Rev. Brasil. Genet. 18, 1, 115-119 (1995)
(Brazil. J. Genetics)

SHORT COMMUNICATION:

Plant transformation mediated by *Agrobacterium rhizogenes*: optimization of the infection process

Luciano Cônsoli, Salete Aparecida Gaziola and Maria Lúcia Carneiro Vieira

ABSTRACT

Because of its ability to transfer part of its R_i plasmid to plant genomes, *Agrobacterium rhizogenes* can be used as a vector for the transfer of genes of interest. The objective of this study was to determine factors for the optimization of the process of plant tissue infection in two species, *Carica papaya* L. and *Centrosema pubescens* Benth. Liquid cultures of the wild *A. rhizogenes* strains LBA 9402, A_4T and 8196 were used to inoculate the explants, obtained 20 days after germination. Three methods were used for explant inoculation: a scapel, a hypodermic needle, and a platinum loop. Strain LBA 9402 was highly efficient in infecting papaya and *C. pubescens* explants, whereas strains A_4T and 8196 only infected *C. pubescens* explants. For both cultures, inoculation methods that promoted wounds in the plant tissue (scalpel and needle) were more efficient than the platinum loop method. Papaya hypocotyl segments were more responsive to infection than leaf and cotyledon explants, with 61% of explants presenting on average 3.9 roots/explant. *C. pubescens* leaf and epicotyledon explants gave similar responses (4.33 and 3.35 roots/explant, with a 54% and 77% rate of explant infection, respectively). When a growth curve was constructed for strain LBA 9402, plotting culture growth phase against infecting ability of the bacteria, the bacteria was found to present a greater ability to infect hypocotyledon and leaf explants of *C. papaya* and *C. pubescens*, respectively, at the end of the log phase (T24-T30).

INTRODUCTION

Agrobacterium rhizogenes is a soil bacterium which promotes the formation of adventitious roots in plant tissues at the site of infection. This ability of the microorganism is coded for by a plasmid, R_i (Moore *et al.*, 1979) that transfers part of its DNA, T-DNA, to the plant genome (White *et al.*, 1982). Root production by the transformed plant cells is related to genes denoted **rol** present in the R_i plasmid (Walden, 1988).

Once it is incorporated into the plant genome, plasmid R_i confers to the transformed tissues the potential ability to synthesize agropine and manopine type opines, depending on the *A. rhizogenes* strain used (Petit *et al.*, 1983; Melchers and Hooykaas, 1987).

Departamento de Genética, ESALQ-USP, Caixa Postal 83, 13418-900 Piracicaba, SP, Brasil. Send correspondence to M.L.C.V.

MATERIAL AND METHODS

Bacterial strains and growth curve

Liquid cultures of the wild strains LBA 9402, 8196 and A_4T of *A. rhizogenes* obtained from EMBRAPA (CENARGEN, Brasília, Brazil) were used to infect explants of *Carica papaya* and *Centrosema pu-*

bescens. Bacterial cultures were established in liquid APM medium (Tepfer and Casse-Delbard, 1987) with orbital shaking at 150 rpm and at 28°C for 16 hours.

A growth curve was constructed for strain LBA 9402 according to the method proposed by Azevedo and Neder (1963). The growth curve was started with an inoculum of approximately 3.6 x 10^4 bacteria/ml, estimated by counting in a Neubauer chamber. Culture samples (1 ml) were removed at 3-hour intervals, diluted in 9 ml liquid APM medium and plated onto three Petri dishes containing solid APM medium (1.5% agar, Difco, w/v). Isolated colonies were obtained from viable cells present in the culture and used for the determination of the number of bacteria/ml present at each culture growth time.

Inoculation of hypocotyledon explants of *C. papaya* (10-14/culture time) and of leaf explants of *C. pubescens* (8 or 10/culture time) was performed by removing aliquots of the original culture at time zero (T0) and at 3-hour intervals, starting from time nine hours.

Explant production

Papaya explants were obtained from plants germinated *in vitro*. The seeds were removed from fruits previously sterilized in 2% NaOCl (v/v) for 30 minutes. The sarcotesta was removed under aseptic conditions. After rinsing in sterilized water, the seeds were inoculated into basic solid MS medium (Murashige and Skoog, 1962) containing 0.9% agar (w/v), 0.5% sucrose (w/v) and 2 mg/l GA_3.

C. pubescens seeds were obtained from the Instituto de Zootecnia de Nova Odessa (Nova Odessa, Brazil). After manual scarification, the seeds were sterilized in 70% ethanol for 40 seconds and in 2% NaOCl (v/v) for 10 minutes and then inoculated into B50 medium (Gamborg *et al.*, 1968).

The seeds germinated under 30 µmol/m^2/s luminous radiation at 27 ± 2°C, with a photoperiod of 16 hours.

Explant inoculation

Leaf and cotyledon explants and hypocotyl and epicotyl segments were excised from axenic plantlets 20 days after germination. The explants were inoculated with the liquid cultures of *A. rhizogenes* by three different methods: 1) a surgical scalpel immersed into the bacterial culture, used to wound the lower epidermis of leaves and cotyledons; 2) a 28-G needle of a plastic syringe containing 1.0 ml bacterial culture used to wound the hypocotyl and epicotyl segments; and 3), alternatively, a platimum loop used to touch the upper end of the hypocotyl and epicotyl segments with the liquid culture.

After inoculation, the explants were placed on filter paper and then immediately plated on half-strength solid MS medium (0.9% agar, w/v), containing 1.5% sucrose, free from phytoregulators and on B50 medium for *C. papaya* and *C. pubescens* cultures, respectively.

The experiment was evaluated 15 days after inoculation by counting the number of adventitious roots per explant.

RESULTS AND DISCUSSION

Bacterial growth curve and infecting ability

Figure 1 shows the bacterial growth curve constructed for strain LBA 9402. The curve showed an initial lag phase (T0-T9) followed by a bacterial growth phase, log (T12-T30), and a third stationary phase (from T30 on). A relationship between growth phase and infecting ability of the bacterium was observed, as measured by the mean number of roots transformed per infected explant (Table I). The data demonstrated that the log phase was ideal for explant infection as this phase gave the best infection rate for the two plant species. A reduction of the infecting ability occurred during the stationary phase.

Explant infection

Figure 2 shows the rate of infection by the three *A. rhizogenes* strains of *C. papaya* and *C. pubescens*

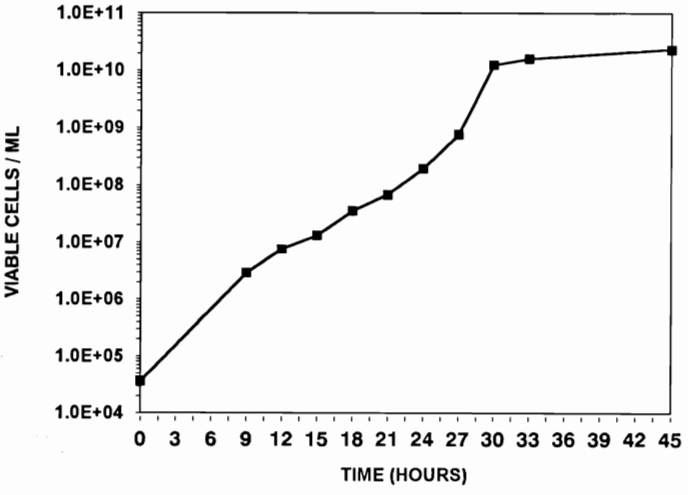

Figure 1 - Growth curve of strain LBA 9402 of *Agrobacterium rhizogenes* from an initial inoculum of 3.6 x 10^4 cells/ml.

Table I - Association between the growth phase of *Agrobacterium rhizogenes* strain LBA 9402 and its ability to infect leaf explants of *Centrosema pubescens* and hypocotyledon explants of *Carica papaya*.

Phase of growth (hours)	Mean number of roots per infected explant*	
	C. pubescens	*C. papaya*
T0-T9	1.60 ± 0.30	4.00**
T12-T30	3.27 ± 0.47	3.70 ± 0.32
T33-T45	2.89 ± 0.70	2.33 ± 0.88

*Mean ± standard deviation of the mean.
**Adventitions root formation observed in one explant.

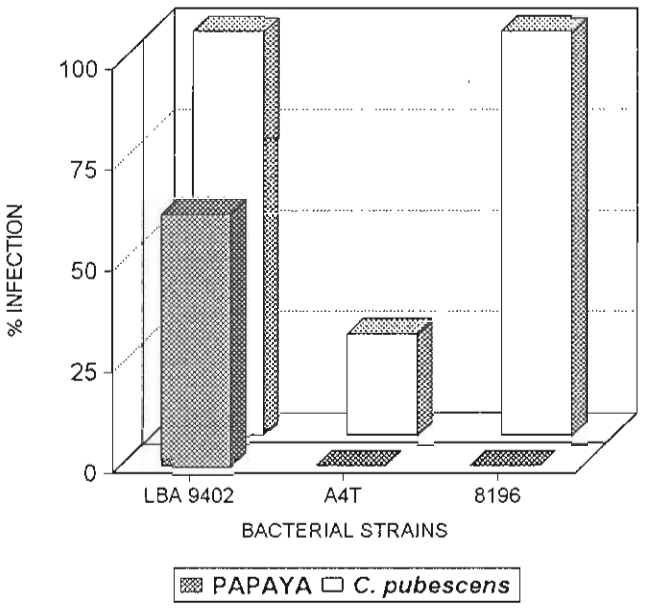

Figure 2 - Percentage of papaya hypocotyledon explants and of *Centrosema pubescens* leaf explants infected with *Agrobacterium rhizogenes* strains.

explants. Only strain LBA 9402 showed a high infecting ability for the two plant species (62.5% for *C. papaya* and 100% for *C. pubescens*). Strain 8196 presented high infecting efficiency for the *C. pubescens* culture only (100% infection).

A comparison of the methods used to inoculate the hypocotyl and epicotyl segments (a platinum loop or wounds induced with a needle or a surgical scalpel) is illustrated in Figure 3. The methods which involved the wounding of explant tissues favored the formation of a larger number of roots (3.91 ± 0.44 roots/explant for *C. papaya* and 3.25 ± 2.22 roots/explant for *C. pubescens*), as well as a higher percentage of infected explants (61% for *C. papaya* and 77% for *C. pubescens*). When the platinum loop was used, the mean number of roots/explant was 2.0 ± 0.71 for *C. papaya* and 1.8 ±

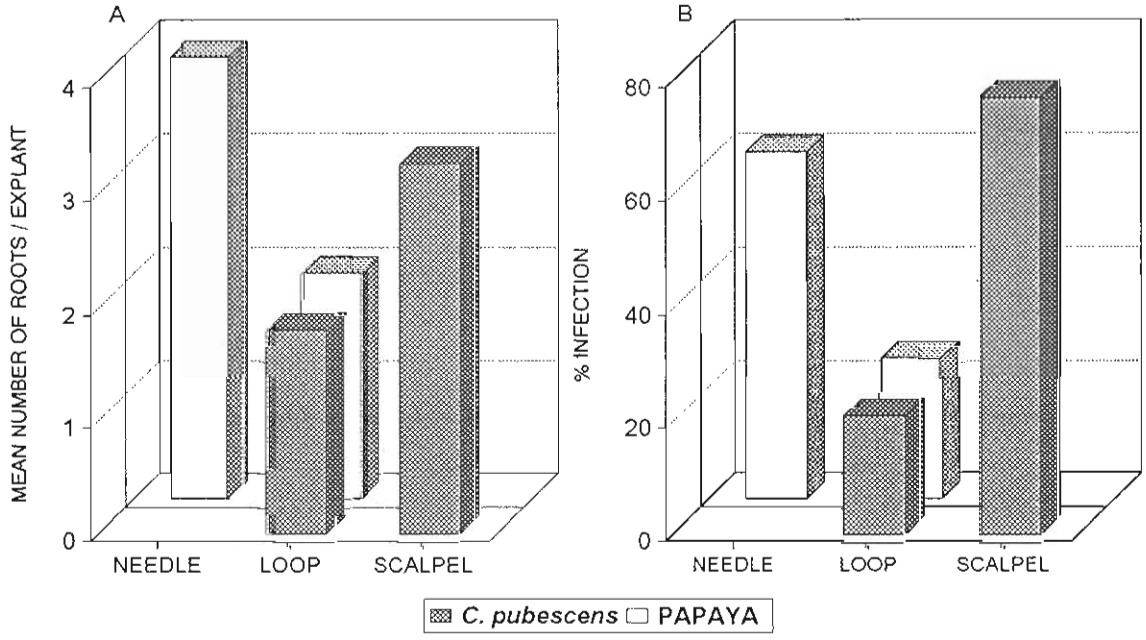

Figure 3 - A, Mean number of adventitious roots and B, percent infection in papaya hypocotyledon explants and *Centrosema pubescens* epicotyledon explants infected with strain LBA 9402 using a needle, a surgical scalpel or a platinum loop to induce infection.

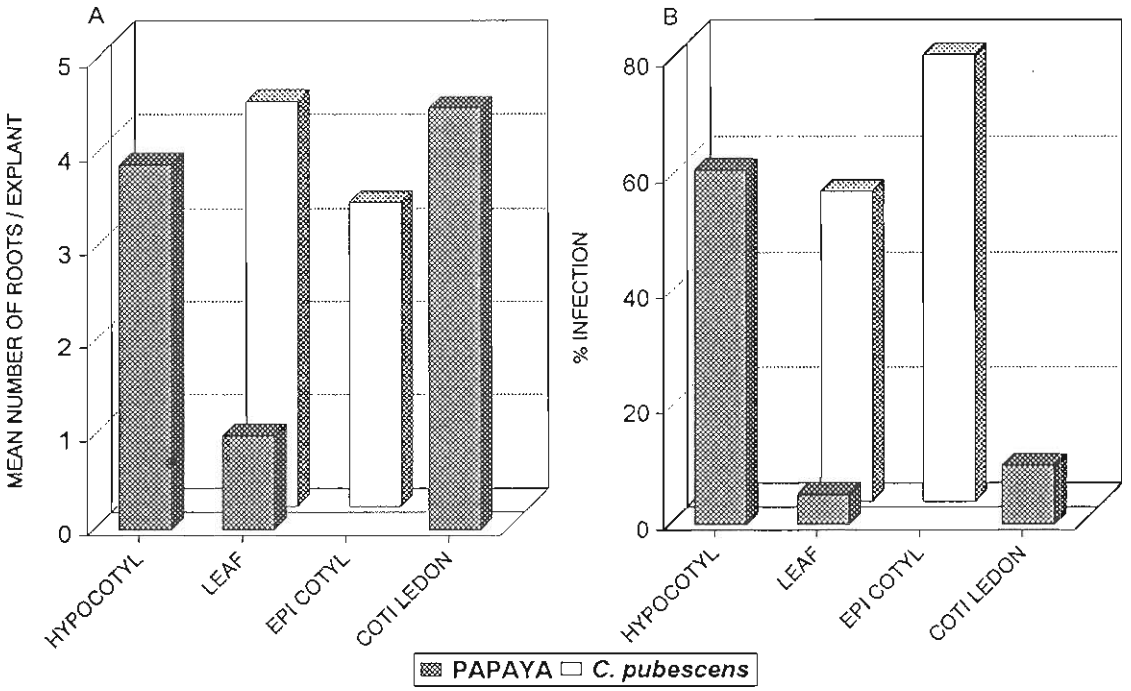

Figure 4 - A, Mean number of adventitious roots per explant and B, percent infection in papaya hypocotyledon , leaf and cotiledon explants and *Centrosema pubescens* epicotyledon and leaf explants infected with strain LBA 9402 of *Agrobacterium rhizogenes*.

0.45 for *C. pubescens*, with a 25% rate of infection of *C. papaya* explants and a 21% rate of infection for *C. pubescens* explants. The wounds ease bacterial access and also induce the appearance of sugars and specific phenolic compounds (acetosyringone, syringone) that stimulate the process of bacterial T-DNA transfer by inducing the expression of some genes present in the region of virulence of plasmid R_i (Lindsey and Jones, 1989).

The infecting ability of strain LBA 9402 is illustrated in Figure 4. The *C. pubescens* culture presented similar responses in terms of mean number of adventitious roots for leaf explants (4.33 ± 3.50) and epicotyledon explants (3.25 ± 2.22). The hypocotyl segments of *C. papaya* were more responsive (3.91 ± 0.44) with a 61.1% rate of infection compared to leaf and cotyledon explants, which presented a rate of less than 10%.

We noted that other factors affect the efficiency of the infection process in addition to bacterial strain and plant species used. It is first necessary to determine the source of explants and the infecting method to be used in order to obtain a reasonable number of possible transformed roots. Another important factor is the determination of the ideal concentration of the bacterial culture at the time of explant infection, taking care to prevent the culture from reaching the stationary phase.

ACKNOWLEDGMENTS

We are grateful to C.A. de Oliveira for technical support, and to M.H.P. Fungaro and J.O. Pereira for helping in the construction of the growth curve.

Research supported by CNPq. Publication supported by FAPESP.

RESUMO

O *Agrobacterium rhizogenes* apresenta a capacidade de transferir parte de seu plasmídio R_i para o genoma da planta, e por isso é utilizado como vetor para a transferência de genes de interesse. Este trabalho teve como objetivo a determinação de fatores para a otimização do processo de infecção do tecido vegetal em duas espécies, *Carica papaya* L. e *Centrosema pubescens* Benth. Culturas líquidas selvagens de *A. rhizogenes* LBA 9402, A_4T e 8196 foram utilizadas para a inoculação dos explantes com 20 dias de idade após a germinação. Foram utilizados três métodos para a inoculação dos explantes: bisturi, agulha de seringa plástica e alça de platina. A linhagem LBA 9402 apresentou alta eficiência na infecção dos explantes de mamão e de *Centrosema*, enquanto as linhagens A_4T e 8196 infectaram apenas explantes de *Centrosema*. Para as duas culturas, os métodos de inoculação que promoveram

ferimentos no tecido vegetal (bisturi ou agulha) apresentaram maior eficiência quando comparados com o método da alça de platina. Os segmentos de hipocótilo de mamão apresentaram-se mais responsivos à infecção, com 61% dos explantes apresentando em média 3,9 raízes/explante. Já para *Centrosema*, os explantes foliares e epicotiledonares apresentaram respostas semelhantes (4,33 e 3,35 raízes/explante com 54% e 77% de explantes infectados, respectivamente. Foi elaborada uma curva de crescimento para a linhagem LBA 9402, associando a fase de crescimento da cultura com a capacidade de infecção da bactéria. Verificou-se que no final da fase log (T24-T27) a bactéria apresentou maior capacidade de infecção dos explantes hipocotiledonares de mamão e foliares de *Centrosema*.

REFERENCES

Azevedo, J.L. and **Neder, K.N.** (1963). *Manual de Genética de Bactérias*. Publicação didática. Instituto de Genética, ESALQ/USP, Piracicaba, pp. 81.

Gamborg, O., Miller, R.A. and **Ojima, K.** (1968). Nutrient requirement of suspension cultures of soybean root cells. *Exp. Cell Res. 50*: 151-158.

Lindsey, K. and **Jones, M.G.K.** (1989). The cell biology of genetic engineering. In: *Plant Biotechnology in Agriculture*. Open University Press, Milton Keynes, pp. 241.

Melchers, L.S. and **Hooyakaas, P.J.J.** (1987). Virulence of *Agrobacterium*. In: *Oxford Surveys of Plant Molecular and Cell Biology* (Miflin, B.J., ed.). Oxford University Press, New York, vol. 4, pp. 167-220.

Moore, L., Warren, G. and **Strobel, G.** (1979). Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes. Plasmid 2*: 617-626.

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

Tepfer, M. and **Casse-Delbart, F.** (1987). *Agrobacterium rhizogenes* as a vector for transforming higher plants. *Microbiol. Sci. 4*: 24-28.

Petit, A., David, C., Dahl, G.A., Ellis, J.G., Guyon, P., Casse-Delbart, F. and **Tempé, J.** (1983). Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Mol. Gen. Genet. 190*: 204-214.

Walden, R. (1988). *Genetic Transformation in Plants*. Open University Press, Milton Keynes, pp. 138.

White, F.F., Guidossi, G., Gordon, M.P. and **Nester, E.W.** (1982). Tumor induction by *Agrobacterium rhizogenes* involves the transfer of plasmid DNA to the plant genome. *Proc. Natl. Acad. Sci. USA, 79*: 3139-3197.

(Received November 11, 1993)