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Effect of *Agrobacterium rhizogenes* infection on *in vitro* rooting of *Vitis vinifera*

T. M. Martins, A. Domingos, C. Novo and P. M. L. Lourenço

Departamento de Biotecnologia, Instituto Nacional de Engenharia e Tecnologia Industrial, Lisboa, Portugal

Summary

Agrobacterium rhizogenes **is known to induce the proliferation of rapid growing, highly branching roots (hairy roots) in most dicotyledonous plants. We report here the effect of *in vitro* infection of *Vitis vinifera* with two *A. rhizogenes* strains (β-glucuronidase transgenic A4 and wild type LBA), with regard to an increase of root mass.**

In vitro**-grown *V. vinifera* explants were infected with two *A. rhizogenes* strains. Both strains induced a significant increase in the number of the developed roots, and of their weight and length. Root number was increased by a factor of 2.9 (strain A4) and 2.7 (strain LBA), length increased 1.9 and 1.6 times (strains A4 and LBA, respectively), while the root weight was more affected by strain A4 (2.8 times increasing) than by LBA strain (1.9 times increasing).**

The transformation status of the developed roots was assessed by two different methods: PCR detection of *rol*B gene in LBA strain-derived roots and quantification of â-glucuronidase activity in A4 strain-derived roots.

K e y w o r d s : *Vitis vinifera*, *Agrobacterium rhizogenes*, rooting, *in vitro* culture.

Introduction

Grapevine (*Vitis vinifera)* is generally propagated by hardwood cuttings. Since grapevines are heterozygous, and since many of its important characters are polygenic, there are difficulties in breeding. Thus non-conventional approaches, including genetic engineering, have been proposed (Guellec *et al*. 1990).

Efficient transformation and regeneration methods are a prerequisite for successful genetic engineering of vegetative propagated plants such as grapevine. To date, regeneration of transgenic grapes has been achieved by both organogenesis and embryogenesis (Mozsar *et al*. 1998, Mezzetti *et al*. 2002). The response of *V. vinifera* to micropropagation and regeneration (in particular the rooting efficiency) is known to vary between cultivars (Péros *et al*. 1998). In order to obtain fully functional regenerated plants, adequate rooting of explants must be achieved. In addition, a well developed root system favours *ex vitro* acclimatization.

The soil bacterium *Agrobacterium rhizogenes* is responsible for the development of hairy root disease in a wide range of dicotyledonous plants. In most cases, these hairy roots have a rapid, highly branching growth on hormone-free media and altered geotropic development (Tepfer and Casse-Delbart 1987).

This phenotype is caused by DNA from *A. rhizogenes* (T-DNA) that is inserted into the nuclear genome of host cells. T-DNA from agropine strains of *A. rhizogenes* is constituted by the two non-contiguous regions TL and TR, both of which can be transferred to plant hosts, individually or together, in a variable number of copies. In TL DNA 4 *loci* (*rol*A, *rol*B, *rol*C, *rol*D) were identified being responsible for the hairy root phenotype, whereas TR DNA shows high homology to T-DNA from *A. tumefaciens* (Tepfer and Casse-Delbart 1987).

A. rhizogenes has been used to improve rooting in a wide range of plants, both *in vivo* and *in vitro*, specially in woody species the root initiation of which is a limiting factor in vegetative propagation (Caboni and Carmini 1994).

A fully functional *A. rhizogenes*-induced root system with an increased root mass could lead to higher water and nutrient uptake and therefore improve plant regeneration and acclimatization.

We report here the effect of the infection with two *A. rhizogenes* strains on root mass of *V. vinifera*.

Material and Methods

Shoot cultures of *V. vinifera* (cv. Ramisco) micropropagated *in vitro* were used in this experiment. The plants were multiplied in modified MS-medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose without phytohormones. Cultures were maintained at 25 °C in a 16 h photoperiod, light intensity 37 µmol m^{-2} s^{-1}.

Before transformation, the cultures of *A. rhizogenes* strain LBA9402 with plasmid pRi1850 (LBA) and strain A4 with plasmid pRiA470Gus (A4) were grown for 48 h at 28 °C, 80 rpm on liquid YMB medium (Hooykaas *et al*. 1977). Microcuttings (length: 1.5-2.0 cm) bearing two leaves, were prepared from 8-week-old shoot cultures. Infections were carried out by dropping bacterial suspension on the fresh wounds at the basal part of the plant material. Infected plants were co-cultivated for 48 h on MS medium without phytohormones and antibiotics. After co-cultivation the explants

Correspondence to: Dr. A. Domingos, Departamento de Biotecnologia, Instituto Nacional de Engenharia e Tecnologia Industrial, Est. do Paço do Lumiar, 1649 038 Lisboa, Portugal. Fax: +351-217-163-636. E-mail: ana.domingos@ineti.pt

were transferred to the same medium containing the antibiotic cefotaxime (500 mg l^{-1}). Uninfected shoots (control) were cultured under the same conditions, except for the inoculum that was substituted for YMB liquid medium.

The experiment was carried out using 15 *V. vinifera* microcuttings as control plants, 29 infected microcuttings with *A. rhizogenes* strain LBA and 27 infected microcuttings with strain A4. The number of roots per plant, the weight of roots and the length of the roots were measured, 40 d after infection.

PCR detection: Genomic DNA used as template on PCR was isolated from roots excised from infected and uninfected plants. The small-scale plant genomic DNA isolation was based on the method described by Rogers and Bendich (1988). The primers used for amplification of *rol*B gene were 5'-ATGGATCCAAATTGCTATTCCTTCC-ACGA-3' and 5'-TTAGGCTTCTTTCTTCAGGTTTACTGC-AGC-3', according to the sequence data of the entire *rol*B gene from *A. rhizogenes* strain LBA9402. The primers used for amplification of *vir*D1 gene were 5'-ATGTCGCAAGGAC-GTAAGCCCA-3' and 5'-GGAGTCTTTCAGCATGGA-GCAA-3' (Hamill *et al*. 1991). PCR was performed in 20 µl reaction volume using 10-300 ng of genomic DNA as template.

GUS assay: All the assays were performed in the whole roots of each plant. Tissues were extracted by freezing (liquid nitrogen) and grinding in a microfuge tube. When a fine powder was obtained, 300 µl GUS buffer (50 mM NaH_2PO_4, pH 7.5; 1 mM EDTA; 0.1 % (v/v) Triton X-100; 10 mM β-mercaptoethanol) was added, and extracts were further homogenised (Topping and Lindsey 1997). The fluorogenic reaction was carried out in 1 mM MUG (4-methyl umbelliferyl glucuronide) GUS buffer with 100 ìl of extract, for a total volume of 1 ml. The fluorescence of MU (4-methyl umbelliferone) was measured on a Hitaschi 3000 spectrofluorometer (excitation at 365 nm, emission at 455 nm, slit widths set at 1.5 nm).

Results and Discussion

Most of the microcuttings treated with both *A. rhizogenes* strains developed roots 8 d after infection. In some of the cuttings *calli* formation was observed in the infection zones. In control experiments the first roots developed only 15 d after infection and *calli* formation was not observed. Roots developed in the *A. rhizogenes*-infected plants were in general thicker than those of control plants. Unlike what is commonly observed in agropine strains-derived roots, in our experiments roots were not able to grow *in vitro* like typical hairy roots (Figure). Similar results were previously described by Guellec *et al*. (1990).

The experimental data showed that all morphological characters in study (number, total weight and total length of the roots) were higher in infected plants than in controls (average values in the Table). The root number in our control is similar to what was previously reported for several *V. vinifera* cultivars (Péros *et al*. 1998)

Although the average, maximum and minimum values of every studied character were higher in A4- than in the LBA-infected plants, the two results are not distinguishable in statistical terms, due to the high standard deviation. The infected plants showed a wider range of values, possibly reflecting the fact that they were not evenly transformed by *A. rhizogenes*. Some roots might not be transgenic while others might derive from cells in which T-DNA was integrated to various degrees.

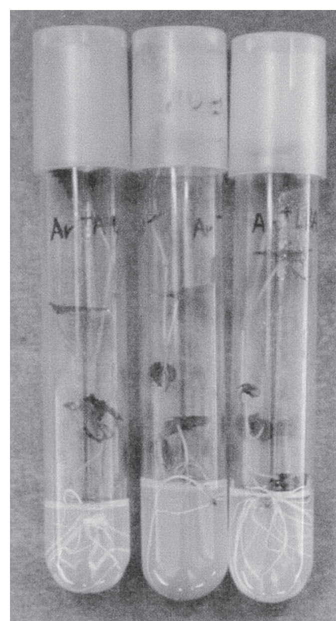

Figure: Plants 30 d after infection with *A. rhizogenes*. A4 (left), control (center) and LBA (right).

Table

Effects of hairy root induction by infection of *V. vinifera* with *A. rhizogenes* strains LBA9402 and A4 on root growth. Average: Arithmetic average of all obtained values for each parameter; SD: Standard deviation; Max: Maximum value; Min: Minimum value

	Control			A4			LBA		
	root number	weight (mg)	length (cm)	root number	weight (mg)	length (cm)	root number	weight (mg)	length (cm)
Average	3.6	58.4	32.4	10.4	161.1	61.8	9.6	112.2	52.3
SD	1.6	48.8	17.7	5.0	130.4	66.7	4.2	65.9	31.2
Max	7.0	192.6	55.5	26.0	479.6	256.8	18.0	248.5	129.5
Min	1.0	11.9	9.5	5.0	26.6	6.1	2.0	18.0	12.0

The average root number was the most strongly affected parameter showing an increase by a factor of 2.9 (strain A4) and 2.7 (strain LBA) while average length increased 1.9 and 1.6 times (A4 and LBA, respectively). Regarding the average root weight the positive effect of *A. rhizogenes* transformation was more pronounced with strain A4 (2.8 times) than with LBA (1.9 times).

Compared with the maximum control value (7 roots), a higher root number was found in 85 % of the plants infected with A4 strain and 72 % infected with LBA. The weight of 37 % of *A. rhizogenes* A4-infected plant roots was above the maximum value of controls (192.6 mg) whereas 20 % of LBA- infected were in that situation. In terms of total length of the roots 30 % of A4-infected and 14 % of LBA-infected plants showed an increased value compared with the maximum of controls (55.5 cm).

In the wild type *A. rhizogenes* the *rol*B gene is one of the genes transferred to the host in the infection process that is considered to be responsible for positively affect rooting. For that reason the confirmation of its presence in the host DNA by PCR, using appropriate primers, could be a proof of transformation. Because it was not possible to ascertain the effective elimination of the bacteria, a non-transferable *A. rhizogenes* gene (v*ir*D1 gene) was chosen to assess the presence of bacterial DNA, and to screen for false positives, as described by HAMILL *et al*. (1991). The PCR amplification of the *rol*B gene in the plants of the group infected with LBA strain of *A. rhizogenes* led to 63 % positive results. These data roughly mirror what was obtained in terms of root number but do not match the length and weight results. In all positive results v*ir*D1 gene PCR amplification was observed, showing that *A. rhizogenes* was present, making inconclusive the evaluation of the root transformation status. DAMIANO *et al*. (1998) obtained a similar result. The best way to obtain reliable results using PCR amplification of transferred genes is by the complete elimination of bacteria. In most cases the transfer after infection of plant material to an antibiotic-containing medium is not sufficient to eliminate the bacteria during the experiment.

Although most of the intensively growing roots corresponded to positive plants, in some cases positive roots showed poor growth parameters while in other cases high vigour was not identified as positive. The fact that *A. rhizogenes* might be present even in non-transformed plants, may explain the PCR positive plants with poor root growth.

A number of marker enzymes such as β-glucuronidase have been used extensively in plants. These markers are extremely useful in transgene expression studies. The A4 *A. rhizogenes* strain used in these experiments bear the β-glucuronidase gene controlled by the 35S eukaryotic promoter (JEFFERSON *et al*. 1986), which is transferred and expressed in host plants, allowing another method to confirm transformation. To detect the presence of this gene and thus to confirm that plants were transgenic, β-glucuronidase was extracted from transformed and untransformed (control) tissues.

The results of GUS assays showed that 60 % of the A4-infected plants were expressing the β-glucuronidase gene. The extracts from control plant roots did not show any activity. This method is potentially free of bacterial interference, taking into account that the gene promoter is only effective in eukaryotic systems. Therefore, the positive results should correspond to transformed plants.

Considering that complete bacteria elimination was not achieved during experiments, the GUS assay seems to be the best method for evaluate successful *A. rhizogenes* transformation. The PCR-based method has shown to be dependent on the contamination level of the samples, leading to false-positive results that would not offer correct information on the transformation status.

While the benefits of *A. rhizogenes* infection on *V. vinifera in vitro* rooting are obvious its effect on the development of the whole plant should be assessed. Since the transformation is supposed to be confined to the infected area, it is not expected for the plant remains to be genetically affected although they could benefit from an improved water and nutrient uptake.

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