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Agrobacterium rhizogenes Mediated-transformation of *Asimina triloba* L. Cuttings

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Abstract: The current research compared rooting of pawpaw, with softwood cuttings from mature stands, cuttings from the terminal 3-4 inches of seedlings and cuttings taken from shoots of rooted cuttings from seedlings. Four treatments were used: *Agrobacterium rhizogenes* strain MSU-1 (A4 wild type), *A. rhizogenes* strain MT232 (TR105 mutant), indole-3-butyric acid at 20,000 mg L^{-1} and control. Only the *A. rhizogenes* treatments induced rooting and only of the seedling cuttings. No statistical differences in rooting percentage were found among the bacterial strains. While the MSU-1 strain produced more roots, these were shorter and thinner than those produced by MT232 strain. No roots resulted from any other treatment. Roots obtained from infected seedlings were analyzed for transformation using polymerase chain reaction targeting the *rolB* and *rolC* vir genes. Roots confirmed as transgenic were 100% for both *rolB* and *rolC*.

Key words: Pawpaw, transformation, IBA, DNA, *rolB*, *rolC*

INTRODUCTION

Many woody plants, economically important for fruit production or ornamental use, are often difficult to root, using both conventional and *in vitro* propagation techniques. Pawpaw (*Asimina triloba* L.) has been no exception and has proven to be a difficult species to root with standard stem cutting propagation methods. Pawpaw, a member of the Annonaceae, is valued for its nutritional edible fruits and ornamental quality (Bonner and Halls, 1974; Callaway, 1992). Germinating papaw from seed is very infrequent (Finneseth *et al*., 1998) and propagation success with stem and root cuttings is limited (Finneseth *et al*., 1998). The ability to propagate pawpaw cultivars from cuttings would present several advantages over using seeds or grafting.

Prior research on rooting of woody ornamentals and fruit trees showed that it was possible to improve *in vivo* rooting with hormone applications, etiolation, or the use of polyamines (Damiano *et al*., 1991; Rugini *et al*., 1993). In spite of these advances, the difficulty of rooting these species is still one of the major obstacles to successful propagation. Recently, a new approach has been implemented to overcome the rooting problem. *Agrobacterium rhizogenes*, a bacterium, has been utilized in woody ornamental and fruit tree stem cutting inoculations for the induction of adventitious root formation.

Root induction is due to the integration and subsequent expression of a portion of bacterial DNA (T-DNA) from the Ri (Root Inducing) plasmid in the bacterium. Four loci involved in root formation were identified in the T-DNA of the Ri plasmids and designated root loci (*rol*) A, B, C, D (Spena *et al*., 1987; Chilton *et al*., 1982). Several authors have reported successful rooting using *A. rhizogenes*-mediated transformation in fruit trees, such as jujube (Hatta *et al*., 1996), apple (Sutter and Luza, 1993), kiwi (Rugini *et al*., 1991), almond (Strobel and Nachmias, 1985; Damiano *et al*., 1995), walnut (Caboni *et al*., 1996) and in other tree genera, such as *Pinus* (Villalobos-Amador *et al*., 2002; Magnussen *et al*., 1994; Mihalievic *et al*., 1996; Burns and Schwarz, 1996; Tzfira *et al*., 1996) and *Eucalyptus* (MacRae and van Staden, 1993). Auxin treatments have also proven useful in promoting initiation of adventitious roots of many difficult-to-root species both in macro- and micropropagation and are now commonly in use in nurseries (Hartmann *et al*., 1990; Hamill *et al*., 1991). The transformation of stem cuttings from seedlings, shoots from adventitious roots or mature stems by *A. rhizogenes* seems to be an excellent way to improve rooting in these trees. The goals of the current research was to determine whether *A. rhizogenes* had the ability to induce root formation on cuttings of *A. triloba* cultivar Mitchell and to compare the response to inoculation with either *A. rhizogenes* strain MT 232 or MSU-1 or with 20,000 ppm indole-3-butyric acid.

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MATERIALS AND METHODS

Plant material: Cuttings from three sources were collected May 11, 2001. These consisted of terminal shoot cuttings obtained from the basal portion of mature trees in a stand located in Huntsville, AL. The second source of cuttings consisted of terminal cuttings excised from seedlings germinated in the spring. Seeds were extracted from ripe fruit collected in the pawpaw orchard at the Western Maryland Research Experiment Station, Keedysville, MD. The third source of shoots were those induced from root cuttings collected February 7, 2001 and allowed to form shoots. The pawpaw cultivar, Mitchell, was used for the propagation/root induction experiments. The cuttings to be inoculated were wounded by making a shallow vertical slice across the base of the stem. Then the wounded sections were scraped against the actively growing bacteria. Inoculated cuttings were placed in Ray Leach Cone-tainers^{TM} (Stuewe and Sons, Inc., Corvallis Oregon, USA), filled with a 1: 1 moistened mixture of Promix ^{TM} and perlite for 24 h at 25°C before being placed under intermittent mist system (4 sec mist every 10 min for the first 6 weeks).

Bacterial inoculum and transformation: Bacteria used in the experiment included two strains of *A. rhizogenes*, MSU-1 (A4 wild type ATCC39207) and MT-232 (TR105 mutant; ATCC 31798). Strain A4 is a wild type which contains three Ri plasmids (pArA4c, pArA4b and pArA4a). TR105 is a mutant which has a plasmid essentially homologous with pArA4b of A4.

A. rhizogenes strains MSU-1 and MT-232 were grown at 28°C on a standard Yeast Mannitol Broth Medium (YMB) (Hooykaas *et al*., 1977) for 24-36 h until an optical density of 0.4 at 600 nm was obtained. The cuttings and inoculations were carried out according to Hatta *et al*. (1996), dipping the basal part of the cuttings for 24 h at 25°C in darkness in 0.5 mL bacterial suspension. The cuttings subcultured for 3 weeks; the most homogeneous shoots were selected and the base cleanly cut. After inoculation, the explants were transferred to the appropriate co-cultivation root induction medium, with or without hormone. Finally cuttings were transferred to a common medium containing the antibiotic cefotaxime (250 mg L^{-1}). Control shoots, were dipped in YMB for 24 h and then cultured under the same conditions as the inoculated plants. After inoculation, the cuttings were cultured in darkness for ten days before being exposed to light.

DNA extraction and PCR: DNA was extracted following the procedures of Edwards *et al*. (1991). The DNA was extracted from single roots, the weight ranging from 0.1 to 0.4 g. The bacteria were removed from the roots by washing in 0.2 M NaOH and 1.0% w/v SDS, for 3 min followed by a short rinse in sterile deionized water on sterile filter paper. Roots were excised and put onto fresh, growth regulator-free MS medium containing 500 mg L^{-1} carbenicillin to eliminate any bacteria and then, placed in liquid nitrogen in storage at -80°C. Samples were then pulverized to a fine powder with liquid nitrogen in a pre-cooled mortar and transferred to a 50.0 mL tube. DNA was isolated using the DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA, USA).

Polymerase Chain Reaction was carried out following the protocol of Hamill *et al*., 1991, in a volume of 50 μL with template DNA standardized to 80-90 ng. A mixture with the following components was used for PCR, 0.0 μL DNA template, 0.0 μL TaqDNA polymerase (1.5 U-Pharmacia), 0.0 10x PCR buffer, 0.0 Forward/Reverse primer and 0.0 ddH_2O. Primers used for the amplification reactions came from internal sequences of the *rolB* gene (780 bp) and from *rolC* gene (540 bp) (Research Genetics, Huntsville, AL, USA). Oligonucleotide primers for PCR detection of sequences homologous to *rolB* and *rolC* genes were designed on the basis of the DNA sequence of these genes described by Furner *et al*. (1986) [*rolB* 5'-GCTCTTGCAGTGCTAGATTT-3' and 5'-GAAGGTGCAAGCTACCTCTC-3'; *rolC* 5'-CTCCTGACATCAAACTCGTC-3' and 5'-TGCTTCGAGTTATGGGTACA-3'] using BLAST (Basic Alignment Search Tools).

The cycling parameters for the amplification with the *rolB* primers were 95°C for denaturation (1 min), 34°C for annealing (2 min) and 72°C for extension (2 min), repeating for 35 amplification cycles. For the *rolC* primers denaturation, annealing and extension temperatures were 95°C (1 min), 55°C (1 min) and 72°C (2 min), respectively, for 35 cycles. The amplified sequences were separated by electrophoresis in a 1.2% agarose gel in Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and observed (photographed) under ultraviolet (UV) light.

Experimental design and data: There were three replicates with 100 single shoot subsamples for each treatment/selection. The cuttings were evaluated for the presence or absence of roots, the roots were counted and the lengths of the roots measured. Data collected were the number of rooted plants, the number of roots per plant and the length of roots. Data were collected 10 weeks from the start of the experiment. Analysis of Variance (ANOVA) was conducted to determine if treatments were significantly different. Means were separated using Tukey's method following ANOVA. Data analysis was conducted using SAS version 9.0 (SAS Institute Inc., 2002).

RESULTS AND DISCUSSION

Table 1 shows the rooting percentages of the cuttings with the different treatments. According to their rooting ability, the cuttings can be divided into two groups: a) those rooting from seedling cuttings only after *A. rhizogenes* inoculation (Fig. 1) and b) those not rooting with auxin or control (Table 1). No roots resulted from any of the other treatments. In general, the addition of an auxin to the media did not improve rooting in pawpaw. Following bacterial inoculation approximately 33% of the cuttings from seedlings rooted (Table 1). However, some differences could be detected. For group (a) no substantial differences were found between hormone-free treatments, while a tendency to decrease the percentage rooting length was observed with strain MT-232 inoculation. Some changes in the root number and root length occurred.

Molecular analyses were carried on a random sample of roots of pawpaw seedlings. PCR was used to detect the integration of the *rolB* and *rolC* genes in the roots induced by *A. rhizogenes* (Fig. 2). Integration of the T-DNA into *A. rhizogenes* genome was confirmed on the molecular level by the PCR with primers constructed on the sequences of *rolB* and *rolC* genes of *A. rhizogenes.* Primers based on the sequence of *rolB* gene amplify the expected fragment of 780 bp and those specific for *rolC* gene amplify the fragment of 540 bp DNA in PCR reaction with DNA isolated from the transformed roots (Fig. 2). The PCR product was absent in non-transformed tissue.

Fig. 1: Rooting of seedling cuttings in response to two *A. rhizogenes* strains 10 weeks after inoculation

Table 1: Rooting ability (means±SE) of cuttings from seedlings in the hormone supplemented medium (IBA), control (check) or infected with two strains of *A. rhizogenes* MSU-1 and MT-232

	Treatments			
Roots (%)	Control (check)	IBA 20,000 mg L^{-1}	MSU-1	MT-232
Number plants with roots	0.0±0.0	0.0±0.0	12.0±0.6	12.0±0.5
% rooting	0.0±0.0	0.0±0.0	33.3±3.0	33.3±3.8
Mean number roots	0.0±0.0	0.0±0.0	2.2±0.7	1.4±0.5
Mean total root length (cm)	0.0±0.0	0.0±0.0	5.2±0.9	9.5±2.5
Mean root Diameter (mm)	0.0±0.0	0.0±0.0	0.8±0.2	1.1±0.3

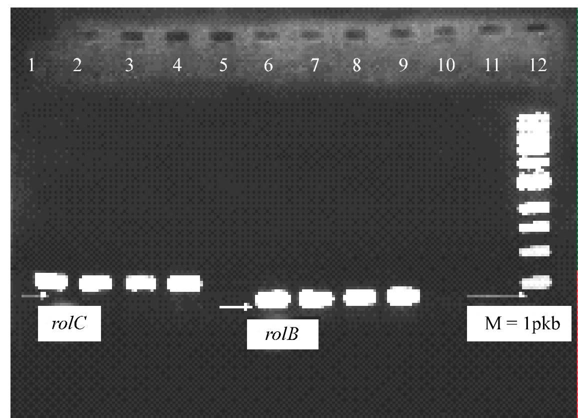

Fig. 2: Identification of rolB and rolC gene fragments in transformed tissue of *Asimina triloba* L. Lanes 1-4, *rolC*; lanes 6-9 *rolB*; lanes 5 and 10 control, lane 11, control without DNA and lane 12, marker (1 kbp)

Amplification of both fragments was obtained in PCR reactions performed with DNA isolated from *A. rhizogenes* cells (Fig. 2). By using DNAs from the roots of the seedling cuttings as template and the non-transformed roots as control, PCR products were amplified with *rolB* primers and *rolC* primers respectively (Fig. 2). Two fragments, with lengths of 780 bp and 540 bp and corresponding to *rolB* and *rolC*, respectively, were amplified only from the hairy root cultures but not from the untransformed (control) roots. In fact, *rolB* and *rolC* could be identified in 100% of the samples (Fig. 2).

Several factors are important for successful *Agrobacterium* mediated transformation of woody plants. These include source material from which cuttings will be taken, bacterial strain to be used and the co-cultivation conditions. Cuttings from seedlings were the only source material that rooted. Rapid growth of the source material is important for transformation of tobacco (*Nicotiana tabacum*; (Furner *et al*., 1986), *Arabidopsis*

(Bechtold *et al*., 1993) and pine (*Pinus radiata*; Li and Leung, 2003) and may contribute to the success observed in this study. Transformation of plant tissue by *Agrobacterium* was earlier confirmed by determination of the production of opines by plant tissue (Petit *et al*., 1983). Recently Henzi (1999) has confirmed integration of the T-DNA to the plant genome using PCR with primers for the NPTII gene cloned earlier to the Ri plasmid of the *A. rhizogenes*. Primers for *rolB* and *rolC* genes used in this work enable molecular confirmation of the transformation with the wild type strains of *A. rhizogenes*. Geneve *et al*. (2003) reported rooting of pawpaw stem cuttings with IBA, however, they used concentrations of 10,000 mgL^{-1} and below with only a 7% of rooting at 10,000 $mg\ L^{-1}$. Our concentration of 20,000 $mg\ L^{-1}$ did not produce any roots.

The results presented in this paper indicate that the use of *A. rhizogenes* can be a successful approach to improve rooting on pawpaw trees, although different responses to inoculation occurred, indicating possible genotype interactions. Furthermore, the regenerated transformed roots did not have a classic hairy-root-appearance (Fig. 1). In this sense, our results agree with the findings of Mihalievic *et al*. (1996) and Villalobos-Amador *et al*. (2002), who showed that the roots induced by *A. rhizogenes* in *Pinus nigra* and *Sequoia sempervirens* had a normal, non-hairy-root appearance.

However, even with the difficult-to-root pawpaw the inoculation always had a positive effect in rooting percentage. From our experiments, it was possible to differentiate two principal behaviors. Most of the samples (i.e., mature stems, stems from roots) did not appear to be affected by inoculation or hormonal treatment even though the bacterial supply may have been sufficient to give good rooting, as occurred with the seedlings. On the other hand, neither IBA nor the control had any effect on rooting of pawpaw.

Further investigations are necessary to better understand the rooting and the transformation processes in pawpaw trees and to improve propagation protocols once roots are initiated. In addition, long-term effects on survival and efficacy of the subsequent root system must still be studied in pawpaw plants derived from cuttings treated with *A. rhizogenes*.

The results of this experiment could significantly affect how hard-to-root tree fruits and woody ornamentals can be clonally reproduced in the future. Previous research using conventional methods of stem or root propagation has shown to be unsuccessful in pawpaw and other recalcitrant woody-fruit trees. If proven an effective method, the use of *A. rhizogenes* to produce adventitious root formation in pawpaw can be a faster method of reproduction leading to application for both commercial nurseries and woody ornamental/fruit researchers.

CONCLUSIONS

A. rhizogenes and cutting sources affected root production in pawpaw. Both strains promoted root formation; however, MSU-1 promoted more rooting than MT-232. *A. rhizogenes* has shown potential to induce roots even on recalcitrant pawpaw. Only cuttings from seedlings were responsive to *A. rhizogenes* treatment; therefore, juvenility should be considered a factor for successful transformation. This also indicates that transformation with *A. rhizogenes* could greatly assist in the establishment of a well-developed root system in difficult-to-root genotypes of pawpaw or other woody tree fruits. A propagation method to produce pawpaw from rooted cuttings could be advantageous to the greenhouse and/orcharding industries because of the shortage of seeds or other clonal propagation material.

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