EJB Electronic Journal of Biotechnology ISSN: 0717-345
Vol.1 No.2, Issue of August 15, 1998.
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Invited review paper/ Received 20 October, 1998.

RESEARCH ARTICLE

In vitro fruit trees rooting by *Agrobacterium rhizogenes* wild type infection

Carmine Damiano[1]

Fruit Trees Research Institute, 00040 Ciampino Aeroporto - Roma, Italia.
Financial support: National Plan for Plant Biotechnology, Ministry of Agriculture
Fruit Trees Research Institute, 00040 Ciampino Aeroporto - Roma, Italia.
Tel + 39 06 7934811- Fax +39 06 79340158
E-mail: isf.propag@mclink.it

Simona Monticelli

Fruit Trees Research Institute, 00040 Ciampino Aeroporto - Roma, Italia.
Financial support: National Plan for Plant Biotechnology, Ministry of Agriculture
Fruit Trees Research Institute, 00040 Ciampino Aeroporto - Roma, Italia.
Tel + 39 06 7934811- Fax +39 06 79340158

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***Agrobacterium rhizogenes* infection at the base of microcuttings *in vitro* can improve the rooting of some fruit species. A study was carried out comparing rooting of almond, apple, plum, *Pyrus pyraster* and two hybrid rootstocks, when infected with *A. rhizogenes* strain 1855, with and without the addition of hormones. Three responses occurred: genotypes rooted without auxins; genotypes rooted only with auxins; genotypes rooted only after infection. All genotypes rooted after bacterial infection. In the first group the auxins increased rooting percentages. No substantial differences were found with and without infection in hormone free media, while the rooting percentages tended to decrease with the combination auxin/infection. In the second group, infection on hormone free media increased rooting; in addition there was a synergistic effect between auxins and infection in pears. In the third group, only infection induced rooting. A random sample of roots obtained from infection was molecularly analysed. Amplification of the sequences of *rolB* and *vir* genes was done using PCR. Roots non-transgenic and confirmed as transgenic were 67 % and 6.8 % respectively. In the remaining 26.2% both genes appeared, thus it was not possible to confirm their transgenic nature. Some microcuttings showed both transformed and non-transformed roots.**

Abbreviations: BAP, 6-benzylaminopurine; IAA, indoleacetic acid; NAA, αnaphthaleneacetic acid; IBA, indole-3-butyric acid; GA_3, gibberellic acid; PCR, polymerase chain reaction; EtBr, ethidium bromide; YMB, yeast-extract mannitol broth; MS, Murashige and Skoog medium; SH, Schenk and Hildebrandt ; QL, Quoirin and Lepoivre.

Many woody plants, economically important for timber and/or fruit production, are often difficult to root, both in conventional and *in vitro* propagation. In some cases, it was possible to improve *in vitro* rooting with hormone application, etiolation, or the use of polyamines (Damiano *et al.*, 1991; Rugini *et al.*, 1993). However, the difficulty of rooting is still one of the major obstacles to successful micropropagation. Recently, many attempts to overcome this problem have been carried out on fruit trees and woody species using *Agrobacterium rhizogenes*. This is a soil gram-negative bacterium that induces adventitious root formation at the site of infection in a large number of plants (Chilton *et al.*, 1982). 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 plant genome. Four loci involved in the root formation were identified in the T-DNA, and called root loci (*rol*) A, B, C, D (Spena *et al.*, 1987).

Several authors reported successful rooting using *Agrobacterium rhizogenes*-mediated transformation in fruit trees, such as almond (Damiano *et al.*, 1995), apple (Sutter and Luza, 1993), kiwi (Rugini *et al.*, 1991), walnut (Caboni *et al.*, 1996), and in woody plants, such as the genus *Pinus* (McAfee *et al.*, 1993; Magnussen *et al.*, 1994; Mihalievic *et al.*, 1996; Burns and Schwarz, 1996; Tzfira *et al.*, 1996), *Larix* (McAfee *et al.*, 1993), *Eucalyptus* (MacRae and Van Staden, 1993). The transformation of microcuttings and seedlings by *A. rhizogenes* seems to be an excellent way to improve rooting in trees. The aim of this work is to determine the ability of *A. rhizogenes* to induce root formation on different species and genotypes of micropropagated fruit trees, both recalcitrant and easy-to-root, in the presence or absence of rooting hormones, and to evaluate the nature of these adventitious roots in order to achieve a successful transfer to the field.

Materials and Methods

Plant material. *In vitro* micropropagated plants were used for the experiments on the almond cultivars Fascionello,

[1] **Corresponding author**

Ferragnes, Tuono; the almond selections M49, M50, M51, M52, M53, M55; the apple cvs. Gala and McIntosh; three seedlings of *Pyrus pyraster* (clones P8, P38 and P50); the plum cultivar Ontario, and two hybrid rootstocks, Citation (plum X peach) and GF677 (almond X peach). The microplants were multiplied on Murashige and Skoog medium (1962), except the pear clones for which Quoirin and Lepoivre (1977) was used and Ontario where Schenk and Hildebrandt (1972) supplemented with hormones was used, as reported in Table 1. Culture conditions were 21±2°C, photoperiod of 16 hrs, with a light intensity of 37 μmol m^{-2} s^{-1}. Preliminary tests were conducted to determine the best root induction medium for each genotype. The auxins concentrations used in the experiments are reported in Table 1.

Bacterial inoculum and transformation. *Agrobacterium rhizogenes* strain 1855 was grown at 28°C on YMB (Hooykaas *et al.*, 1977) for 24-36 hrs until an optical density of 0.4 at 600 nm was obtained. The infections were carried out according to Damiano *et al.* (1995), dipping the basal part of the plants for 24 hours in darkness in 0.5 ml bacterial suspension. The microcuttings were prepared after subculturing for three weeks; the most homogeneous shoots were selected and the base cleanly cut. After infection, the explants were transferred to the appropriate co-cultivation root induction medium, with or without hormone, and finally transferred to the same medium containing the antibiotic cefotaxime (250 mg/l). Control uninfected shoots were dipped in YMB for 24 hrs and then cultured under the same conditions as the infected plants. After infection, microcuttings of Fascionello, Ferragnes, Tuono, M49, M50, M52, M53 M55, P50 were cultured in darkness for ten days before being exposed to light. Some of the explants of Ferragnes and Tuono were maintained with the basal part in darkness after the exposure to light.

DNA extraction and PCR. The DNA was extracted from single roots, whose weight ranged from 0.1g to 0.4g (Damiano *et al.*, 1995). The bacteria were removed from the roots by washing in NaOH, 200 mM and SDS 1 % w/v, for some minutes and then rinsing in sterile deionised water. The roots, dried on sterile filter paper, were quickly frozen in liquid nitrogen and ground to a fine powder in a mortar containing insoluble 40% w/v PVPP (Damiano *et al.*, 1995). Each PCR reaction was carried out on 20-30 ng of total DNA with 1.2U TaqDNA polymerase (Pharmacia) in a volume of 30μl (Hamill *et al.*, 1991). Primers used for the amplification reactions came from internal sequences of the *rol*B gene (0.78kb) and from *vir*D1 gene (0.45kb) (Hamill *et al.*, 1991). The amplification temperatures for *rol*B gene were 95°C for denaturation (duration 1 min.), 34°C for annealing (2 min.) and 72°C for extension (2 min.), with 25 amplification cycles. For *vir*D1 gene amplification, denaturation, annealing and extension temperatures were respectively 95°C (1 min.), 55°C (1 min.) and 72°C (2 min.) for 25 cycles. The amplified sequences were made visible on agarose/EtBr gels.

Experimental design and data. There were three replicates with 8-10 shoots per plot for each cultivar or selection. Data was recorded after 35 days from the start of the experiment. Data collected was the number of rooted plants, the number of roots per plant and the length of roots in mm. Results, expressed as percentages, were normalised according to the formula arcsine√% before statistical analysis.

Results

Table 2 shows the rooting percentages of the genotypes exposed to the different treatments. According to their rooting ability, the genotypes can be divided into three groups: a) those rooted without auxins: Citation, GF677, M51, Ontario, Gala, McIntosh (Fig. 1); b) those rooted only with auxins: M49 (Fig. 2), M50, M52, M53, M55, P38, P50 (Fig. 3); c) those rooted only after *A. rhizogenes* infection: P8, Fascionello, Ferragnes, Tuono (Fig. 4). In general, the addition of auxins to the media improved rooting in group (a). After bacterial infection all genotypes rooted. 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 was observed with the combination of auxin and infection. With group (b) the positive effect of infection on shoots on hormone free media should be noted and also the synergism between the hormones in the media and infection in the cases of P38 and P50 (Fig. 3). No comments are needed on the results of group (c). With Ferragnes and Tuono darkening of the plant bases tended to decrease the rooting, although not significantly (Fig. 4).

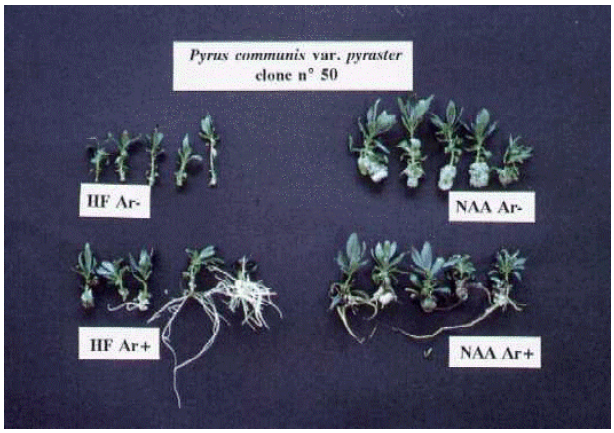


Figure 1. Response of the apple cv McIntosh to the treatments. HF = hormone free medium; IAA = medium supplemented with IAA 0.5 mg/l; Ar- = microcuttings not infected; Ar+ = microcuttings infected with *A. Rhizogenes*

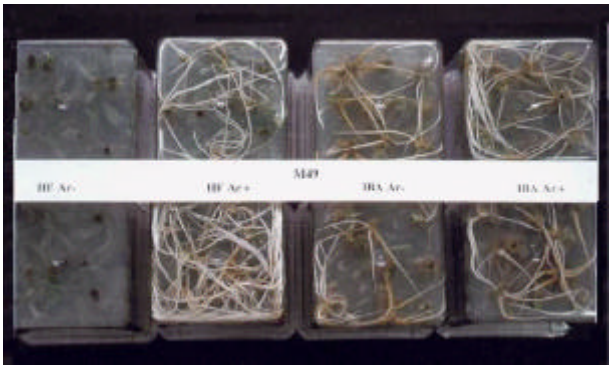


Figure 2. Response of the almond rootstock M49 to the treatments. HF = hormone free medium; IBA = medium supplemented with IBA 2 mg/l; Ar- = microcuttings not infected; Ar+ = microcuttings infected with *A. Rhizogenes*

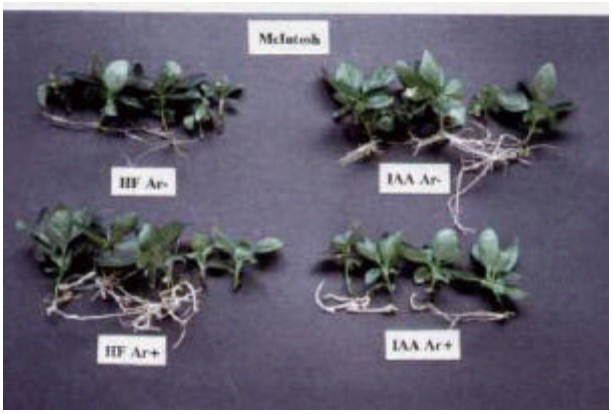


Figure 3. Response of wild pear clone P50 to the treatments. HF = hormone free medium; NAA = medium supplemented with NIAA 0.5 mg/l; Ar- = microcuttings not infected; Ar+ = microcuttings infected with *A. Rhizogenes*

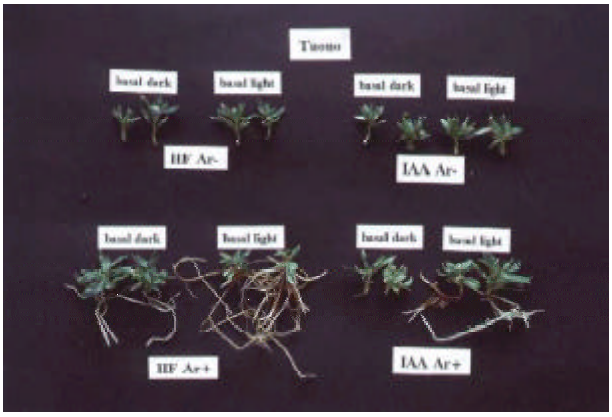


Figure 4. Response of the almond cv Tuono to the treatments. HF = hormone free medium; IAA = medium supplemented with IAA 2 mg/l; Ar- = microcuttings not infected; Ar+ = microcuttings

Some changes in the roots number and root length occurred, but more evident was a reduced geotropic response with several infected plants, particularly almond; this growth behaviour was reported as typical in transformed roots (Leguè *et al.*, 1996). Molecular analyses were carried on a random sample of roots of some almond genotypes and GF677. PCR was used to detect the integration of the *rol*B gene in the roots induced by *A. rhizogenes* (Fig.5). The *vir*D1 amplification by PCR was done to avoid false positives (Fig. 6). In fact with 26.2 % of the samples it was not possible to exclude the bacterial contamination (Fig. 6, lanes 6-9-14-17), thus making the confirmation of the transgenic nature of the roots impossible. In the remaining samples, 67 % of the roots were not transformed (Fig. 5, lanes 1-2-3-4-5-7-8-10-11-12-13-15) and only 6.8 % were positively transgenic (Fig. 6, lane 16). Some roots with negative geotropism were subjected to PCR analysis in order to correlate the reduced geotropic response with the transgenic nature of the roots. This was not possible, as not all the roots growing upwards appeared to be transgenic. Both transformed and non-transformed roots differentiated on plants cultured in hormone free medium or in the presence of hormones. In some cases the same microcutting showed both transformed and non-transformed roots (Fig. 5, lanes 15-16).

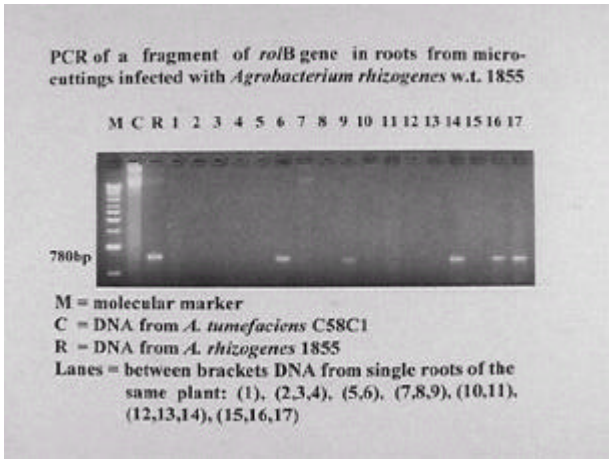


Figure 5

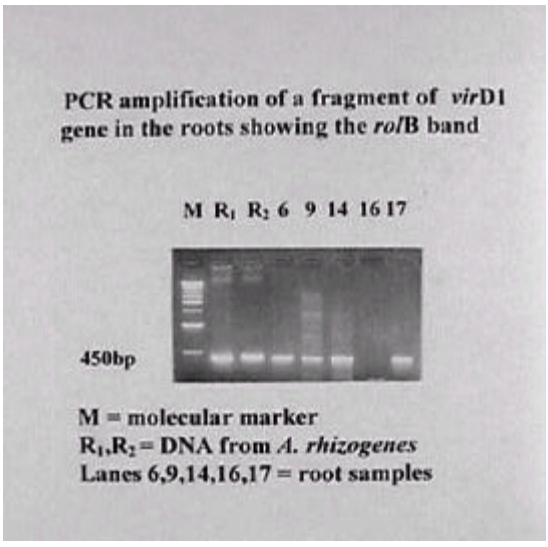


Figure 6

Table 1. Cultural media used

	Basal composition		Vitamins (mg/l)	Hormonal composition (mg/l)	
	Multiplication	Rooting		Multiplication	Rooting
Citation	MS	MS 1/2	MS, ascorbic acid 10, citric acid	BAP 0.55, GA_3 0.2, NAA 0.02	IAA 6
Fascionello	Msmod	BN	MS/BN	BAP 0.25, IAA 0.125	IAA 2
Ferragnes, Tuono	MS	BN	MS/BN	BAP 0.2, IAA 0.05	IAA 2
M49, M50, M52, M53, M55	MS	MS	MS	BAP 0.2, IAA 0.05	IBA 2
Gala, McIntosh	MS	MS 1/2	nicotinic acid 0.5, pyiridoxine 0.5, thiamine 1, inositol 100	BAP 0.5, IBA 0.1	IAA 0.5
GF677	MS	MS	MS	BAP 0.35, GA_3 0.1, NAA 0.01	IBA 1
M51	MSmod	MS 1/2	MS	BAP 0.3, IAA 0.05	IAA 0.75
Ontario	SH	SH	MS	BAP 0.15, GA_3 0.2, IBA 0.1	IAA 0.75
P38, P8 and P50	QL	MS 1/2	nicotinic acid 0.5, pyiridoxine 0.5, glycine 2, thiamine 0.5, biotin 0.1, inositol 200, riboflavin 0.5, calcium pantotenate 1	BAP 0.65, IBA 0.05	IBA 2 NAA 1

BN, Bourgin and Nitsch (1967); MS, Murashige and Skoog (1962); QL, Quoirin and Lepoivre (1977); SH, Schenk and Hildebrandt (1972); MSmod: MS modified (NH_4NO_3 660 mg/l, KNO_3 1662 mg/l)

Table 2. Rooting percentages (± S.E.) of microcuttings infected with *A. rhizogenes* (Ar+) and not infected (Ar-) in hormone free medium (HF) or in the hormone supplemented medium (WH)

	HF Ar-	WH Ar-	HF Ar+	WH Ar+
	% ± S.E.	% ± S.E.	% ± S.E.	% ± S.E.
Citation	69.1± 5.6	82.2 ± 3.8	63.6 ± 5.7	69.2 ± 7.7
GF677	58.7 ± 5.7	84.8 ± 5.2	56.2 ± 7.3	71.8 ± 12.3
M51	42.0 ± 11.2	50.0 ± 4.1	52.6 ± 6.3	51.9 ± 3.3
Ontario	35.2 ± 4.1	49.4 ± 10.3	16.5 ± 5.6	16.5 ± 8.0
Gala	26.2 ± 8.1	55.4 ± 5.1	15.7 ± 3.4	34.4 ± 3.2
McIntosh	22.1 ± 8.1	35.7 ± 3.6	25.3 ± 12.5	31.4 ± 13.2
M49	0.0 ± 0.0	26.1 ± 3.5	41.1 ± 4.2	28.1 ± 4.9

M50	0.0 ± 0.0	26.0 ± 6.8	27.7 ± 6.5	6.9 ± 4.4
M52	0.0 ± 0.0	33.8 ± 3.8	75.0 ± 15.0	33.8 ± 3.8
M53	0.0 ± 0.0	70.2 ± 3.8	79.4 ± 3.2	80.9 ± 3.3
M55	0.0 ± 0.0	65.0 ± 8.8	57.8 ± 4.3	67.8 ± 8.2
P38	0.0 ± 0.0	32.1 ± 6.0	26.3 ± 8.6	73.4 ± 5.8
P50	0.0 ± 0.0	16.7 ± 5.1	27.7 ± 9.0	63.7 ± 8.7
P8	0.0 ± 0.0	0.0 ± 0.0	50.8 ± 2.8	15.0 ± 6.4
Fascionello	0.0 ± 0.0	0.0 ± 0.0	30.0 ± 4.5	25.3 ± 4.6
Ferragnes	0.0 ± 0.0	0.0 ± 0.0	52.3 ± 3.9	55.0 ±3.4
Tuono	0.0 ± 0.0	0.0 ± 0.0	46.4 ± 6.2	33.3 ± 4.9

Discussion

The results presented in this paper indicate that the use of *A. rhizogenes* can be a successful approach to improve rooting on fruit trees, although different responses to infection occurred. While with the difficult-to-root genotypes the infection always had a positive effect, sometimes, in association with a hormonal treatment, the responses were larger for the easy-to-root ones. From our experiments, it is possible to differentiate two principal behaviours. Most of the species did not appear to be affected by infection and the hormonal supply may be sufficient to give good rooting, as it occurred with the hybrids Citation and GF677, the apple cultivars Gala and McIntosh and the almond selection M51. On the other hand, the infection had a detrimental effect on rooting, with or without hormone, on the plum cv. Ontario.

The first step in the infection process is the host/pathogen interaction, where several factors contribute to the establishment of this relationship (Winans, 1992). Sugars and phenolic compounds released from the plants and membrane-binding protein can differ according to plant genotype, thus producing different responses.

By now the hypothesis of a direct auxin release from auxin-glucoside esters by the action of the *rol*B products has been abandoned (Maurel *et al.*, 1994). Changes in the auxin sensitivity of the *rol*B in transformed tobacco cells seems to be a consequence of an altered permeability of the membrane auxin binding protein (Filippini *et al.*, 1994; Maurel *et al.*, 1994). Thus, the different responses observed could result from alterations of the auxin reception-transduction pathway. Again, Capone *et al.* (1994) identified some regulatory domains of the *rol*B promoter region, responding to endogenous plant regulatory proteins whose presence and/or concentration can differ in different cell types, and maybe in different genetic backgrounds. According to Di Cola *et al.* (1996), the presence and expression of *rol*B are necessary but not sufficient to induce rhizogenesis in transformed cells, because a high rooting potential should be associated with the presence of pre-committed cells, whose potentiality could be amplify by *rol*B.

An interesting result emerging from the molecular analysis is that only a few roots were shown to be transgenic. Magnussen *et al.* (1994) found the induction of normal adventitious roots in infected conifers, but not in untreated seedlings. No correlation could be established between the transformation rate and the genotypes or the treatments, because of the size of the sample. The washing step must be changed to eliminate the high percentage of doubtful results. Our results seem to suggest that the rooting process involves more than the transformed cells. McAfee *et al.* (1993) proposed a role for the bacterium in modifying the root environment by means of organic acid secretions and lowering the pH. Finally, Guivarc'h *et al.* (1996a, b) suggested the existence of diffuse factors associated with the expression of *rol*A and *rol*C, implying that the sites of action and expression of these genes are physically separated.

Further investigations are necessary to better understand the rooting and the transformation processes in fruit trees and to improve propagation protocols. Furthermore the field behaviour of plants rooted by localised infection must be investigated
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