INSERTION OF *Agrobacterium rhizogenes rolB* GENE IN MANGO

Marleny Chavarri, Ariadne Vegas García, Asia Y. Zambrano†, Zulay Gutiérrez y Jhonny R. Demey

SUMMARY

*Transgenic mango (*Mangifera indica *L.) plants were regenerated from somatic embryos inoculated with a wild* Agrobacterium rhizogenes *strain. The bacteria were grown in Luria Bertani medium supplemented or not with acetosyringone until an optical density of 1.0-1.5 was reached. Incubation time of somatic embryos from the Kent, Haden and Madame Francis varieties in bacterial suspension was 1h, 15 and 5min. Somatic embryos were transferred into Gamborg Miller Ojima semisolid medium for 48h, and finally placed on the same medium containing cefotaxime/carbencillin, in darkness at 27 ±1°C. In the first treatment,* A. rhizogenes *was eliminated after 20 washings with cefotaxime/carbencillin, only in the Kent variety; while 1-5 washes were needed for the other treatments. There were no differences between preculture treatments of* A. rhizogenes *with or without acetosyringone. After infection embryos formed callus, then secondary embryos and, finally, plants were regenerated but hairy roots were not induced. The presence of rolB gene in leaf tissue was confirmed by PCR. An expected band of 720bp, corresponding to rol B gen amplification, was obtained only in the transformed plants. An efficient protocol has been developed for successful production of transgenic somatic embryos and plants of the Kent variety using* A. rhizogenes. *Twenty transgenic clones per 0.5g of inoculated tissues with 80% embryo survival were produced. The results constitute the first report of transgenic mango plants mediated by A. rhizogenes as an alternative tool for breeding improvement of this crop.*

Introduction

Mango (*Mangifera indica* L.) is a member of the *Anacardiaceae* family, and the fourth most widely grown fruit crop in the world. The fruits are used for fresh consumption and pulp production. In Venezuela, it constitutes one of the most important fruit crops. A large proportion of the production is consumed locally, and a small percentage is exported to USA and Europe. Mango has high vitamin C content in addition to Ca and K (Avilán and Leal, 1996).

Mango plants are seriously affected by pathogens and pests. Anthracnosis caused by *Collectotrichum gloesporoides* Penz presents high incidence during flowering and fruit ripening, and can be controlled by application of chemicals and hydrothermal treatments to fruits. The fruit flies are the most important pest worldwide, and tramps and hydrothermal treatments are used for their control (Lizada, 1993). Another problem in mango production is that fruits are climacteric and highly perishable due to over ripening. Mature fruits show increased susceptibility to diseases (Gómez-Lim and Litz, 2004).

Mango tissue culture presents some difficulties to regenerate complete normal plants, and only a low proportion of somatic embryos develop into plants. Moreover, shoot growth is slow and induction of normal roots in shoots and somatic embryos is limited (DeWald *et al*., 1989a, 1989b; Rivera, 2006). Transformed mango plants regenerated after infection of *Agrobacterium rhizogenes* will be expected to have a higher number of roots, and the possibility exists to regenerate plants through organogenesis or somatic embryogenesis from hairy roots (Cabrera *et al*., 1996; Welander and Zhu, 2006). Another possibility in mango is the use of *A. rhizogenes* to improve rooting, by simple inoculation of stems, as has been done in the production of transformed roots of almond, olive and apple rootstocks (Welander and Zhu, 2006).

A. rhizogenes is the causal agent of hairy root disease in dicotyledonous plants. For the transfer of DNA from bacteria to plant cells, three blocks of gene families are necessary: the T-DNA, the virulence (*vir*) genes and the chromosomal virulence (*chv*) genes. In the TR-DNA are located the genes for agropine and auxin synthesis (*aux1* and *2*). The TL-DNA harbors four *rol* genes (root loci): *rolA*, *rolB*, *rolC* and *rolD*, distinguishing the Ri plasmid from the Ti plasmid of *A. tumefaciens*. The *rol* genes have been shown to be responsible for the Ri phenotype (Schmülling *et al*., 1989; Bettini *et al*., 2003). The *rolB* has been found to have indoxyl-β-oxidase activity, suggesting a role in the release of active auxin (Filippini *et al*., 1996; Marsilio *et al*., 2001; Altamura, 2003). The *rol*B gene plays a central role in hairy

KEYWORDS / Genetic Transformation / *Mangifera indica* / Oncogenes / Somatic Embryogenesis /

Received: 04/20/2009. Modified: 05/17/2010. Accepted: 05/24/2010.

Marleny Chavarri. Doctor in Agricultural Sciences, Universidad Central de Venezuela, (UCV). Professor, UCV, Venezuela. e-mail: chavarrim@agr.ucv.ve

Ariadne Vegas García. Doctor in Agricultural Sciences, UCV, Venezuela. Researcher, Instituto Nacional de Investigaciones Agrícolas (INIA), Venezuela. Dirección: Unidad de Biotecnología Vegetal. Centro Nacional de Investigaciones Agropecuarias (CENIAP). Apartado 5653. Maracay 2105, Venezuela. e-mail: avegas@inia.gob.ve

Asia Y. Zambrano. Doctor in Agricultural Sciences, UCV, Venezuela. Researcher, CENIAP-INIA, Venezuela. †12/2009.

Zulay Gutiérrez. Agricultural and Livestock Technician, Instituto Universitario de Tecnología Alonso Gomero, Venezuela. Research Associate, CENIAP-INIA, Venezuela. e-mail: zgutierrez@inia.gob.ve

Jhonny R. Demey. Doctor in Applied Multivariate Statistics, Universidad de Salamanca, España. Biostatitician, Fundación Instituto de Estudios Avanzados, Venezuela. e-mail: jdemey@idea.gob.ve

INSERCIÓN DEL GEN *Agrobacterium rhizogenes rolB* EN MANGO

Marleny Chavarri, Ariadne Vegas García, Asia Y. Zambrano†, Zulay Gutiérrez y Jhonny R. Demey

RESUMEN

*Plantas transformadas de mango (*Mangifera indica *L.) fueron regeneradas a partir de embriones somáticos inoculados con una cepa silvestre de* Agrobacterium rhizogenes*. La bacteria creció en un medio Luria Bertani suplementado o no con acetosiringona. El tiempo de incubación de los embriones somáticos de las variedades Kent, Haden y Madame Francis en la suspensión bacteriana fue de 1h, 15 y 5min. Los embriones somáticos fueron transferidos a un medio semisólido de Gamborg Miller Ojima por 48h y finalmente se colocaron en el mismo medio conteniendo cefotaxima/carbencillina, en oscuridad a 27 ±1°C. En el primer tratamiento,* A. rhizogenes *fue eliminado después de 20 lavados con cefotaxima/carbencillina, solo en la variedad Kent; mientras para los otros tratamientos fueron necesarios entre 1 y 5 lavados. No hubo diferencias entre los tratamientos precultivo of* A. rhizogenes *con y sin acetosiringona. Después de la infección, en las plantas regeneradas no se logró inducir la formación de las raíces de cabellera típicas. La presencia del gen rolB en tejido de hojas se confirmó mediante PCR. Se obtuvo una banda de 720pb, correspondiente a la amplificación del gen rol B, solo en las plantas transformadas. En este trabajo se produjeron 20 clones por 0,5g de tejido inoculado, con un 80% de sobrevivencia. Estos resultados constituyen el primer trabajo en transformación de mango mediada por* A. rhizogenes*, como una herramienta para el mejoramiento genético del cultivo.*

INSERÇÃO DO GEN *Agrobacterium rhizogenes rolB* NO MANGO

Marleny Chavarri, Ariadne Vegas García, Asia Y. Zambrano†, Zulay Gutiérrez and Jhonny R. Demey

RESUMO

*Plantas transformadas de mango (*Mangifera indica *L.) foram regeneradas apartir de embriões somáticos inoculados com uma cepa silvestre de Agrobacterium rhizogenes. A bactéria cresceu em um meio Luria Bertani suplementado ou não com acetoseringona. O tempo de incubação dos embriões somáticos das variedades Kent, Haden e Madame Francis na suspensão bacteriana foi de 1h, 15 e 5min. Os embriões somáticos foram transferidos a um meio semisólido de Gamborg Miller Ojima por 48h e finalmente se colocaram no mesmo meio contendo cefotaxima/carbencillina, no escuro a 27 ±1°C. No primeiro tratamento,* A. rhizogenes *foi eliminado depois de 20 lavagens com cefotaxima/carbencilina, somente na variedade Kent; enquanto que para os outros tratamentos foram necessários entre 1 e 5 lavagens. Não houve diferenças entre os tratamentos precultivo of* A. rhizogenes *com e sem acetoseringona. Depois da infecção, nas plantas regeneradas não se alcanzou induzir a formação das raízes de cabeleira típicas. A presença do gen rolB em tecido de folhas se confirmou mediante PCR. Obteve-se uma banda de 720pb, correspondente à amplificação do gen rol B, somente nas plantas transformadas. Neste trabalho se produziram 20 clones por 0,5g de tecido inoculado, com um 80% de sobrevivencia. Estes resultados constituem o primeiro trabalho em transformação de mango mediada por* A. rhizogenes*, como uma ferramenta para o melhoramento genético do cultivo.*

root induction, while *rolA*, *rolC*, *rolD* and other ORFs act in synergy to promote root induction (Welander and Zhu, 2006; Scorza *et al*., 1998).

Rol genes, in combination or individually, have been demonstrated to modify morphological, physiological and developmental processes in plants, such as dwarfism, increased branching, early flowering and improved rooting. The *rol* genes offer great opportunities to manipulate plant architecture traits such as plant height, branching, leaf form and color, flowering, fruiting, and root system for the horticultural, agricultural and forestry industries. Transgenic tomato plants with the *rolB* gene had wider and shorter leaves and reduced apical dominance than the wild-type (Van Alvorst *et al*., 1992).The use of *rolB* transgenic rootstocks in combination with untransformed scions would allow adapting to various soil conditions with limited or no transgenic spreading (Welander and Zhu, 2006).

Transformation efficiency can be increased enhancing virulence of the bacterium by adding phenolic compounds such as acetosyringone (AS). In a number of plant species and their mediating *Agrobacterium* an exogenous supplement of AS had been found to enhance the efficiency of transformation, applied via pretreating plant explant or *Agrobacterium* culture, by inclusion of the AS in the coculture medium, and by combining the pretreatment of explant and *Agrobacterium* culture. AS precultured *A. rhizogenes* was potent in term of transformation efficiency of tobacco leaves, suggesting that activation of *Agrobacterium* vir genes governed a highly efficient transformation (Godwin *et al*., 1991; Shih-Cheng *et al*., 2007).

The development of genetic manipulation techniques has provided new opportunities for plant improvement. Plant transformation has made possible to modify just one or two desired agronomic traits, while retaining the unique characteristics of the original cultivar. Genetic transformation of mango has the potential for improving disease and pest resistance, alteration of tree architecture, and prolonging fruit shelf life (Mathews and Litz, 1992; Gómez-Lim, 1999; Gómez-Lim and Litz, 2004).

Mango transformation has been reported using *A. tumefaciens* (Mathews *et al*., 1993) and particle bombardment (Cruz-Hernández *et al*., 1997, 2000; Chavarri *et al*., 2004). The mango ACC synthase and ACC oxidase genes have been used in mango transformation experiments (Cruz-Hernández *et al*., 1997).

Herein is reported an efficient method of mango transformation through *A. rhizogenes* as a potential tool for improvement of disease resistance and prolongation of fruit shelf life, as well as for

the enhancement of *in vitro* rooting in shoots.

Materials and Methods

Establishment of somatic embryogenesis

Immature fruits (30-45 days after flowering) of the Haden, Kent and Madame Francis varieties were taken from the germplasm collection of Instituto Nacional de Investigaciones Agrícolas (INIA), Venezuela. The fruits were cleaned and washed in soap water, disinfected by soaking in 70% alcohol for 10min and in 3% sodium hypochlorite for 30min, then washed three times in sterile distilled water, and dissected under sterile conditions (modified from Dewald *et al*., 1989a). The zygotic embryos were recovered and placed in the induction medium. After proliferating, somatic embryo masses were separated from zygotic embryos and cultured for development of secondary embryos. Callogenesis and somatic embryogenesis from zygotic embryos of Haden, Kent (both monoembryonic) and Madame Francis (polyembrionic) varieties were induced *in vitro*, in semisolid culture medium (induction medium) with half MS (Murashige and Skoog, 1962) salts supplemented with 10% coconut-water, 0.4mg·l^{-1} thiamine, 100mg·l^{-1} myo-inositol, 0.5mg·l^{-1} nicotinic acid, 400mg·l^{-1} glutamine, 1mg·l^{-1} 2.4-D, 1mg·l^{-1} BAP, 30·gl^{-1} sucrose, and 7g·l^{-1} agar, adjusting pH to 5.8, incubating in dark conditions at 25 ±1°C and 70-80% relative humidity. Sections of 0.5cm from zygotic embryo calli were transferred to B5 medium (Gamborg *et al*., 1968) supplemented with 0.4mg·l^{-1} thiamine, 0.5m·l^{-1} nicotinic acid, 100mg·l^{-1} myo-inositol, 400mg·l^{-1} glutamine, 30g·l^{-1} sucrose, 7g·l^{-1} agar, adjusting pH to 5.8, at 25 ±1°C with periods of 16h light (32µmol·m^{-2}·s^{-1}) and 8h dark, and 70-80% of relative humidity (Salazar, 1997).

Bacterial strain

A. rhizogenes wild type provided by Tatsuji Sakamoto, Osaka Prefecture University, Japan, was used in this study.

Transformation

A. rhizogenes wild type was inoculated in Luria Bertani (LB) medium (Draper *et al*., 1988) and grown at 27 ±1°C for 15h, until an optical density of 1.0-1.5 was obtained at 560nm (Lin *et al*., 1994). The bacteria were suspended in LB medium (50ml) supplemented or not with 200µM AS.

Somatic embryos were precultured on B5 semisolid medium without hormones for 48h, after which they were macerated and dipped in the bacterial suspension for 1h, 15min and 5min. The first treatment (1h) was applied to all three varieties and the second and third treatments only to the Kent variety.

Then, tissues were incubated on preculture medium for 48h., in the dark at 27 ±1ºC, after which they were washed 3 times in sterilized distilled water and dried on sterilized paper towel. Finally, macerated somatic embryos were transferred to the same medium containing cefotaxime (250mg·l^{-1}) and carbenicillin (500mg·$^{-1}$), and incubated in the dark at 27 ±1°C. Control somatic embryos were used without inoculation with *A. rhizogenes* and cultured under the same conditions as above.

To evaluate infection and bacterial growth, tobacco (*Nicotiana tabacum* L.) leaf sections of 1cm^2 were co-cultivated simultaneously, dipping both sides in the bacterial suspension, previously injured with a sterile needle, and placing them in the same culture media used for the mango explants.

A completely randomized experimental design was used, with ten treatments (three varieties, one bacterium strain, supplemented or not with AS, one incubation time; one variety, one bacterium strain, with or without AS, two incubation times) and five replications per treatment, for a total of 10-30 Petri dishes per variety, each with 0.5g of somatic embryos.

Histological assay

Histological sections of somatic embryos regenerated after inoculation with the wild strain of *Agrobacterium rhizogenes* were made and compared with control somatic embryos, both with >3 months cultured in B5 medium. Cuts were done by hand, stained with 0.5% toluidine blue and observed under a light microscope.

Confirmation of transformation

For the polymerase chain reaction (PCR) analysis, genomic DNA was extracted from somatic embryos transformed and non-transformed according to Zambrano *et al.* (2002).

PCR was performed using a MJ Research PTC 200 thermalcycler with oligonucleotide primers of the *rolB gene* (100nM). The primers used were the following 5'-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3' and 5'-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3' (Furner *et al.,* 1986). These primers amplified a fragment of ~720bp.

Each PCR reaction was carried out according Hamill *et al.* (1991) in a total volume of 50µl containing 10mM Tris HCl (pH 9.0), 50mM KCl, 0.1% Triton® X-100, 1.5mM $MgCl_2$, 200µM of each dNTPs, 100ng of genomic DNA and 1U of *Taq* DNA polymerase (Promega). The reaction mixture was overlaid with 20µl of mineral oil.

Amplification was performed according to Hamill *et al.* (1991) under the following program: 92°C for denaturation (duration 1min), 55°C for annealing (1min) and 72°C for extension (1.5min), with 35 amplification cycles. The amplified sequences were made visible on 1.5% agarose etidium bromide gels.

Results and Discussion

Establishment of somatic embryogenesis

Somatic embryos developed from zygotic embryos calli in three weeks for Madame Francis and in four weeks for Haden and Kent varieties, when cultured in the induction medium. These results are in concordance with that obtained by Salazar (1997) for Kent and Haden varieties.

Transformation

Inoculation of somatic embryos from Haden, Kent and Madame Francis varieties with *A. rhizogenes* by maceration and dipping for *1h*. After 48h bacterial growth was evident on somatic embryos tissues of the three varieties. Washes and subcultures in B5 medium with antibiotics cefatoxime and carbenicillin were done every 24h for the first week, and every 48h thereafter for one month. The bacterium was only eliminated from Kent variety after 20 washings and subcultures. Inoculated embryos formed callus and ultimately embryos and plants in culture medium B5 without antibiotics (Figure 1). Similar results were obtained with or without AS.

Inoculation of Kent somatic embryos with *A. rhizogenes* by dipping for *15min*. Bacterial growth was observed in the somatic embryos 48h after inoculation. Bacteria were eliminated after 5 washings of tissues and placing them in fresh B5 medium with cefotaxime and carbencilin. Afterwards, some embryos developed calli and secondary embryos. Similar results were obtained with or without AS.

Inoculation of Kent somatic embryos with *A. rhizogenes* by dipping for *5min*. Bac-

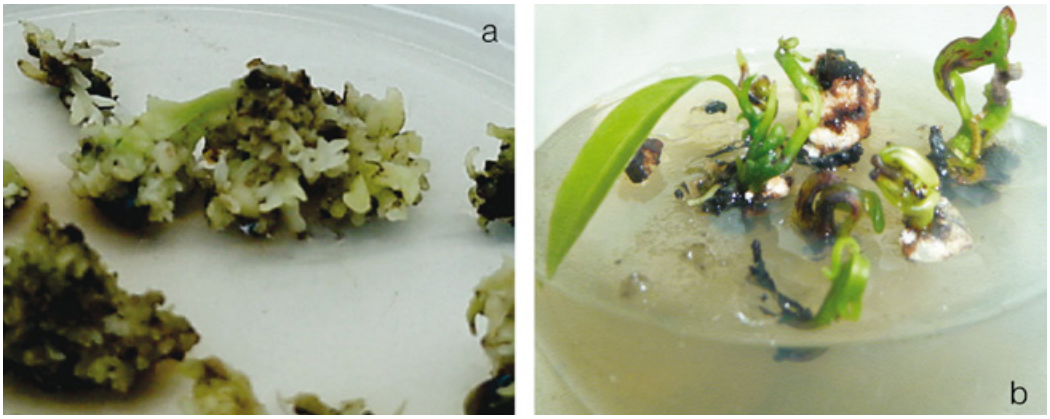

Figure 1. Somatic embryogenesis and plantlet regeneration of mango cv. Kent, after infection with wild strain of *A. rhizogenes* a: somatic embryo, b: *in vitro* mango plants with and without roots obtained from somatic embryos.

terial growth was observed in the somatic embryos 72h after inoculation. Bacteria were eliminated after the first wash. Afterwards, the majority of embryos developed calli and secondary embryos. Similar results were obtained with or without AS.

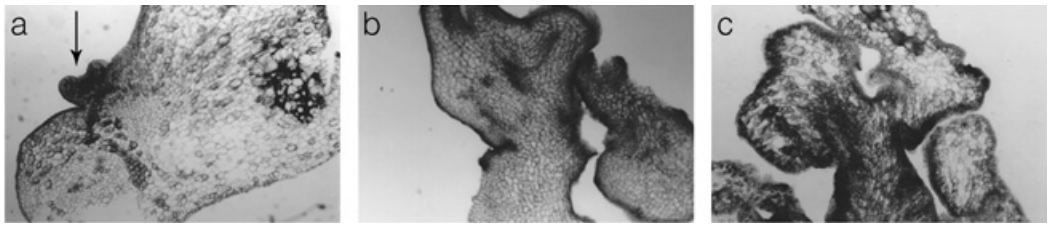

Figure 2. a: section of a somatic embryo at heart stage (indicated by arrow) on B5 medium from zygotic embryo (control), b: sections of cotyledonary embryos developed after infection with wild strain of *A. rhizogenes*, showing root formation, c: sections of cotyledonary embryos developed after infection with wild strain of *A. rhizogenes* showing foliar structures. 40×.

In all treatments, *A. rhizogenes*-mediated mango transformation process was not dependent on the presence of AS, suggesting that in the present system AS did not enhanced activation of *Agrobacterium vir* genes.

All Kent secondary embryos obtained after infection with wild *A. rhizogenes* were small, with a tendency to rosette phenotype (Figure 1a), compared with control embryos, which had normal appearance and larger size, with the same time of culture. These results agreed with those reported by Jasik *et al.* (1997), Jouanin *et al.* (1978) and Tepfer (1984; 1990) who mentioned that apical dominance was reduced or lost in plants transformed using *A. rhizogenes*.

Some mango somatic embryos regenerated into plants with roots, although they did not show typical hairy roots (Figure 1b), as compared to tobacco leaf sections inoculated with the same strain, which formed calli and typical hairy roots. These results agree with Spiral *et al.* (1993) in *Coffea* somatic embryos and Molphe and Ochoa (1998) in lime (*Citrus aurantifolia* Christm) shoots segments inoculated with *A. rhizogenes*. In these two species, indirect and direct embryogenesis took place, respectively, and the new plants did not developed hairy roots.

In histological sections, both transformed and control embryos had defined protodermis, vascular organization, asymmetric form without a defined axis (Fig. 2A, 2B, 2C, 40X magnification). These results are in concordance with DeWald *et al.* (1989b), who reported that non transformed mango somatic embryos were asymmetric. On the other hand, Wu *et al* (2007) induced direct somatic embryogenesis from nucelli and immature cotyledon cuts from mango cv. Zihua, and histological studies revealed that normal symmetric embryos formed from epidermal cells. In papaya, Cabrera *et al* (1995) showed bilateral asymmetry in transformed somatic embryos, using the same system as the present one, but non-transformed embryos were normal.

Confirmation of transformation

In the agarose etidium bromide gels, the expected band (720bp) corresponding to the amplification of rol B gene, was observed in the transformed embryos, while no band was formed from non-transformed embryos (Figure 3).

The successful production of transgenic somatic embryos of Kent variety using *A. rhizogenes* mediated transformation is demonstrated. Twenty clones were produced per 0.5g of inoculated tissue with 80% of survival of the embryos. Using the same mango variety and the biobalistic procedure the *Bar* gene was incorporated and only 3 clones per 0.5g of tissue were obtained with 4% survival (Chavarri *et al.*, 2004). This low efficiency was probably due to the intense herbicide selection applied after the transformation process (Chavarri *et al.*, 2004).

A. rhizogenes transformation method is time consuming, laborious, and seems to be dependent upon the variety used and time of co-culture with the bacterium; nevertheless, standardized procedures can produce a high number of transformed rooted plantlets. In both methods, biobalistic and *A. rhizogenes*,

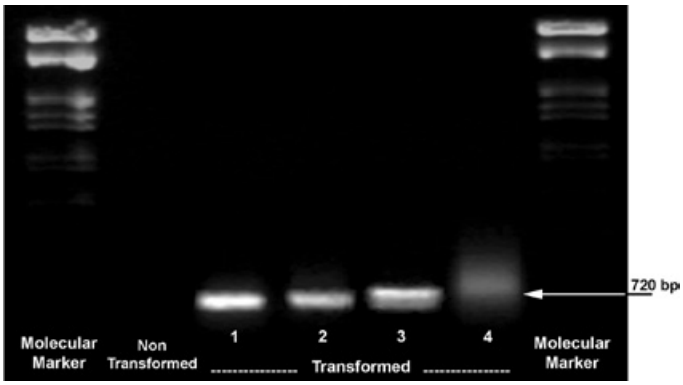

Figure 3. PCR analysis of *Mangifera indica* L. transformed somatic embryos by wild *A. rhizogenes* (lanes 3, 4, 5 and 6) and non-transformed (lane 2). Molecular Marker DNAλ/ *Eco*RI+*Hind* III (M) (lanes 1 and 7). Arrow shows amplified fragments of *rolB* gene (720bp).

only the Kent variety showed positive results. The Haden and Madame Francis varieties did not regenerated transformed somatic embryos through any of the two methods, demonstrating the high dependence of the response on the genotypes involved.

Although mango has been transformed using *A. tumefaciens* (Mathews *et al*., 1993) and particle bombardment (Cruz-Hernandez *et al*., 2000; Chavarri *et al*., 2004), the present results constitute the first report on regeneration of transgenic mango plants from somatic embryos mediated by *A. rhizogenes.*

In conclusion, an efficient system was developed for gene transfer in mango Kent variety using *A. rhizogenes*, regenerating complete plants from somatic embryos, without hairy root formation. Transgenic embryos produced by this system rooted easily in culture medium without growth regulators. This response is probably due to the *rol* genes transferred to mango tissues, which could induce other valuable characters for production of plants. The transgenic lines obtained and reported in this paper must be further evaluated *in vitro* and *in vivo* for the selection of promising commercial varieties of mango.

ACKNOWLEDGEMENTS

The authors are grateful to Tatsuji Sakamoto, Osaka Prefecture University, Japan, for providing the wild type *Agrobacterium rhizogenes*. This research was carried out at the Centro Nacional de Investigaciones Agropecuarias (CENIAP), Instituto Nacional de Investigaciones Agrícolas-(INIA), Maracay, Venezuela, and was supported in part by CDCH-UCV and Fundacite Aragua grant 1995-FCT-01-05-01-4.

REFERENCES

Avilán L, Leal F (1996) *El Comercio Mundial de Frutales y las Perpectivas de la Fruticultura Nacional*. INIA. CENIAP-FONAIAP. Maracay, Venezuela. 36pp.

Altamura MM (2003) *Agrobacterium rhizogenes rolB* and *rolD genes*: regulation and involvement in plant development. *Plant Cell Tiss. Org. Cult. 4718*: 1-13.

Bettini P, Michelotti S, Bindi D, GIannini M, Capuana R, Buiatti M (2003) Pleiotropic effect of the insertion of the *Agrobacterium rhizogenes rolD* gene in tomato (*Lycopersicon esculentum* Mill.) *Theor. Appl. Genet. 107*: 831-836.

Cabrera J, Vegas A, Herrera L (1996) Regeneration of transgenic papaya plants via somatic embryogenesis induced by *Agrobacterium rhizogenes. In Vitro Cell. Dev. Biol. Plant 32*: 86-90.

Chavarri M, Vegas A, Zambrano AY, Demey JR (2004) Transformación de embriones somáticos de mango por biobalística. *Interciencia 29*: 261-266.

Cruz-Hernández A, Litz RE (1997) Transformation of mango somatic embryos. *Acta Hort. 455*: 292-298.

Cruz-Hernández A, Town L, Cavallaro A, Botella J (2000) Transient and stable transformation in mango by particle bombardment. *Acta Hort. 509*: 237-242.

DeWald S, Litz RE, Moore G (1989a) Optimizing somatic embryo production in mango. *J. Amer. Soc. Hort. Sci. 114*: 712-716.

DeWald S, Litz RE, Moore G (1989b) Maturation and germination of mango somatic embryos. *J. Amer. Soc. Hort. Sci.* 114: 837-841.

Draper J, Roderick S, Philop A, Walden R (1988) *Plant Gene Expression. A Laboratory Manual*. Blackwell. Boston, MA, USA. 355 pp.

Filippini F, Rossi V, Marin O, Trovato M, Constantino P, Downey PM, Lo Schiavo F, Terzi M (1996) A plant oncogene as phosphatase. *Nature 379*: 499-500.

Furner IJ, Huffman GA, Amasino RM, Garfinkel DJ, Gordon MP, Nester EW (1986) An *Agrobacterium* transformation in the evolution of the genus nicotiana. *Nature 319*: 422-427.

Gamborg OL, Miller RA, Ojima K (1968) Plant cell cultures. I. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res. 50*: 150-158.

Gómez-Lim MA (1999) Physiology and molecular biology of fruit ripening. In Paredes-López O (Ed.) *Molecular Biotechnology for Plant Food Production*. Technomic. Landcaster, PA, USA. pp 303-342.

Gómez-Lim MA, Litz RE (2004) Genetc transformationof perennial tropical fruits. *In Vitro Cell. Dev. Biol. Plant 40*: 412-449.

Godwin I, Todd G, Ford-Lloyd B, Newbury HJ (1991) The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. *Plant Cell Rep. 9*: 671-675.

Hamill JD, Rounsley S, Spencer A, Todd MJC Rhodes G (1991) The use of the polymerase chain reaction in plant transformation studies. *Plant Cell Rep. 10*: 221-224.

Jasik J, Boggetti, B, Caricato G, Mantell S (1997) Characterization of morphology and root formation in the model woody perennial shrub *Solanum aviculare* Forst expressing rol ABC genes of *Agrobacterium rhizogenes. Plant Sci. 124*: 57-68.

Jouanin L, Vilaine F, Tourneurt C, Pautot V, Muller JF, Caboche M (1978) Transfer of 4,3-kb fragment of the TL-DNA of *Agrobacterium rhizogenes* strain A4 confers the pRi-transformed phenotype to regenerated tobacco plants. *Plant Sci. 53*: 53-63.

Lin J, García N, Kuo J (1994) Effects of *Agrobacterium* cell concentration on the transformation efficiency of tobacco and Arabidopsis thaliana. *Focus 16*: 72-77.

Lizada C (1993) Mango. In Seymour G, Taylor J, Tucker G (Eds.) *Biochemistry of Fruit Ripening*. Chapman & Hall. London, UK. pp. 255-271.

Marsilio S, Tornielli GB, Pomponi M, Pezzotti M, Constantino P, Cardarelli M (2001) Effect of rolB gene of *Agrobacterium rhizogenes* on gamete development and embryogenesis in model species. *2nd Int. Apomixis Conf.* APO2001. Italy. Abstracts, p. 52.

Mathews H, Litz RE (1992) Mango. In Hammerschlag FA, RE Litz RE (Eds.) *Biotechnology of Perennial Fruit Crop Species*. CABI. Wallingford, UK. pp 433-448.

Mathews H, Wilde HD, Litz RE, Wetzstein HY (1993) Genetic transformation of mango. *Acta Hort. 341*: 93-97.

Molphe E, Ochoa A (1998) Regeneration of transgenic plants of Mexican lime from *Agrobacterium rhizogenes*-transformed tissue. *Plant Cell Rep. 17*: 591-596.

Murashige T, Skoog F (1962) A revised medium for a rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant. 15*: 473-497.

Rivera M (2006) La biotecnología en plantas y aspectos biotecnológicos del mango. *Interciencia 31*: 95-100.

Salázar E (1997) La propagación *in vitro* de plantas. En *1er Encuentro de Productores con la Biotecnología*. Maracaibo. Venezuela. pp. 53-65.

Scorza R, Zimmerman TW, Cordts JM, Footen KJ (1998) Horticultural characteristics of transgenic tobacco expressing the *rolC* gene from *Agrobacterium rhizogenes. J. Am. Soc. Hort. Sci. 119*: 1091-1098.

Schmülling T, Schell J, Spena A (1989) Promoters of the *rolA, B* and *C* genes of *Agrobacterium rhizogenes* are differentially regulated in transgenic plants. *Plant Cell 1*: 665-670.

Shih-Cheng C, Hui-Wen L, Kung-Ta L, Takashi Y (2007) High-efficiency *Agrobacterium rhizogenes*-mediated transformation of heat inducible sHSP18.2-GUS in *Nicotiana tabacum*. *Plant Cell Rep. 26*: 29-37.

Spiral J, Thierrey C, Paillard M, Petiard V (1993) Obtention de plantules de *Coffea canephora* Pierre (robusta) transformées par *Agrobacterium rhizogenes. C. R. Acad. Sci. Par. t.316, Ser. III.* pp 1-6.

Tepfer D (1984) Transformation of several species of higher plants *Agrobacterium rhizogenes*: sexual transmissions of the transformed genotype and phenotype. *Cell 37*: 959-967.

Tepfer D (1990) Genetic transformations using *Agrobacterium rhizogenes. Physiol. Plant. 79*: 79-146.

Van Alvorst AC, Bino RJ, Van Dijk AJ, Lamers AM, Lindhout WH, Van Der Mark F, Dons JJ (1992) Effects of the introduction of *Agrobacterium rhizogenes rol* genes on tomato plant and flower development. *Plant Sci. 83*: 77-85.

Welander M, Zhu M (2006) *Rol* genes: Molecular biology, physiology, morphology. *Breed. Rev. 26*: 79-103.

Wu Y, Huang X, Chen Q, Li X, Engelmann F (2007) Inducción and cryopreservation of embryogenic cultures from nucelli and immature cotyledon cuts of mango (*Mangifea indica* L. var Zihua). *Plant Cell Rep. 26*: 161-168.

Zambrano AY, Demey JR, Martínez G, Fuenmayor F, Gutiérrez Z, Saldaña G, Torrealba M (2002) Método rápido, económico y confiable de minipreparación de ADN para amplificaciones por RAPD en bancos de germoplasma. *Agron. Trop. 52*: 235-243.