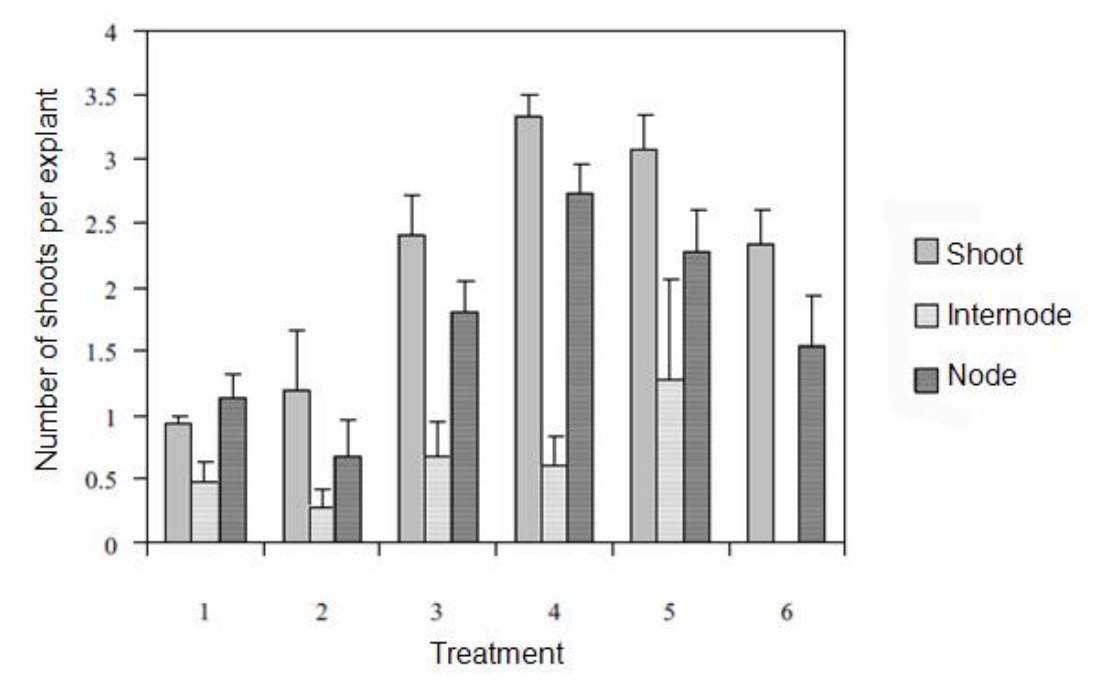


Figure 3. Number of shoots per explant obtained from different plant parts cultured under different conditions for 5 weeks: 1 = MS; 2 = MS + 1 µM BA; 3 = MS + 2 µM BA; 4 = MS + 3 µM BA; 5 = MS + 4 µM BA; 6 = MS + 5 µM BA.

Differences in root induction percentage from shoots were recorded (Figures 4 & 5). The maximum root induction percentage at 83.3 was obtained on the half-strength MS medium supplemented with 8 µM NAA.

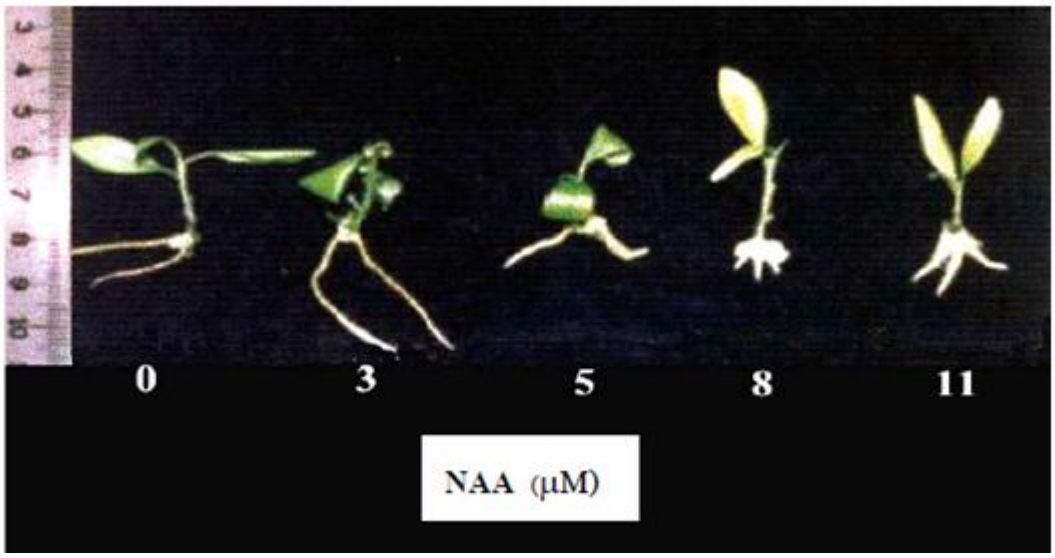


Figure 4. Newly developed roots obtained from shoots cultured under different conditions for 4 weeks: 1 = ½ MS; 2 = ½ MS + 3 µM NAA; 3 = ½ MS + 5 µM NAA; 4 = ½ MS + 8 µM NAA; 5 = ½ MS + 11 µM NAA.

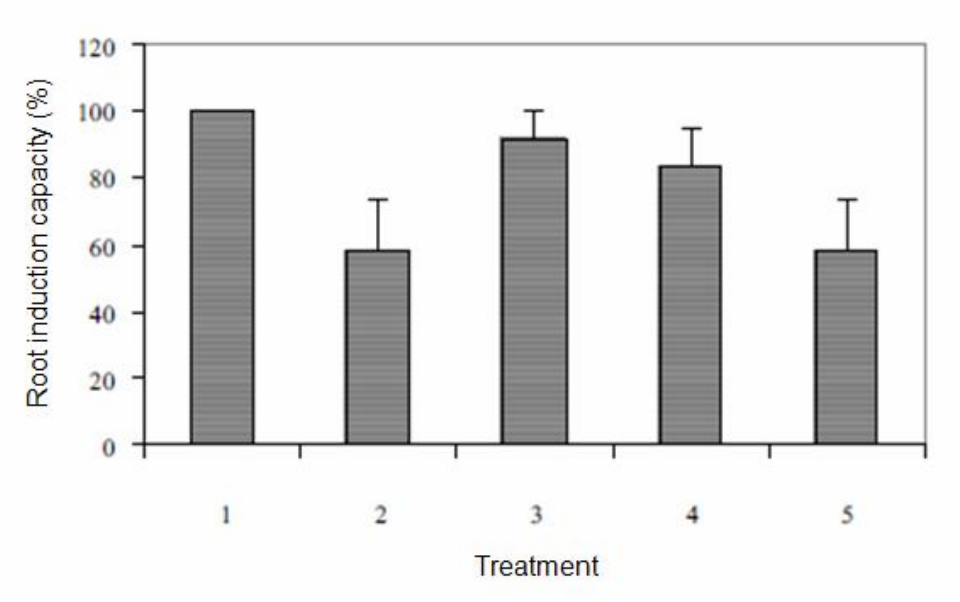
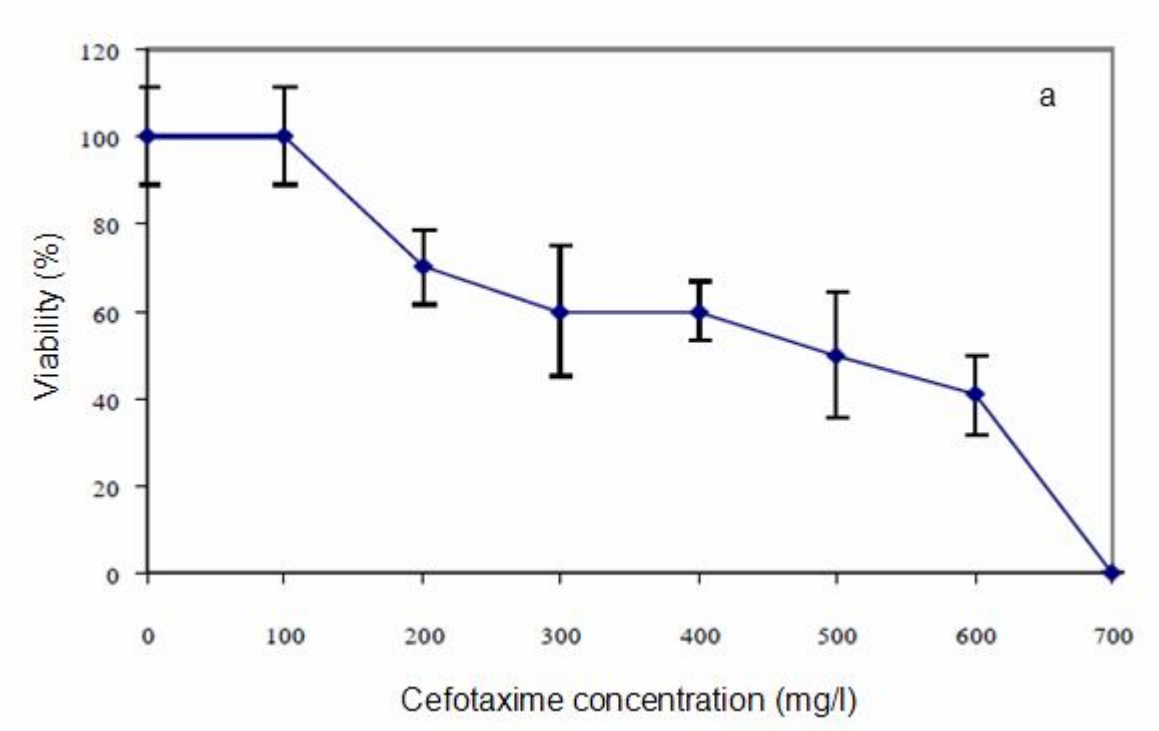


Figure 5. New root induction percentage obtained from shoots cultured under different conditions for 4 weeks: 1 = ½ MS; 2 = ½ MS + 3 µM NAA; 3 = ½ MS + 5 µM NAA; 4 = ½ MS + 8 µM NAA; 5 = ½ MS + 11 µM NAA.

Effect of antibiotics on shoot regeneration. Antibiotics used in the study strongly reduced regeneration capacities of *C. reticulata* shoots. In the presence of 100-700 mg/l cefotaxime and 10-25 mg/l hygromycin, a slight inhibitory effect was observed. The highest dose of cefotaxime that yielded surviving embryoids was 200 mg/l (Figures 6a & 7a). The lowest dose of hygromycin that completely inhibited embryoid growth was 20 mg/l (Figures 6b & 7b). All of the shoots turned brown and finally died in five weeks after they were transferred to the selective medium.



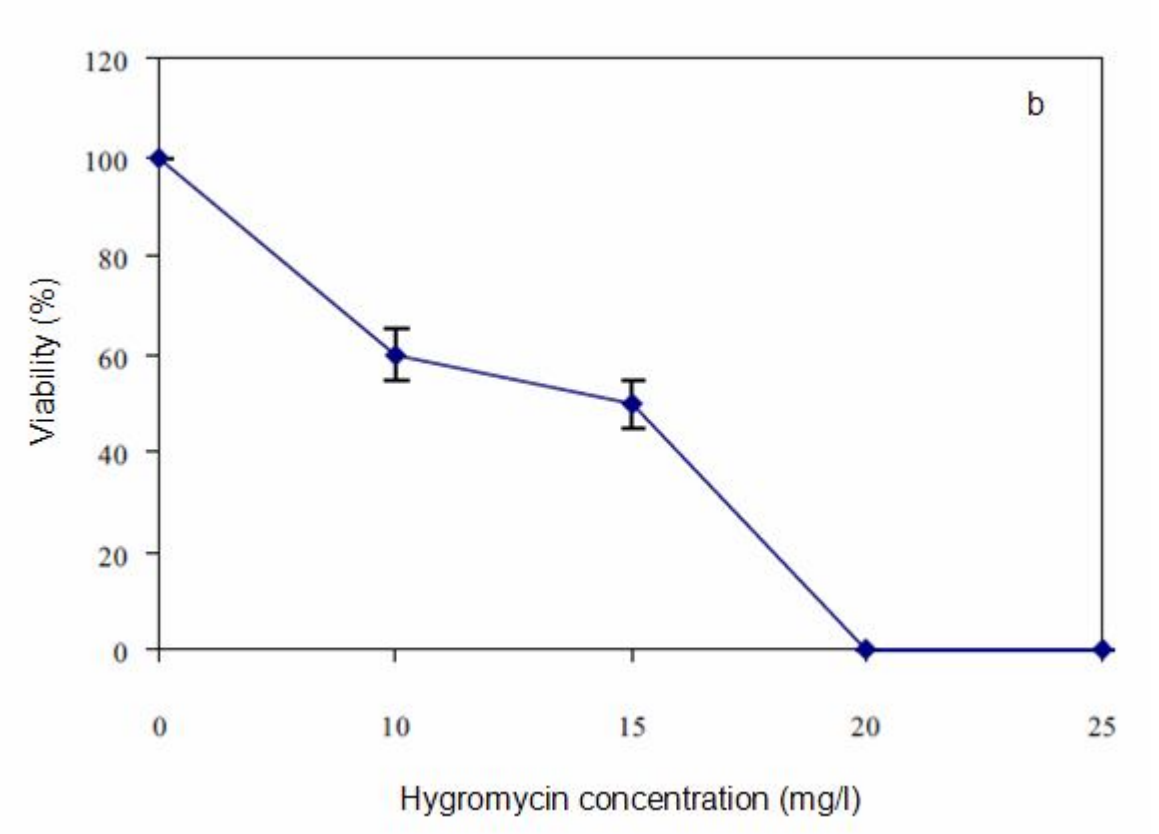


Figure 6. Effect of different concentrations of cefotaxime (a) and hygromycin (b) on *C. reticulata* shoot growth.

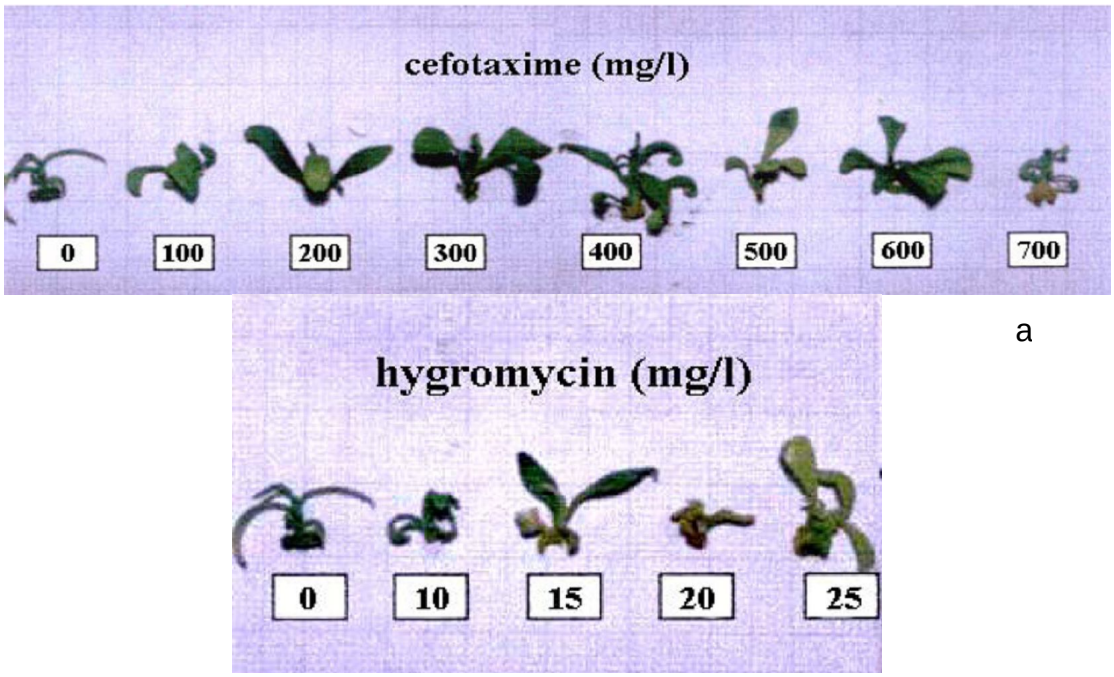


Figure 7. Shoots on the MS medium containing different concentrations of cefotaxime (a) and hygromycin (b).

Agrobacterium-mediated transformation. Differences in levels of GUS activities in shoots after being co cultivated with *A. tumefaciens* for 0-25 minutes were detected (Figure 8a). The optimal co cultivation time for the maximum GUS activities was 15 min. *Agrobacterium*-mediated transformation of *C. reticulata* showed a maximum percent expression (100%) (Figure 8B).

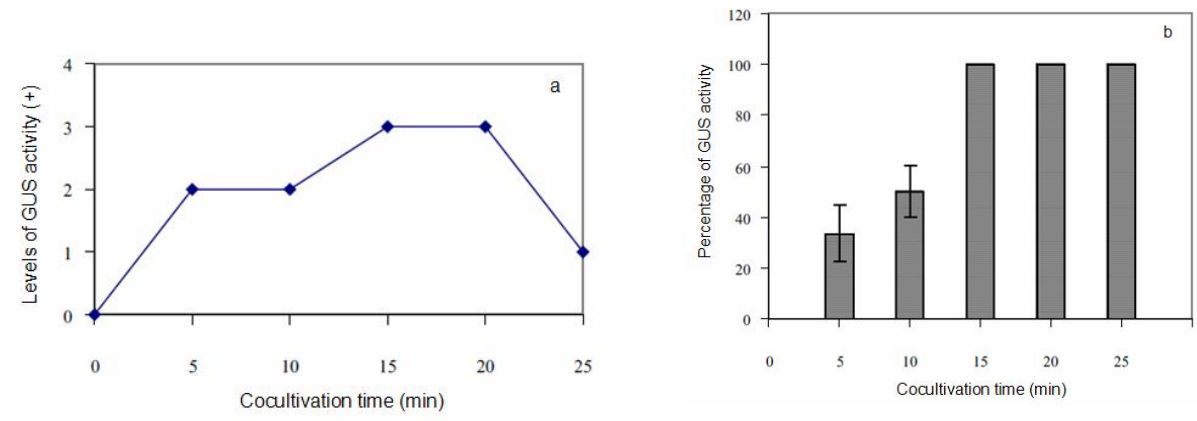


Figure 8. Levels (a) and percentages (b) of GUS expression in shoots after being cocultivated for 0-25 min.

To determine the integration of T-DNA fragments in hygromycin-resistant plantlets, polymerase chain reaction (PCR) analysis was carried out. It was found that the size of amplified fragment was 180 bp for NOS, whereas non-transformed control plantlets did not show any expected band size (Figure 9).

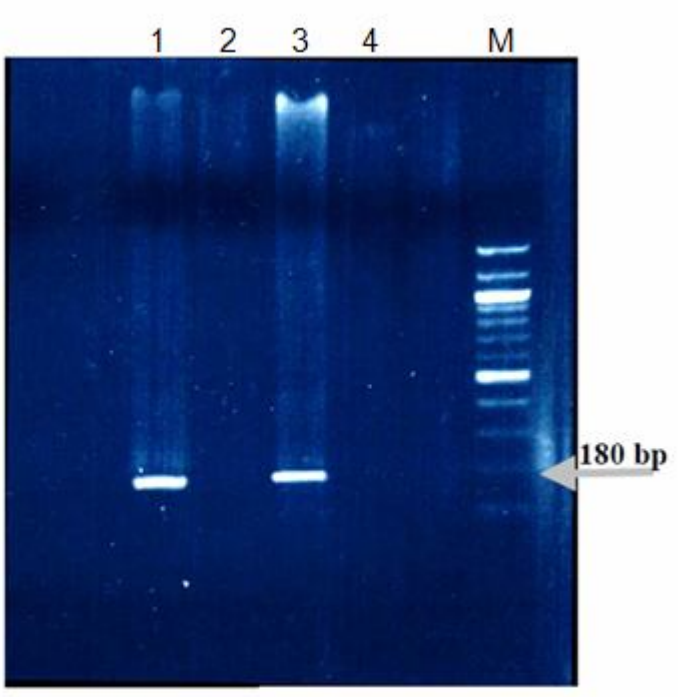


Figure 9. PCR analysis in transformed shoots in *C. reticulata* using primers to detect NOS; lane M: 100 bp ladder, lane 1: transformed plantlets, lane 2: non-transformed control plantlets, lane 3: pCAMBIA1305.1 (positive control), and lane 4: negative control.

Discussion. Addition of plant growth regulators in the medium is considered essential for shoot- or root-induction in citrus. In the present study, 3 µM BA in the MS medium was optimal for shoot induction. Higher concentrations of BA resulted in low percentage of shoot initiation. Our study was in agreement with Gill et al (1995) who reported that elevated concentrations of cytokinin inhibited callus growth in citrus. Moreover, our results indicated that shoots of *C. reticulata* were easily induced by BA, producing a number of new shoots per explant. Our findings conformed to Barlass & Shene (1982) who reported that different tissues showed different responses to cytokinin. Apart from that, our results suggested that addition of high concentrations of NAA in the half-strength MS medium resulted in low capacity of root induction and roots obtained were generally short and flesh.

A selective agent plays an important role in the selection of the transformants and for avoiding development of undesirable numbers of the escapes. It was suggested that hygromycin is an excellent selective agent and needs to be optimized for each plant species (Datta et al 1999). In this report, *hpt*II encoding resistance to hygromycin was used in the production of transgenic citrus. Hygromycin are aminoglycoside antibiotics which cause harmful death to plant cells by inhibiting transcription and translation.

Eliminating *A. tumefaciens* after cocultivation by adding antibiotics in the medium is necessary. In the present study, cefotaxime showed strong inhibition of the regeneration potential in *C. reticulata*. However, it was found that the concentration of 200 mg/l did not inhibit the regeneration of explants and was thus considered optimal for eliminating *A. tumefaciens* while maintaining explant growth and development. Our findings are in agreement with Sriskandarajah et al (1994) who reported that cefotaxime concentration of 200 mg/l did not inhibit the regeneration of explants in *Malus sylvestris* var. 'Delicious'.

In the present study, a number of transformed *C. reticulata* were produced using an *Agrobacterium*-mediated transformation system. The outcomes confirmed that shoots can be used as explants for this transformation system. In addition, our results showed that the CaMV35S promoter was useful for genetic transformation of *C. reticulata*.

Conclusions. To conclude, this report described the use of *A. tumefaciens* strain EHA105 (pCAMBIA 1305.1) to deliver screenable and selectable marker genes into *C. reticulata* and displayed molecular evidence of primary transgenic plants which showed stable integration of transgenes. We recommend that shoots of *C. reticulata* are the suitable target tissues for *Agrobacterium*-mediated transformation.

Acknowledgements. The authors would like to thank the Graduate School, Khon Kaen University for financial support, and the Department of Biology, Faculty of Science, Khon Kaen University for granting us permission to use the devices necessary for the study.

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Received: 10 February 2013. Accepted: 07 March 2013. Published online: 11 March 2013.
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How to cite this article:
Bunnag S., Tangpong D., 2013 Delivery of an antisense ACC oxidase gene into *Citrus reticulata* Blanco. mediated by *Agrobacterium tumefaciens*. AAB Bioflux 5(1):29-38.