Genetic transformation of Soursop (*Annona muricata*) via *Agrobacterium tumefaciens*

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ABSTRACT

A protocol for *in vitro* shoot induction and *Agrobacterium*-mediated transformation of *Annona muricata* L. was developed in this study. High number of shoots (12 shoots per explant) and *in vitro* plant regeneration was achieved using hypocotyls segments from 30 day-old seedlings planted in Murashige and Skoog (MS) media supplemented with 1 mg/L of zeatin. For the development of the genetic transformation protocol, a GFP reporter gene and a phosphinothricin (PAT) selection system were used. Different pre-culture, co-cultivation conditions, and three *A. tumefaciens* strains were tested. The best treatment for the regeneration of transgenic shoots was preculture of explants for two days on shoot-induction medium, co-cultivation with *Agrobacterium* strain EHA105 for two days, followed by transfer to selective medium with 3 mg/l of PAT. The presence of the transgenes in regenerated shoots was reconfirmed by genomic PCR and GFP fluorescence. These results offer a potential alternative for the rapid mass propagation and genetic improvement of soursop (*A. muricata L).*

INTRODUCTION

The Soursop, *Annona muricata L.* is a tropical tree recognized for the economic potential, and the nutritional properties of its pulp. It is available as fresh fruit or used in the manufacturing of beverages, jams, nectars and other preserves. The seeds have anticancer potential as well as some medical applications (Pinto et al., 2005). To maintain and increase its market share, steps that the industry must take include developing high quality *Annona* varieties that are adaptable to the diverse abiotic and biotic conditions, meet the demands of the market place, and produce acceptable returns to the grower. The major goal of this research was to develop a genetic transformation method for this tropical tree.

MATERIALS AND METHODS

IN VITRO CULTURE SYSTEM

Seeds of *Annona muricata* obtained from fresh harvested fruit were initially treated with 100 mg of Vitavax 300 (Proficol) for a minimum of one day. Seeds were then sterilized with 25% bleach for 20 min, and rinsed with sterilized water. The seed coats were manually removed with pliers. The seeds were germinated on MS media supplemented with 1 mg/l GA_3, and incubated at RT under dark conditions for a period of one month. When the seedlings were approximately 10-12 cm in length, hypocotyls segments of about 0.5 cm were dissected and cultured on shoot induction medium supplemented with different hormones (Table 1). The hypocotyls were incubated at RT, under a light intensity of 45 μmol $m^{-2}s^{-1}$ for sixteen hours. Developing shoots were transferred to a shoot elongation medium (MS supplemented with 1.5 mg/ml meta-Topolin). When shoots were 2 to 3 cm in length, they were transferred to different rooting induction media (Table 2). The rooted plants were transferred to jiffy pots in a high humidity chamber before transfer to ambient greenhouse conditions. The steps on the regeneration of shoots from hypocotyl explants is illustrated in Figure 1.

AGROBACTERIUM TRANSFORMATION SYSTEM

For the establishment of the *Agrobacterium* mediated transformation system a modified binary vector pDM805 described by Tingay et al. (1997) was used. This modified binary vector contains a selectable marker gene (*bar*) encoding phosphinothricin acetyl transferase (*PAT*), and the Green Fluorescent Protein (GFP) reporter gene driven by *Ubi1* and *Act* promoters respectively (Fig. 2). *Agrobacterium* strains EHA105, AGLO1 and GV3101 carrying the modified pDM805 were used in the transformation experiments. Bacterial strains were grown for 24-48 h on a shaker (225 rpm) at RT in YEP media supplemented with 50 μM acetosyringone (3'-5'-Dimethoxy-4-hydroxyacetophenon), 100 mg/l spectinomycin, and the cultures were pelleted and re-suspended in liquid MS medium. Hypocotyls of 0.5 cm in length were pre-cultured on solidified MS supplemented with 1 mg/l zeatin for two days, before inoculation with Agrobacterium. Then, the hypocotyls were submerged in *Agrobacterium* suspension at a concentration of 10^{10} *Agro*-cells/ml for 20 min on a shaker at 50 rpm. The *Agrobacterium* treated explants were then blotted on sterile filter paper and transferred to solidified MS supplemented with 1 mg/l zeatin. After two days of co-cultivation, explants were transferred to the same medium, but supplemented with 300 mg/l timentin, and 3 mg/l phosphinothricin for shoot initiation (Fig. 3). The explants were transferred to the same selection medium every two weeks following removal of dead and dying tissue. After ten weeks shoots were transferred to shoot elongation media (MS supplemented with 1.5 mg/ml meta-topolin).

Table 1. Regeneration of adventitious shoots in hypocotyl explants cultivated on MS media with differentconcentrations of hormones. Shoot induction was determined after 9 weeks.

Media	Shoots/explant
MS	0.2
MS + 0,5 mg/l BAP	2.3
MS + 1 mg/l BAP	4.0
MS + 2 mg/l 2ip	3.6
MS + 4 mg/l 2ip	3.6
MS + 0.5 mg/l Meta-topolin	2.9
MS + 1 mg/l Meta-topolin	3.6
MS + 1.5 mg/l Meta-topolin	2.7
MS + 0.5 mg/l CPPU	1.4
MS + 2 mg/l TDZ	1.5
MS + 0.5 mg/l NAA	0.5
MS + 1 mg/l Zeatin	**12.0**
MS + 2 mg/l Zeatin	1.0
MS + 3 mg/l Zeatin	3.7

Table 2. Rooting of shoots under different media treatments.

Media composition	Rooting shoots (%)
MS Media (+) IBA	0
MS Media	0
MS Medium (+) Galactose (+) IBA	0
MS Medium (+) Galactose	0
WPM Medium (+) IBA	0
WPM Medium	0
MS Medium (+) Charcol (+) IBA	1
MS Medium MS (+) Charcol	0
Plants treated with IBA 200 mg/l	70

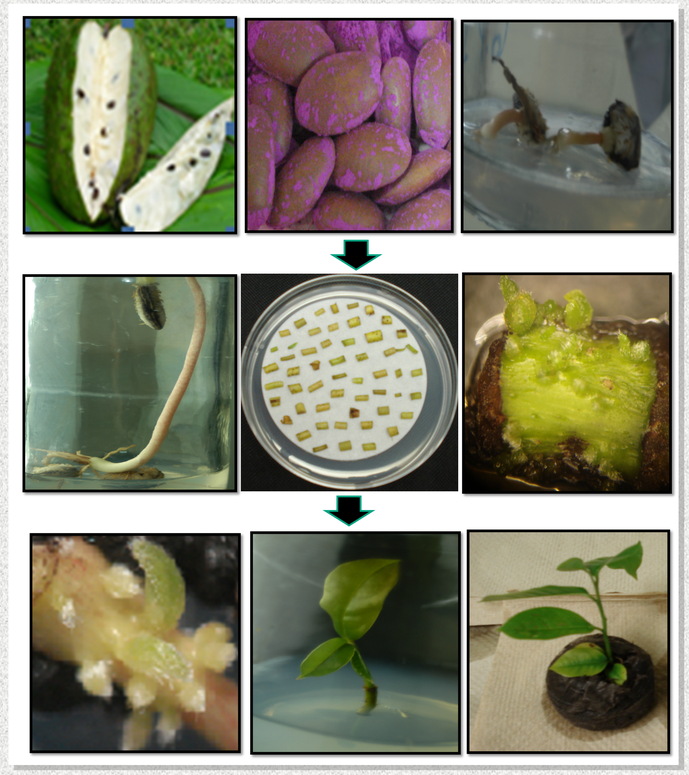


Figure 1. Regeneration of shoots and rooted plants from hypocotyl segments.

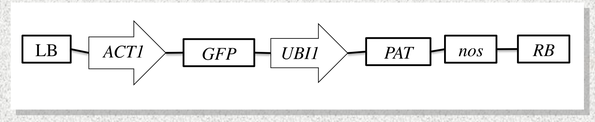


Figure 2. Map of the modified binary vector pDM805. The GFP gene from pCAMBIA 1302 was cloned between ACT1 rice promoter and the the UBI1 maize promoter.

Figure 3. Regeneration of transgenic shoots from hypocotyl explants treated with Agrobacterium. A, Untreated hypocotyl growing on non-selective medium (control); B-C, Hypocotyl explants treated with *Agrobacterium* strains AGLO1 (C), GV3101 (D) and EHA105 (E), planted on selective medium with 3 mg/l phosphinothricin; E-F, Shoot regeneration on selective medium.

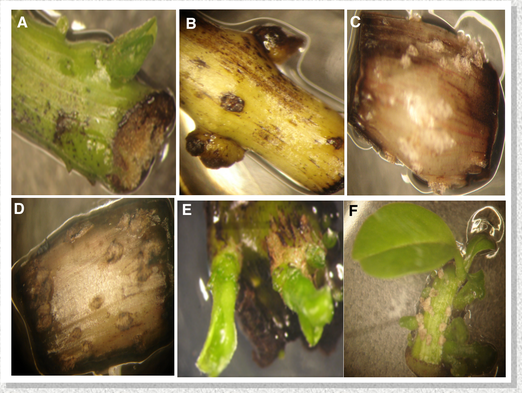


Figure 4. Detection of green fluorescent protein (*GFP)* in tissue from transgenic shoots. A-B, stem cross sections of shoots growing on phosphinothricin selective medium; C, GFP fluorescence in shoots developing from a hypocotyl explant treated with Agrobacterium; D, cross section through the stem of a wild type, non-transformed shoot showing only red chlorophyll autofluorescence.

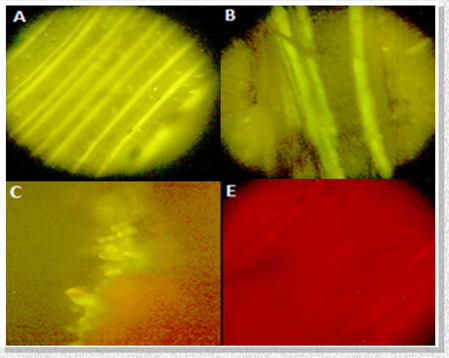


Figure 5. Genomic PCR analysis of leaves from soursop shoots regenerated on selective medium. Analysis was performed using primers against phosphinothricin acetyl transferase (*PAT*) gene. The plasmid DNA was used as a positive control. The amplification product of 528 bp corresponds to the selective amplification of *PAT*. WT, wild type DNA (negative control); NTC, non-template control.

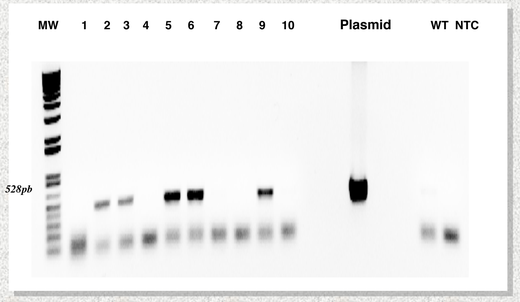
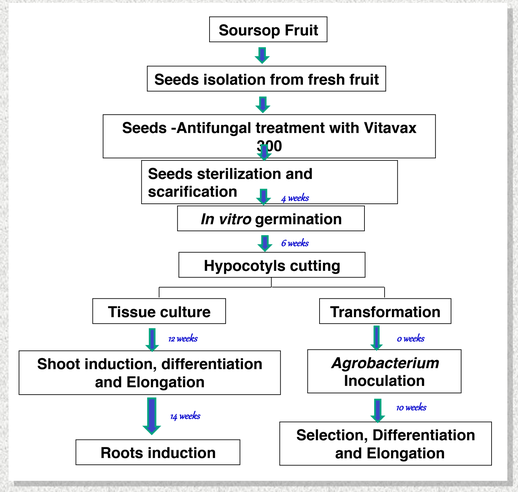


Figure 6. *In vitro* culture and transformation of *Annona muricata* L. via *Agrobacterium tumefaciens*



RESULTS AND DISCUSSION

The major goal of this research was to develop a genetic transformation method for *A. muricata*. Every transformation method consist of two important subsystems. The first is a regeneration system that allows for the development of organized tissues from single cells, usually through techniques based on tissue culture. The second is a transformation system that utilizes one of several methods to physically deliver new genetic material into plant cells. Research to optimize conditions for both of these subsystems was conducted (Fig. 6). An efficient organogenic regeneration system based on the use of hypocotyl explants from zygotic seedlings that produced genetically true-to type adventitious shoots was established (Fig. 1). The highest number of shoots (12 shoots per explant) and *in vitro* plant regeneration was achieved using 30-day-old seedlings germinated on MS media supplemented with 1mg/l of zeatin (Table 1). Rooting of regenerated shoots was best (70%), when shoots were immersed in a solution containing 200 mg/l IBA for 30 minutes and then incubated in MS media for 2 weeks (Table 2).

The next step was to establish the genetic transformation system using *Agrobacterium* mediated transformation. Phosphinothricin tolerant shoots were regenerated from hypocotyl explants inoculated with the *Agrobacterium* strain EHA105. The putative transgenic shoots were screened for GFP expression and the presence of the *PAT* gene. Stem cross sections of transgenic shoots showed GFP fluorescence (Fig. 4). Genomic PCR analysis showed amplification of a 528 bp fragment corresponding to the coding region of the *PAT* gene in five out of ten independently regenerated shoots (Fig. 5). Future studies will be conducted to improve the efficiency of the methodology for the genetic transformation of *Annona muricata*. The *in vitro* culture and genetic transformation systems developed for *A. muricata* in this work could be applied for the improvement of this tropical fruit tree. This is the first report in the literature showing the genetic transformation of Soursop.

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