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Evidence of migration and endophytic presence of *Agrobacterium tumefaciens* in rose plants

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Abstract

Agrobacterium tumefaciens was isolated from stem tumors of several rose cultivars showing that the bacterium is the causal agent of aerial galls in rose plants. No differences were observed in the characteristics of the *Agrobacterium* isolates from crown or aerial galls. Stem inoculation of ten rose cultivars showed that all of them were susceptible to *A. tumefaciens* but differences in the size of the resulting tumors were observed. The movement of *A. tumefaciens* in rose plants was demonstrated using two wild type strains and two antibiotic resistant mutants. Three months after inoculation, the inoculated strains were recovered in the roots, crown and below and above the inoculation site but low numbers of pathogenic *Agrobacterium* cells were isolated. New tumors appeared in 5% of the noninoculated wounds. *A. tumefaciens* was isolated from the stem at different distances from the tumor in naturally infected plants. In symptomless commercial plants, the isolation from the roots, crown and at different stem levels demonstrated the existence of systemic and latent infections in rose. Direct isolation using a nonselective and selective media with or without a previous enrichment step were efficient methods for isolating tumorigenic *Agrobacterium* from the different parts of rose plants.

Introduction

Systemic movement of *A. tumefaciens*[1] inside plants has been demonstrated in several hosts (Riker, 1923; Hill, 1928; Suit and Eardley, 1935; Stapp et al., 1938; Lehoczky, 1968; Miller, 1975; Bouzar et al., 1995) but has not previously been demonstrated in rose plants. Secondary tumors arising at a distance from an inoculation site have been observed on some hosts (Braun, 1941; Braun and Stonier, 1958; El-Khalifa et al., 1973). These were not initially attributed to *A. tumefaciens* due to the difficulties in isolating living bacteria, but the demonstration of systemic movement of *A. tumefaciens* suggested that the so called secondary tumors could be caused by pathogenic *Agrobacterium* strains.

A. tumefaciens, causal agent of crown gall, has been reported to cause significant losses in rose crops (Dickey, 1969; López et al., 1981; Otha and Nishiyama, 1984; Farkas and Haas, 1985). The problem is economically important at the nursery level because of regulations against trade of infected plants. Different rose cultivars, growing in heated greenhouses or in subtropical conditions show with relatively high frequency plants with aerial tumors, that do not show any other symptom of the disease. This suggests that in rose, internal translocation of *A. tumefaciens* from crown or root tumors is not the only source of inoculum for aerial tumors. Another hypothesis is that *A. tumefaciens* can be latent inside the plant and develop stem galls at different sites under appropriate environmental conditions. Latent infections in stone fruit trees inoculated with *A. tumefaciens* have been reported

[1] In order to simplify the interpretation of the results the classic nomenclature (Holt et al., 1994) is used in this paper although a new proposal has been made to rename strains of *Agrobacterium* from biovar 1 and 2 as species (Sawada et al., 1993).

(Moore, 1976) but demonstrating their occurrence on roses can be of great importance in developing new methods for sanitary selection in roses multiplication.

The purpose of this study was to obtain additional information on the life cycle of this bacterium in rose, on which we have previously reported (Martí et al., 1991), and to determine: (i) the etiology of aerial tumors observed on rose plants, (ii) whether *A. tumefaciens* can migrate inside the stem of rose plants. (iii) the endophytic presence of this bacterium in symptomless plants. The new information provided by this model could be applied to the detection of the systemic migration and the latent presence of *Agrobacterium* in other hosts.

Materials and methods

Isolation of A. tumefaciens *from aerial and crown galls in rose plants*

Eighty five rose samples were collected from nurseries at different locations in Spain and one from Colombia. The samples analysed are described in Table 1. Most of these plants had been treated with *Agrobacterium radiobacter* K84 to prevent *A. tumefaciens* infection. Crown and aerial tumors from plants were collected, washed with soapy water and flamed superficially. The epidermis was removed aseptically and small pieces were comminuted in sterile distilled water. After 30 min maceration, comminuted tissues were plated on media selective for biovar 1 (Schroth et al., 1965), biovar 2 (New and Kerr, 1971), and *A. vitis* (Roy and Sasser, 1983), and on PYGA medium (Bacto-peptone $3\,g\,l^{-1}$, yeast extract $5\,g\,l^{-1}$, glycerol $10\,ml\,l^{-1}$, agar $20\,g\,l^{-1}$). Before being characterised, typical *Agrobacterium* colonies were selected and purified by subculturing twice on PYGA medium. Each strain studied originated from a single colony. The methods described for biovar determination (Kerr and Panagopoulos, 1977) were used. Production of 3 ketolactose, utilization of litmus milk, acid production from sucrose, melezitose and erythritol, and alkali from tartrate, malonate, propionate and mucid acid were assayed. Sensitivity to agrocin 84 and bacteriocin production were studied according to Stonier (Stonier, 1960). Strain C58 of *A. tumefaciens* was used to check bacteriocin production by the strains. All the strains were inoculated on tomato cv. Roma according to the method described by López et al. (1981) and on five rose plants grown in greenhouse at 25 °C. Tumors appearance was observed 15 and 30 days after tomato inoculation or 60 and 90 days after rose inoculation.

Susceptibility of different rose cultivars to A. tumefaciens

Cultivars Sonia, Candia, Silva, Carte Blanche, Dallas, Red Success, Yonina, Cocktail, Aurelia and Samantha

Table 1. Characterization of *Agrobacterium* strains from rose tumors

Reference	Rootstock or cultivar	Tumor location	Tumors analysed	Pathogenic isolates studied	Biovar	Agrocin 84 sensitivity (strains tested)[1]	Bacteriocin production (strains tested)
939	—	Crown	12	33	2	R[1](28)	+(11)
961	Indica	Crown	5	44	2	R(34)	+(20)
974	—	Crown	9	20	2	R(18)	+(13)
1118	—	Crown	3	8	N.T.[3]	R(3)	N.T.
1139	Indica	Crown	7	9	2	R(7)	N.T.
1140	—	Crown	5	6	2	R(5)	N.T.
1156	Manetti	Crown	4	3	N.T.	N.T.	N.T.
1159	Manetti	Crown	3	1	N.T.	N.T.	N.T.
1165	Indica	Crown	6	6	N.T.	N.T.	N.T.
1169	—	Crown	6	2	N.T.	N.T.	N.T.
962	Majita	Aerial	4	21	2	R(20)	+(18)
973	—	Aerial	8	6	2	R(5)	+(4)
976	—	Aerial	6	23	2	R(23)	+(4)
1126	Candia	Aerial	1	1	N.T.	S[2](1)	N.T.
1166	Laura	Aerial	6	5	N.T.	N.T.	N.T.

[1]R = Resistant to agrocin 84; [2]S = Sensitive to agrocin 84; [3]N.T. = Not tested.

were used to determine susceptibility to *A. tumefaciens*. Four *A. tumefaciens* strains isolated from crown and aerial rose tumors and characterized as biovar 2 were used as inoculum. The strains were named, 30, 66, 75 and 33 and were deposited at the IVIA collection. Rose plants were wounded at 10–20 cm above the grafting site and 10 μl of bacterial suspensions (10^9 cfu ml^{-1}) were inoculated into each wound. About fourteen inoculations were performed for each strain and cultivar. Plants were grown in greenhouse under controlled conditions at 20–24 °C and 60–70% relative humidity (RH). Tumors appearance was recorded 45 days after inoculation and isolations according to the procedure described above were made from two tumors of each combination. Statistical analysis of the tumor size was performed in two ways (strains by cultivars) ANOVA followed by a Duncan multiple average test for means comparison.

Migration of A. tumefaciens *on artificially inoculated plants*

Two mutants of *A. tumefaciens* strains 30 and 66 resistant to streptomycin (str) and rifampicin (rif) were obtained. The two mutants and two biovar 2 strains, 265 and 13-O, isolated from aerial rose tumors were used. To obtain the mutants 30R and 66R 0.5 ml of a 10^9 cfu ml^{-1} suspension of isolates 30 and 66 were added to 100 ml of peptone, yeast extract, glycerol liquid medium (PYGB) amended with 500 μg ml^{-1} str. After 48 h shaking, 100 μl of each suspension was plated onto PYGB amended with 500 μg ml^{-1} str. Viable colonies were selected, plated in PYGB + str medium once again and inoculated to tomato. Pathogenic colonies were incubated in shaken cultures in PYGB medium amended with 100 μg ml^{-1} rif for 48 h. One hundred microliters of each suspension was plated on PYGB medium with 500 μg ml^{-1} str and 100 μg ml^{-1} rif. Colonies were selected and inoculated on tomato and rose and pathogenic colonies were stored at −80°C. To study the stability of the resistant mutants fifteen serial transfers were performed with seven days old cultures on PYGB medium. The surviving colonies after the final transfer were plated on PYGB + str + rif and inoculated on tomato plants.

To study the systemic movement of *A. tumefaciens* in rose plants 22, 27 and 12 plants of cultivars Sonia, Samantha and Candia respectively, were inoculated with the four *A. tumefaciens* strains or mutants. Twenty microliters of a 10^9 cfu ml^{-1} suspension of the mutants 30R and 66R and of the strains 265 and 13-O were added to small wounds made 15 cm above the grafting site. Wounds were covered with parafilm immediately after inoculation. Two, three or five plants per cultivar and three or four stems per plant and bacterial isolate were used for this purpose. At the same time the plants were aseptically wounded on the stems every 10 cm above and below the inoculation point to observe if secondary tumors formed at these sites. Two plants of each cultivar were inoculated with sterile water and wounded. Plants were grown in two greenhouses under controlled conditions at 20–24 °C and 60–70% RH with drip irrigation and were not manipulated during the experiments. Tumor readings were made 60 and 90 days after inoculation. Plants were harvested three months after inoculation when tumors of ≥2 cm diameter were observed. Isolations from ten secondary tumors appearing in the noninoculated wounds were performed according to the procedure described above. Biovar 2 selective medium (New and Kerr, 1971) or PYGA + str + rif were used.

To follow the migration of the bacteria, asymptomatic samples from root, crown, stem below the grafting point, 5 cm below the inoculation point and at 5, 10, 15, 20 or 25 cm above the inoculation point were collected aseptically. Each plant was externally disinfected with 70% ethanol, washed with bactericide soap, and the selected pieces were aseptically taken, the epidermis was aseptically removed and 0.5 × 0.5 cm fragments of vascular and pith tissue of such asymptomatic samples were comminuted in sterile water. After 30 min, 50 μl of each was plated on selective media (Schroth et al., 1965; New and Kerr, 1971) for strains 26S and 13-O or on PYGA + str + rif medium for mutants 30R and 66R. Furthermore 0.5 ml of each comminuted preparation was added to 4.5 ml liquid selective medium (New and Kerr, 1971; Schroth et al., 1965) or PYGB + str + rif media. After five days these enrichment cultures were plated on the same media containing agar. Controls inoculated with sterile water were analysed in the same way and by PCR using primers FGP tmr 530 and FGP tmr 701' of the T-DNA region genes (Nesme et al., 1989) and a standard protocol (Cubero et al., 1997; Cubero et al., in press). Putative colonies of *Agrobacterium* grown on the plates were purified, inoculated on tomato cv. Roma and rose plants cv. Sonia grown in the greenhouse and stored at −80 °C. The pathogenic colonies obtained from plants inoculated with strains 265 and 13-O were serologically characterized to confirm their identity

using antisera for biovar 2 in double immunodiffusion (DID) to observe specific precipitation lines for each strain. A standard protocol (Alarcón et al., 1987) was followed, using pure cultures and 1 : 2 diluted antisera in a Gelman support (Gelman Instrument Co., Ann Arbor, Mich. USA).

Extraction of xylem fluids from nine stems from inoculated plants, was performed by analysing stem portions of 10 cm length after external disinfection, taken 20 cm above the tumor and using the pression chamber soil moisture mod. 3000 (Equipment Corp. Santa Barbara, USA). After extraction, 100 µl of the xylem fluids were plated on the described selective media. Contaminations were avoided by using the precautions described above.

Isolation of A. tumefaciens *from naturally infected rose plants from stem samples and from the tumors*

Twenty rose plants of the cv. Ferdy grafted on Indica were analysed. Plants all showed typical crown gall symptoms. Two samples from each plant were analysed: one from the tumor and the other from the grafted cultivar, taken 10–20 cm above the tumor. These pieces were taken with similar precautions as indicated before and 50 µl of each extract were plated on selective and nonselective media or subjected to enrichment as described above. Putative *Agrobacterium* colonies on the media were collected, purified and inoculated on tomato.

Isolation of A. tumefaciens *from noninoculated rose plants without crown gall symptoms*

Twenty four plants without crown gall were analysed. They were all grafted on Indica and sixteen were of cv. Aurelia, six of cv. Red Sucess, and two of cv. Silva. Five samples from each plant were collected with similar precautions as before, to be analysed. The samples were taken from the crown, and at 5, 10, 15 and 20 cm above the crown and processed as above indicated.

Results

Isolation of A. tumefaciens *from aerial and crown galls in rose plants*

Table 1 shows that *A. tumefaciens* was isolated from 60 crown galls and the 25 aerial tumors from different rose samples. The efficiency of the detection of pathogenic *Agrobacterium* was similar in crown and aerial galls. All the strains pathogenic on tomato also produced tumors on rose plants. Most of the pathogenic 188 isolates studied were biovar 2 and resistant to agrocin 84. Only one of the isolates was sensitive to agrocin 84, and 70 of them produced unidentified agrocin-like substances. No differences were observed between isolates that originated from crown and aerial tumors, nor differences between the characteristics of strains isolated from different rose cultivars or from different geographic origins.

Susceptibility of different rose cultivars to A. tumefaciens

Table 2 shows the susceptibility of the different cultivars to *A. tumefaciens* inoculation. Tumors formed in most of the aerial inoculations after 45 days. Significant differences among cultivars were observed because in Sonia, Candia and Samantha average tumors size was significantly different from the other cultivars and among them. Tumors obtained with strains 30, 66 and 75 were not significantly different, but those obtained with strain 33 were smaller. *Agrobacterium* type colonies were isolated from the analysed tumors.

Migration of A. tumefaciens *on artificially inoculated plants*

Strain 265 and 13-O and mutants 30R and 66R were used to study migration in rose plants. Mutants 30R and 66R were obtained and proved to be stable because after fifteen transfers they retained their antibiotic resistance and pathogenicity. After inoculation of the rose plants, tumors appeared at all the sites of inoculation and in 23 out of 448 wounds. Such secondary tumors were observed at sites distal to the points of inoculation, as shown in Table 3. This table summarizes combined results from the two greenhouses where the same rose cultivars and bacterial strains were used, as no significant differences were observed among them. In Sonia and Candia secondary tumors were observed with the four strains studied, but in Samantha only mutant 30R was able to cause one secondary tumor. Isolations from 10 secondary tumors confirmed the presence of *A. tumefaciens*. No tumors appeared on plants inoculated with sterile water.

The results of isolation of systemic *A. tumefaciens* out of the wounds, above and below the inoculation site

Table 2. Average tumor size (mm diameter) obtained after 45 days of aerial inoculation with four *A. tumefaciens* strains in ten rose cultivars

A. tumefaciens strain	Sonia	Candia	Silva	C. Blanche	Dallas	Red Success	Yonina	Cocktail	Aurelia	Samantha	Mean	Duncan Grouping
30	20,0 ± 2,0	14,9 ± 1,2	12,4 ± 2,3	8,7 ± 1,3	7,5 ± 0,3	7,3 ± 1,0	7,0 ± 0,0	4,7 ± 0,6	2,8 ± 1,3	1,0 ± 0,4	9.260	A
66	21,1 ± 2,6	13,8 ± 1,1	7,8 ± 0,9	10,5 ± 2,5	4,1 ± 0,9	6,0 ± 1,0	5,6 ± 1,0	6,6 ± 0,6	4,7 ± 0,7	0,7 ± 0,1	8.849	A
75	15,0 ± 0,0	16,4 ± 1,5	9,7 ± 0,3	10,0 ± 0,0	7,2 ± 1,0	5,0 ± 0,0	5,1 ± 0,7	5,4 ± 0,6	4,7 ± 0,8	1,4 ± 0,5	8.063	A
33	N.I.[1]	N.I.	5,7 ± 0,9	2,7 ± 0,3	7,2 ± 1,3	5,7 ± 1,4	4,0 ± 0,4	N.I.	4,0 ± 0,0	0,5 ± 0,0	4.220	B
Mean	19.963	15.204	8.632	8.231	6.250	6.158	5.577	5.518	4.125	0.883		
Duncan Grouping	A	B	C	C	C D	C D	C D	C D	D	E		

Sq. error mean square = 3.84.
[1]N.I. = Not inoculated.

are shown in Table 4. At 5 cm below and 5–25 cm above the inoculation points cells of pathogenic *Agrobacterium* were observed in the isolations from all of the cultivars assayed, demonstrating the presence of living bacterial cells. The exception was the mutant 66R in Samantha which was not found in any plant above the inoculation point. At the grafting site and the crown, the four strains studied were isolated only in Samantha. In Candia, strains 265 and 66R were not isolated from the grafting site and strain 66R was not in crown or in roots. In Sonia, only strain 13-O was isolated in crown and the grafting site. Strain 265 in Samantha, mutant 66R in Sonia and both mutants in Candia were not isolated from roots. No differences were observed in the behavior of the mutant strains compared with the wild type strains. Table 5 and 6 compare the results of the isolation of *A. tumefaciens* mutants 30R and 66R and strains 265 and 13-O at different sites with and without an enrichment step in selective liquid media. Some differences were observed in the isolation by direct plating of the comminuted plant material or enrichment in the liquid media. Numbers of *Agrobacterium* colonies isolated varied between 1 and 250 per plate. A highly variable percentage (1.7–100%) of the isolated agrobacteria cells obtained were pathogenic on rose and tomato (data not shown). According to results in Tables 5 and 6 the number of plants from which *A. tumefaciens* was isolated above and below the inoculation site was variable, in different media and cultivars. In isolation of the mutants 30R and 66R direct plating and enrichment showed similar efficiency but in some

Table 3. Secondary tumors observed at noninoculated wounds on three rose cultivars that were inoculated with *A. tumefaciens* 15 cm above the grafting site. Results are expressed as number of tumors observed divided by non inoculated wounds made[1,2]

Rose cultivar	Strain	Tumors observed/wounds performed
Sonia	265	1/43
	13-O	1/33
	30R	1/31
	66R	2/25
Candia	265	7/66
	13-O	5/68
	30R	4/63
	66R	1/46
Samantha	265	0/18
	13-O	0/25
	30R	1/15
	66R	0/15

[1]Combined results from two greenhouses.
[2]*A. tumefaciens* strains 265 and 13-O and mutants 30R and 66R were inoculated 15 cm above the grafting site. At the same time, the plants were aseptically wounded on the stems every 10 cm above and below the inoculation point. Readings were performed after three months.

Table 4. Recovery of *A. tumefaciens* from rose plants from fragments out of the wounds, at different noninoculated sites in plants inoculated above the grafting site[1]

Isolate inoculated	Rose cultivar	Isolation of *A. tumefaciens* [2]				
		Root	Crown	Grafting site	5 cm below the inoculation	5–25 cm above the inculation
265	Candia	+	+	—	+	+
13-O	Candia	+	+	+	+	+
30R	Candia	—	+	+	+	+
66R	Candia	—	—	—	+	+
265	Sonia	+	—	—	+	+
13-O	Sonia	+	+	+	+	+
30R	Sonia	+	—	—	+	+
66R	Sonia	—	—	—	+	+
265	Samantha	—	+	+	+	+
13-O	Samantha	+	+	+	+	+
30R	Samantha	+	+	+	+	+
66R	Samantha	+	+	+	+	—

[1]*A. tumefaciens* strains 265 and 13-O and mutants 30R and 66R were inoculated 15 cm above the grafting site. Asymptomatic samples of roots, crown, 5 cm below and 5–25 cm above the inoculation were analysed 90 days after inoculation. A piece of each sample was externally disinfected, the epidermis was aseptically removed and was comminuted in sterile water.
[2]+ = Pathogenic *Agrobacterium* strains recovered; — = No *Agrobacterium* strains recovered.

Table 5. Recovery of *A. tumefaciens* mutants from rose plants, at noninoculated points in plants inoculated 15 cm above the grafting site.[1] Results are expressed as plants from which *A. tumefaciens* were isolated divided by the plants inoculated with each mutant

Mutant	Cultivar	Root		Crown		Grafting site		5 cm below inoculation site		5–25 cm above inoculation site	
		Dir.[2]	Enr.[3]	Dir.	Enr.	Dir.	Enr.	Dir.	Enr.	Dir.	Enr.
30R	Candia	0/6	0/6	0/6	**1/6**	0/6	**2/6**	**6/6**	**4/6**	**2/6**	**2/6**
66R	Candia	0/6	0/6	0/6	0/6	0/6	0/6	**5/6**	**5/6**	**3/6**	**3/6**
30R	Sonia	**1/4**	0/4	0/4	0/4	0/4	0/4	**3/4**	**2/4**	**1/4**	**2/4**
66R	Sonia	0/6	0/6	0/6	0/6	0/6	0/6	**6/6**	**2/6**	**3/6**	**2/6**
30R	Samantha	**1/3**	0/3	0/3	**1/3**	**1/3**	0/3	**1/3**	**2/3**	0/3	**1/3**
66R	Samantha	**1/2**	0/2	**1/2**	0/2	0/2	**1/2**	**2/2**	**2/2**	0/2	0/2

[1]Inoculation were the same as described in Table 4.
[2]Dir. = Direct plating on PYGA with streptomycin and rifampicin.
[3]Enr. = Enrichment in selective medium for 5 days and plating on the same medium.
Boldface numbers indicate the isolations where pathogenic colonies were detected.

Table 6. Recovery of *A. tumefaciens* strains from rose plants, at noninoculated points in plants inoculated 15 cm above the grafting site. Results are expressed as plants from which *A. tumefaciens* was reisolated divided by the plants inoculated with every strain[1]

Strain	Cultivar	Root			Crown			Grafting site			5 cm below the inoculation site			5–25 cm above the inoculation site		
		[2]P	[3]N		P	N		P	N		P	N		P	N	
		Dir.	[4]Dir.	[5]Enr.	Dir.	Dir.	Enr.	Dir.	Dir.	Enr.	Dir.	Dir.	Enr.	Dir.	Dir.	Enr.
265	Candia	0/8	**2/8**	**2/8**	0/8	**2/8**	**1/8**	0/8	0/8	0/8	**7/8**	**7/8**	**5/8**	**3/8**	**3/8**	**5/8**
13-O	Candia	**1/7**	0/7	**3/7**	0/7	0/7	**1/7**	0/7	**1/7**	**1/7**	**6/7**	**6/7**	**5/7**	**4/7**	**3/7**	**4/7**
265	Sonia	0/6	**1/6**	0/6	0/6	0/6	0/6	0/6	0/6	0/6	**3/6**	**4/6**	**1/6**	**4/6**	**3/6**	0/6
13-O	Sonia	0/6	**1/6**	**1/6**	0/6	**1/6**	0/6	**1/6**	**1/6**	**1/6**	**5/6**	**5/6**	**1/6**	**1/6**	**1/6**	**2/6**
265	Samantha	0/4	0/4	0/4	0/4	**1/4**	**2/4**	0/4	**1/4**	**1/4**	**2/4**	**3/4**	**3/4**	**2/4**	**2/4**	**1/4**
13-O	Samantha	0/3	0/3	**2/3**	2/3	**1/3**	**1/3**	0/3	**1/3**	**1/3**	**3/3**	**2/3**	**1/3**	**2/3**	**1/3**	**1/3**

[1]Inoculations were the same as in Table 4.
[2]P = PYGA (peptone yeast extract glycerol agar medium).
[3]N = Biovar 2 selective medium (New and Kerr, 1971).
[4]Dir. = Direct plating on the different media.
[5]Enr. = Enrichment in selective liquid for 5 days and plating on the same medium.
Boldface numbers indicate the isolations where pathogenic colonies were detected.

samples the results were not the same confirming the need of using both protocols. Isolations were more successful from stem sections below and above the inoculation site than from roots, crown or the grafting site indicating short distance migration. In isolations of strains 265 and 13-O the results were also quite similar following plating on non selective or selective media or after enrichment. As before, a large number of reisolations were successful below and above the inoculation site. The identity of the pathogenic colonies isolated from plants inoculated with strains 26S and 13-O was confirmed by their specific pattern of precipitation lines in DID and from the plants inoculated with the mutants by their antibiotic sensitivity. No *Agrobacterium* type colonies were isolated from the controls inoculated with sterile water and the PCR analysis of them was always negative.

The systemic movement of the bacteria inside rose plants was also demonstrated by isolating pathogenic agrobacteria after plating the fluids extracted by

vacuum from three over the nine analysed stems. No *Agrobacterium* type colonies were isolated from the negative controls.

Isolation of A. tumefaciens *from tumors and stem sections from naturally infected roses*

Table 7 shows that *A. tumefaciens* was isolated from 13 tumors of the 20 plants naturally galled and from symptomless tissues of eight galled plants. Only in one plant pathogenic *Agrobacterium* was isolated 10 cm above the tumor but not in the tumor. In the other samples, when *A. tumefaciens* was found above the tumor it was found in the tumor as well. In this experiment the addition of an enrichment step did not consistently increase the sensitivity of the detection but in some samples according to the number of *Agrobacterium* cells present we were unable to isolate *A. tumefaciens* by direct plating while the enrichment method succeeded. Low populations of *A. tumefaciens* were detected outside the tumor site and in general less than 10 colonies appeared on the isolation plates.

Isolation of A. tumefaciens *from noninoculated plants without crown gall symptoms*

Table 8 shows that *A. tumefaciens* was isolated from 14 out of the 24 plants without tumors. Pathogenic *Agrobacterium* isolates were found in the plants of cv. Silva at different sites from the crown to 15 cm above it. In Aurelia and Red Success *A. tumefaciens* was found in the crown and 5 cm above it, but not at other sites. In seven plants of Aurelia out of 16, five of Red Success out of six and in the two analysed plants of Silva *A. tumefaciens* was isolated in one or more sites in symptomless plants.

Table 7. Isolation of *A. tumefaciens* from 20 naturally infected plants in samples taken from tumors and stem fragments 10 cm above them.[1] Results are expressed as number of plants from which *A. tumefaciens* were isolated

Sites of isolation	Isolation medium and technique						
	[2]P	[3]S		[4]N		[5]R	
	[6]Dir.	Dir.	[7]Enr.	Dir.	Enr.	Dir.	Enr.
Tumor	4	1	0	11	1	2	0
10 cm above	4	0	0	1	7	0	1

[1] Fragments were externally disinfected and the epidermis was aseptically removed before being comminuted in sterile water.
[2] P = PYGA medium (peptone, yeast extract, glycerol, agar).
[3] S = Biovar 1 selective medium (Schroth et al., 1965).
[4] N = Biovar 2 selective medium (New and Kerr, 1971).
[5] R = Biovar 3 selective medium (Roy and Sasser, 1983).
[6] Dir. = Direct plating on the different media.
[7] Enr. = Enrichment in selective medium for 5 days and plating on the same medium.

Discussion

This is the first report on the isolation of *A. tumefaciens* from aerial rose tumors, the systemic movement of this pathogen in rose plants and on the isolation of tumorigenic bacteria from symptomless plants. As it was first necessary to demonstrate the bacterial etiology of the aerial tumors observed in rose plants, *A. tumefaciens* was isolated from such tumors from several cultivars and origins and such bacterium was demonstrated to be the causal agent. The results, comparing the success in isolating *A. tumefaciens* from aerial and crown galls, showed that the frequency of isolation of living bacteria was similar. Furthermore, small pieces of crown and stem tumors were grafted onto rose and bean plants and tumors were observed in both hosts at most of the grafting sites (data not shown). As it is relatively common to not be able to isolate *A. tumefaciens* or to isolate only nonpathogenic agrobacteria from tumors of different plants (Vicedo et al., 1993; Peñalver, 1994; Vicedo, 1995) the relatively low efficiency observed in *A. tumefaciens* isolation was not surprising. Perhaps the survival of bacterial cells is lower in aerial than in underground tumors, because those located on the stem suffer desiccation, and UV radiation. Strains isolated from all the studied tumors had similar characteristics: biovar 2, resistant to agrocin 84 and producers of an uncharacterized agrocin-like substance. A majority of strains resistant to agrocin 84 was observed, probably due to the treatment of most of the rose cuttings with strain K84 before planting, that favoured the selection of strains resistant to this antibiotic.

The results on the susceptibility of ten rose cultivars to aerial inoculations with several strains of *A. tumefaciens* showed that all the cultivars were sensitive to the bacterium and developed aerial tumors. Nevertheless significant differences in tumor size were observed among cultivars and tumors on Samantha were smaller than in other cultivars. Variability in crown gall sensitivity in rose rootstocks was observed previously (Boelema, 1969; López et al., 1981), but as far as we know no data on scion susceptibility was available.

The movement of *A. tumefaciens* in three rose cultivars was demonstrated by using two wild type strains

Table 8. Isolation of *A. tumefaciens* in noninoculated plants without crown gall symptoms. Results are expressed as plants with pathogenic isolates divided by analysed plants[1]

Rose cultivar/ rootstock	Isolation site (cm above the crown)	[2]P	[3]S		[4]N		[5]R	
		Dir.	[6]Dir.	[7]Enr.	Enr.	Dir.	Dir.	Enr.
Aurelia/Indica	0	**2/16**	**1/16**	0/16	1/16	**1/16**	**3/16**	1/16
	5	**2/16**	**1/16**	0/16	0/16	0/16	0/16	0/16
	10	0/16	0/16	0/16	0/16	0/16	0/16	0/16
	15	0/16	0/16	0/16	0/16	0/16	0/16	0/16
	20	0/16	0/16	0/16	0/16	0/16	0/16	0/16
Red Success/ Indica	0	**1/6**	**2/6**	**2/6**	**1/6**	**1/6**	**3/6**	**3/6**
	5	0/6	0/6	0/6	0/6	**2/6**	**1/6**	0/6
	10	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	15	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	20	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Silva/Indica	0	0/2	**1/2**	0/2	**1/2**	**1/2**	0/2	0/2
	5	**1/2**	0/2	0/2	**1/2**	**2/2**	**1/2**	0/2
	10	**1/2**	0/2	0/2	0/2	0/2	0/2	**1/2**
	15	0/2	0/2	**1/2**	0/2	**1/2**	0/2	0/2
	20	0/2	0/2	0/2	0/2	0/2	0/2	0/2

[1]Samples were taken at different sites above the crown, externally disinfected and the epidermis was aseptically removed before being comminuted in sterile water.
[2]P = PYGA medium (peptone, yeast extract, glycerol, agar).
[3]S = Biovar 1 selective medium (Schroth et al., 1965).
[4]N = Biovar 2 selective medium (New and Kerr, 1971).
[5]R = Biovar 3 selective medium (Roy and Sasser, 1983).
[6]Dir. = Direct plating on the different media.
[7]Enr. = Enrichment in selective medium for 5 days and plating on the same medium.

and two antibiotic resistant mutants. All were biovar 2, the most frequent *Agrobacterium* biovar in Spain in rose plants but our unpublished data suggest that biovar 1 strains could have similar behavior (Cubero and López, unpubl.). The mutants were able to induce tumors in rose and tomato plants, indicating that these mutations did not affect virulence. The results of migration of *A. tumefaciens* inside the plants, by observation of secondary tumors appeared in wounds performed out of the inoculation points showed that tumors appeared in 5% of the wounds not inoculated with the bacterium. Furthermore, *A. tumefaciens* was isolated from secondary tumors appearing in such wounds even at 25 cm from the inoculation point showing the movement of the bacteria inside the plant. The observed differences between secondary tumors and *Agrobacterium* detection in Tables 3, 4 ,5 and 6 shows that the presence of tumorigenic strains is not always correlated with tumor appearance. Differences between the amount of plants with *Agrobacterium* living cells and secondary tumors have also been reported by El Khalifa (1973) in castor bean leaves and by Jones and Raju (1988) in chrysanthemum. Both found higher amount of plants without secondary tumors but with pathogenic *Agrobacterium* strains.

The recovery of the resistant mutants in symptomless tissue at distal sites from the inoculation point demonstrated that like in some other hosts (Riker, 1923; Hill, 1928; Stapp et al., 1938; Tarbah and Goodman, 1987) *A. tumefaciens* can move in the inoculated rose plants. The incidence of the recovery of *A. tumefaciens* ranged from 78% to 89% from stems of different cultivars three months after inoculation. All the strains and mutants inoculated were reisolated above and below the inoculation sites. These results suggest although that the frequency of bacterial translocation in rose plants is not very high, the bacteria can move in both directions after introduction. Living cells of *A. tumefaciens* were also obtained by plating fluids extracted by vacuum in three inoculated plants. Movement of *A. tumefaciens* through the xylem was described on tomato (Suit and Eardley, 1935) and grapevine (Tarbah and Goodman,

1987) but such experiments were performed by inoculation of the base of the stems or cuttings with bacterial suspensions. We have demonstrated similar movement in rose, a new systemic host, but using a procedure closer to natural infection, by puncture inoculation of the stem. Our results demonstrate that *A. tumefaciens* can be systemic on roses like in other host plants but the bacteria appear to be translocated only short distances following artificial inoculations. The differences observed could be attributed to the different ability among bacterial strains and cultivars to favor migration inside plants. Cultivar Samantha showed the smallest tumors in the experiment of comparative susceptibility and the lower number of tumors at noninoculated wounds, in plants inoculated with different strains of *Agrobacterium*. The incidence of systemic movement in rose can be variable as in *Chrysanthemum* where similar variability in the frequency of systemic movement has been described (Miller, 1975; Jones and Raju, 1988).

Furthermore, the recovery of *A. tumefaciens* outside tumors in naturally infected plants support the hypothesis of the systemic migration of the bacteria. In eight over 20 studied plants *A. tumefaciens* was isolated at 10 cm above the tumor. No clear differences in the frequencies of isolation were observed in the analysed plants.

Isolation of *A. tumefaciens* from symptomless plants, at different sites, demonstrates that *A. tumefaciens* can live as an endophytic bacterium. Its presence was detected in most cases in small numbers at the soil level but in some plants pathogenic *Agrobacterium* cells were also detected at different levels in the same plant. It is possible that using theoretically more sensitive techniques like PCR we could be able to detect lower amounts of pathogenic bacteria (Nesme et al., 1989; Sawada et al., 1995), but in these experiments our goal was to demonstrate the presence of living tumorigenic bacteria. Surprisingly, the frequency of isolation of *A. tumefaciens* from symptomless plants of several rose cultivars was very high, because such bacteria were detected in 58% of the analysed plants. It was possible to isolate *A. tumefaciens* at crown and at 5, 10, 15 and 20 cm above it in apparently healthy plants, but the pathogenic bacteria were not regularly distributed inside them.

In contrast with these results, in the other experiments described in this paper the analysed control plants were *Agrobacterium* free. They came from a different origin than those analysed in Table 8. This suggests that plants from different sources could be affected by different inoculum sources (mother plant, soil water) and have different or no *Agrobacterium* contamination.

The presence of endophytic bacteria was previously described on roses because large numbers of bacteria were detected in the stems of cut rose flowers of cv. Sonia (Van Doorn et al., 1991) but the identified bacterial genera were predominantly *Pseudomonas* and *Enterobacter*. As far as we know *Agrobacterium* has not been previously described as an endophytic bacterium in roses but isolates of this genus were found in other plants like sweet corn, cotton (Mc Inroy and Kloepper, 1995) potato (Sturz, 1995) and artichoke (Peñalver et al., 1994). The species *A. tumefaciens* was not identified in these studies of endophytic populations, but *A. radiobacter* (that only differs by the absence of Ti plasmid or other factors of virulence) was detected.

Conventional plating techniques and a selective enrichment before plating were used for studying the movement of the bacteria inside rose plants and for isolating *A. tumefaciens* from apparently healthy plants. Enrichment was performed only in selective media because interference of the *Agrobacterium* growth with the accompanying bacteria was previously observed in nonselective PYGB medium. The results show that both direct plating and selective enrichment were useful in isolating *A. tumefaciens* in different experiments but the frequency of success of each method was variable. At this point it is difficult to suggest the best protocol for isolation of *A. tumefaciens* from the stems of rose plants but a combination of direct plating and selective enrichment has given good results. Due to the low populations of *Agrobacterium* cells detected in the different parts of the plants the techniques are near their limit of sensitivity and some *Agrobacterium* cells could be detected by only one or more techniques. A variable proportion of these *Agrobacterium* colonies recovered was not pathogenic on tomato (data not shown). The instability of the virulence of the agrobacteria inside the plants has been reported and can be a complex phenomenon (Belanger et al., 1992; Fortin et al., 1993; López et al., 1997; Llop and López, 1998).

It is difficult to visually assess whether rose cuttings or mother block plants are infected with *A. tumefaciens* because this bacterium can survive in the vascular system of the rose plants without causing tumors. The control of latent infections of *A. tumefaciens* is difficult because biological control and external disinfection

would not be effective. Prevention is the only solution by early detection in the mother plants and cuttings. Sensitive methods to detect low levels of tumorigenic bacteria inside rose plants could help in sanitary selection for propagation of *A. tumefaciens* free plants. Isolation with a previous enrichment step, and PCR were compared with this purpose in other hosts (Cubero et al., 1996; Cubero et al., in press).

The translocation of *A. tumefaciens* in rose plants and the evidence of a latent period in this host must be taken into consideration for an integrated control of the disease. Its existence has been observed in several rose cultivars grafted on Indica, but similar situation could happen in other combinations. Latent infections and systemic movement can be considered a relatively frequent event in rose plants but unknown abiotic or physiological factors can increase the frequency of such bacterial translocation. This could be favoured under subtropical conditions where the incidence of aerial tumors seems to be higher than in the warm areas.

Our results enlarge the number of species where the movement of *A. tumefaciens* through the vessels of the xylem has been demonstrated and suggest that this bacterium can move in many other hosts. As earlier stated (Suit and Eardley, 1935) the systemic migration can be influenced by the transpiration stream, the number and size of the vessels and the vascular anatomy of the host.

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