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RESEARCH PAPERS

Efficacy of the non-pathogenic *Agrobacterium* strains K84 and K1026 against crown gall in Tunisia

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Summary. The non-pathogenic *Agrobacterium radiobacter* strain K84 and its genetically modified (GEM) strain K1026 were tested for their effectiveness against local Tunisian strains and two reference strains (C58 and B6) of the crown gall bacterium *Agrobacterium tumefaciens*. Tests *in planta* were carried out on herbaceous plants (tomato and tagetes) and on some sensitive rootstocks (bitter almond, peach×almond hybrid GF677 and quince BA29). *In vitro* tests showed that both K84 and K1026 were effective and that the difference between these strains was not statistically significant. On tomato and tagetes, strain K84 was effective against all crown gall isolates with the exception of the *A. tumefaciens* reference strain B6. GEM strain K1026 was very effective against all isolates from Tunisia and against the reference strains. Both antagonistic strains significantly reduced the percentage of galled plants as well as the number of galls per plant. Under field conditions, both antagonists controlled crown gall effectively. Best results were obtained on the bitter almond-tree rootstock. Antagonist effectiveness was less evident on quince BA29 and peach×almond GF677 rootstocks. The genetically modified strain K1026 is of interest in controlling crown gall disease in Tunisia.

Key words: *Agrobacterium tumefaciens*, biocontrol, agrocin 84, ALS 84, rootstock.

Introduction

Various *Agrobacterium* species cause crown gall, a neoplastic disease widespread in temperate areas, and especially in Mediterranean countries. Crown gall affects mainly stone and pome fruit-trees, grapevines, roses and some ornamental plants. The crown-gall bacterium *A. tumefaciens* has a wide host range including 140 plant genera from 100 different families (De Cleene and De Ley, 1976). The disease it causes is especially troublesome in nurseries, losses in orchards are sporadic.

Crown gall is caused by a large plasmid in *Agrobacterium* species called pTi (tumour inducing plasmid) (Ream, 1989). In infection, a region of the pTi, the T-DNA, is transferred from the bacterium to the plant cell genome where it is integrated (Gelvin,

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1992). After integration into the plant genome, the T-DNA genes encode enzymes responsible for the uncontrolled synthesis of the plant hormones auxin and cytokinin, which account for the abnormal tissue proliferation and gall formation on the crown, roots and in some cases the stems.

Good sanitation and cultural practices are necessary for the control of crown gall. Disease-free stocks and pathogen-free soil in the nursery are essential to avoid the disease and rootstocks with low crown gall susceptibility should be combined with management practices that minimise wounding.

For three decades the bacterial antagonist strain *A. radiobacter* K84 has been very effective in preventing crown gall on stone and pome fruit trees (New and Kerr, 1972; Bazzi and Mazzucchi, 1978). The strain is used on the seeds and roots, as well as on the cuttings of propagation material. It is effective against crown gall in various hosts and countries all over the world (Süle and Kollanyi, 1977; López *et al*., 1989; Moore and Canfield, 1996). Nevertheless, the use of K84 has certain problems (Moore and Canfield, 1996). The failure of this strain is mainly due to the transfer of genes controlling agrocin 84 production (pAg84). Resistance can be transferred from strain K84 to a pathogenic *Agrobacterium* recipient, resulting in the loss of biocontrol (Panagopoulos *et al*., 1979) because the recipient then becomes resistant to agrocin 84 and remains pathogenic. In order to avoid this transfer and ensure the biocontrol of crown gall, the transfer Tra region has been deleted by genetic engineering to produce a Tra^- mutant of strain K84, termed K1026 (Jones *et al*., 1988). Therefore except for a small portion of DNA removed from K84, the two strains are essentially identical and have the same characteristics. The removal of this genetic material prevents strain K1026 from transferring a piece of DNA to the bacteria that cause crown gall disease and reduces the likelihood of those bacteria becoming resistant.

In Tunisia, crown gall is an important disease of stone fruit trees (Zouba and Hammami, 1988, Rhouma *et al*., 2001). The disease has spread rapidly with the expansion of fruit tree cultivation and the establishment of new nurseries without adequate phytosanitary standards. Tunisian farmers are now facing problems in producing crown-gall free nursery stocks, due to the lack of information about the disease and difficulties in identifying diseased stocks at an early stage. In spite of the preventive measures, that are being taken, crown gall continues to cause important damage in nurseries and in the field. This study was conducted to test the efficacy of non-pathogenic *A. radiobacter* strains K84 and K1026 against crown gall in the laboratory and in the field.

Materials and methods

Bacterial isolates and strains

Ninety-nine isolates recently obtained from tumours on the collar and roots of fruit trees from different regions of Tunisia were used. The isolates came from different rootstocks: 26 from bitter almond (*Prunus amygdalus*), 25 from GF677 (*Prunus persica*×*P. amygdalus*), 17 from peach

Table 1. Strains of *Agrobacterium tumefaciens* and *A. radiobacter* used in the antagonism assay.

Bacterial species	Strain	Biovar	Origin
Agrobacterium tumefaciens	B6	1	Laboratory of Microbial Ecology (Lyon, France)
	C58	1	Laboratory of Microbial Ecology (Lyon, France)
	MS	1	Mixed strains (LS) from Tunisia isolated from different rootstocks
	AA40	1	Bitter almond
	GF66	1	GF677
	MT6	1	BA29
	MYR7	1	Myrobolan
Agrobacterium radiobacter	K84	2	Biocare Technology (NSW, Australia)
	K1026	2	Biocare Technology (NSW, Australia)

(*Prunus persica*), 11 from Myrobolan (*Prunus cerasfera*), 3 from MM106 (*Malus domestica*), 7 from BA29 (*Cydonia oblonga*), and also from olive trees (*Olea europea*) (5 isolates) and from soil (5 isolates).

For biocontrol of crown gall, two reference strains of *A. tumefaciens* (B6 and C58) and some virulent strains, separately or mixed, were used (Table 1). The experiments were carried out with the two antagonistic *A. radiobacter* strains K84 and K1026.

Isolation of *Agrobacterium* from galls

Galls were washed under running tap water to remove adhering soil particles, surface-sterlised by dipping into 0.5% v:v sodium hypochlorite for 2 min, rinsed three times with sterile distilled water, and blotted dry on sterile filter paper. Small portions were aseptically removed from each gall and macerated in sterile distilled water. The resulting suspension was left to stand for 30 min, then a loopful was streaked on D1 medium plate (Kado and Heskett, 1970) and incubated for 4 days at 27°C.

Circular olive-green colonies indicated the probable occurrence of *Agrobacterium* colonies. *Agrobacterium*-like colonies were selected and purified on YPGA medium (yeast extract 5 g, peptone 5 g, glucose 10 g, agar agar 20 g, distilled water 1000 ml) according to the description of Moore *et al*. (1988). Purified agrobacterial colonies were stored in sterile distilled water at 4°C until use.

Biovar affiliation

Twenty four cultures of the obtained isolates were tested for urease and esculine. The isolates found to be *Agrobacterium* by their positive reaction to these tests, were separated into biovars using different biochemical and physiological tests (Moore *et al*., 1988), including Gram staining, oxidation of lactose to 3-ketolactose, growth on Simmon's citrate sodium medium, growth on L-tyrosine, tolerance to 2% (w:v) sodium chloride (NaCl), growth and pigmentation on ferric ammonium citrate medium and acid production of erythriol.

Pathogenicity determination

The gall-forming ability of isolates was determined following the method of Moore *et al*. (1988), by inoculating three wounded stems of four-week-old seedlings of two indicator plants, tomato (*Lycopersicum esculentum*) and *Kalanchoe daigremontiana,* with a dense suspension of 48-h-old bacterial cultures (10^8 cfu ml^{-1}). Gall formation was assessed by visual inspection 3 weeks after inoculation. The reference strains C58 and B6 were used as positive controls.

Effect of the antagonists K84 and K1026 *in vitro*

In vitro sensitivity of the local isolates and reference strains to the substances produced by strains K84 and K1026 was tested by the method of Vidaver (1976) and Stonier (1960). For sensitivity to the Antibiotic-Like Substance ALS84, isolates were grown in a Mannitol-glutamate (MG) medium (Moore *et al*., 1988) and the test was performed according to Peñalver *et al*. (2001). MG or stonier's medium plates were inoculated by spreading a loopfull of the antagonistic strains K84 and K1026, at the centre of each dish. Dishes were incubated for 48 h at 27°C, and then exposed to chloroform to kill the antagonistic strains. A suspension in water (100 μl) of the isolate tested (10^8 cfu ml^{-1}) was mixed with 4.5 ml of molten soft agar (0.6% agar in 20 mM phosphate buffer [pH 7]) at 45°C and overlaid on MG medium or Stonier's medium. If after 48 h of incubation at 27°C, a translucent inhibition zone appeared around the antagonist, the isolate tested was considered sensitive to agrocin 84 and ALS84. Isolates that did not produce an inhibition zone were considered resistant, while isolates producing inhibition zones with a diameter of <1.5 cm, of 1.5–3.0 cm, and of >3 cm were considered to have low, medium and high sensitivity respectively.

Effect of antagonists K84 and K1026 *in planta*

Agrobacterium radiobacter strains K84 and K1026 were tested against pathogenic strains of *Agrobacterium* under field conditions on herbaceous plants (tomato and tagetes) and on three stone fruit tree rootstocks.

Field experiments were conducted in a nursery located in the region of Shbika (centre of Tunisia) specialised in the cultivation of stone-fruit trees. Tests were performed with 1-year-old rootstocks of bitter almond and quince (BA29), pro-

vided by the Coopérative Centrale des Semences et des plantes Sélectionnées) and with the rootstock peach×almond (GF677), which was purchased from Italy.

Herbaceous plant tests

For root and stem inoculation of tomato (*Lycopersicum esculentum* L.) and tagetes (*Tagete patula* L.), bacterial suspensions were prepared by suspending the 24-h-old agrobacterial cultures in sterile distilled water to a final concentration of 10^8 cfu ml^{-1}.

Tomato and tagetes plants were root-inoculated simultaneously with one of the antagonists (K84 or K1026) and with the pathogenic *Agrobacterium* strain. Inoculation was performed by soaking the roots of the plants for 30 min in the antagonist suspension and in the bacterial suspension of the pathogen for further 30 min. Inoculated plants were transplanted to pots and placed in the field. Control plants were treated only with sterile distilled water.

The stems were inoculated with the antagonists and the pathogenic strains by introducing 10 μl suspension into 1 centimeter longitudinal wounds made with a sterile scalpel. Control inoculations were with sterile distilled water.

Rootstock experiments

Effect of the antagonists on potted rootstocks

The same rootstocks used in naturally contaminated field conditions were tested in pots containing sterile soil. Fifty plants per rootstock were root-inoculated with the potential antagonists (*A. radiobacter* K84 and K1026) and with the pathogenic strains by soaking for 30 min in the bacterial suspension (10^8 cfu ml^{-1}) and were then transplanted to pots. Tumor development was determined after 9 months.

Effect of the antagonists in the field

The test was conducted in a field naturally contaminated with *A. tumefaciens* in a nursery of the region of Chbikha (Kairouan). The occurence of pathogenic bacteria in this field was monitored by evaluating tumour formation on *Agrobacterium*-free plants (propagated *in vitro*).

The pruned roots of rootstocks were soaked in bacterial suspensions of the antagonistic strains K84 and K1026 at 10^8 cfu ml^{-1} and then transplanted to field plots. After 9 months, the percentage of infected plants, and the number and weight of the galls was determined.

Data analysis

Data were subjected to analysis of variance (ANOVA). The significance of the mean differences was determined by Duncan's test, and responses were judged significant at the 5% level (P=0.05).

Results

Biovar affiliation and pathogenicity

All the ninety-nine isolates were identified as *Agrobacterium* since they reacted positively to urease and esculine. On the basis of their reaction to

Table 2. Results of biovar affiliation and pathogenicity tests for *Agrobacterium* isolates.

Host	Isolate No.	Biovar affiliation	Pathogenicity	
			No. of virulent isolates on tomato	No. of virulent isolates on kalanchoe
Bitter almond	26	19 B1, 5B2, 5B1/2	18	12
GF677	25	15 B1, 3 B2, 7 B1/2	14	11
Peach	17	10 B1, 1 B2, 4 B2	10	7
Myrobolan	11	8B1, 3 B1/2	8	8
MM106	3	1 B1, 2 B1/2	1	0
BA29	7	5 B1, 1 B2, 1 B1/2	4	3
Olive tree	5	3 B1, 2 B1/2	3	3
Soil	5	3 B1, 2 B1/2	2	2

the various tests, the isolates were divided into three groups (Table 2):

1. Members of group 1 oxidized lactose to 3-ketolactose, produced pigments on ferric ammonium citrate and acid from sucrose, and grew on nutrient agar supplemented with 5% NaCl. Members of this group varied in the extent to which they utilised sodium citrate in Simmon's citrate medium and reduced propionic acid to alkali compounds. The reactions of these isolates were identical to those of the *A. tumefaciens* reference strains C58 and B6 (biovar 1);

2. Members of group 2 did not oxidise lactose to 3-ketolactose, or produce pigments from ferric ammonium citrate. They did not oxidise sucrose to acid or reduce propionic acid to alkali compounds. They did not grow on nutrient agar supplemented with 5% NaCl. They did however utilise sodium

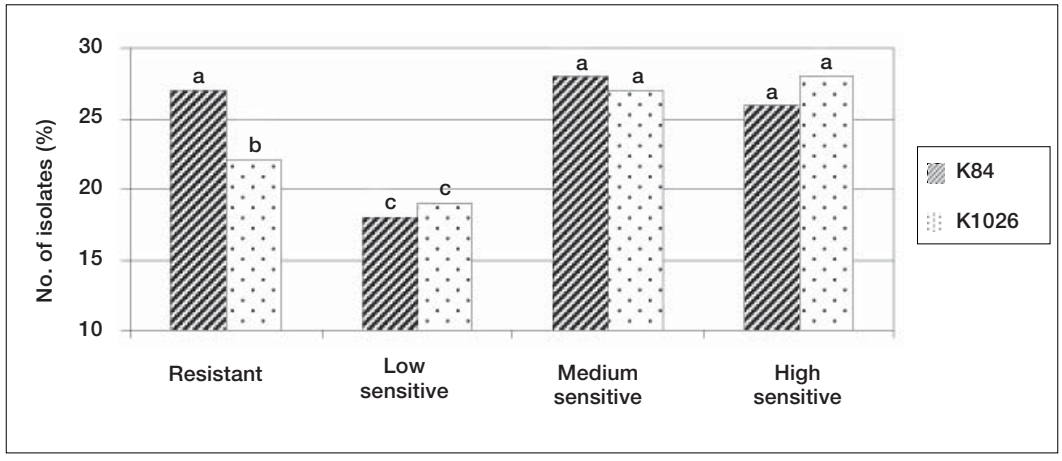

Fig. 1. Distribution of the isolates according to their sensitivity to *Agrobacterium radiobacter* strains K84 and K1026. Bars with the same letters are not significantly different at P=0.05.

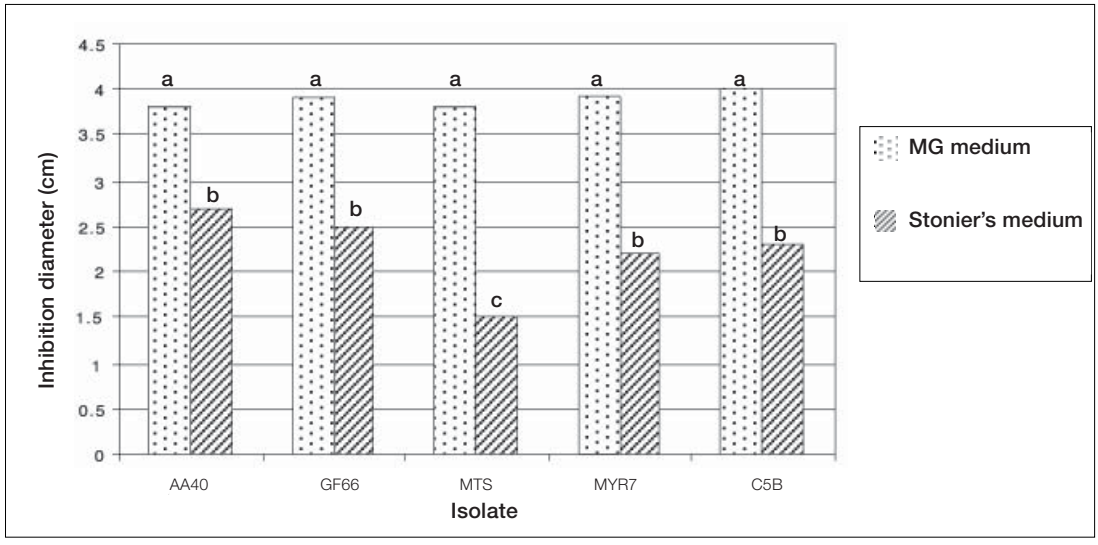

Fig. 2. Inhibition diameter on MG and Stonier's media for some *Agrobacterium tumefaciens* isolates sensitive to *A. radiobacter* strain K84. Bars with the same letter are not significantly different at P=0.05.

citrate in Simmon's citrate medium. The reactions of these isolates were identical to those of K84 and K1026 (biovar 2);

3. Members of group 3 were allocated to an intermediate biovar since their reactions to the different tests were different from those of the other two groups.

When bacterial suspensions were applied to the wounded stems of tomato or kalanchoe seedlings, 59 isolates induced tumours on tomato

Table 3. Effectiveness of the antagonistic *Agrobacterium radiobacter* strains K84 and K1026 in preventing tumours on tomato and tagetes roots.

Strain	Treatment	Tomato			Tagetes		
		No. of plants analysed	No. of plants with galls (%)	Index of biocontrol efficiency[a]	No. of plants analysed	No. of plants with galls (%)	Index of biocontrol efficiency [a]
MS	Untreated	15	100		13	100	
	K84	15	0	100	15	0	100
	K1026	14	0	100	15	0	100
C58	Untreated	15	90		14	100	
	K84	15	0	100	15	0	100
	K1026	15	0	100	15	0	100
B6	Untreated	15	100		14	93	
	K84	14	64	36	15	66	29
	K1026	15	0	100	15	0	100

[a] Index of crown gall biocontrol = 100% - ([% of disease incidence in treatment×100) / [% of disease in corresponding untreated control]) according to Peñalver and López (1999).

Table 4. Effectiveness of the antagonistic *Agrobacterium radiobacter* strains K84 and K1026 in preventing tumours on tomato and tagetes stems.

Strain	Treatment	Tomato			Tagetes		
		No. of plants analysed	No. of plants with galls (%)	Index of biocontrol efficiency[a]	No. of plants analysed	No. of plants with galls (%)	Index of biocontrol efficiency [a]
MS	Untreated	15	95		15	100	
	K84	15	0	100	15	0	100
	K1026	15	0	100	15	0	100
C58	Untreated	15	100		15	86	
	K84	15	0	100	15	0	100
	K1026	15	0	100	15	0	100
B6	Untreated	15	100		15	95	
	K84	15	40	60	15	46.66	50.88
	K1026	15	0	100	15	0	100

[a] See Table 3.

seedlings after 3 weeks, while 46 isolates induced tumours on kalanchoe after 4 weeks. Some isolates induced tumours on both tomato and kalanchoe.

Effect of antagonist strain K84 and K1026 *in vitro*

Figure 1 shows the distribution of isolates in accordance with their sensitivity to K84 and K1026. A clear inhibition zone formed around the K84 and K1026 colonies with 77% of isolates. Twenty-seven per cent of isolates were resistant to strain K84 and 22% to K1026. Except for resistant isolates, strain K1026 was as efficient as strain K84. Other than in the resistant isolates, there were no significant difference between the two antagonists.

The inhibition diameter (Fig. 2) was significantly longer in a Mannitol-glutamate medium than in Stonier's medium. These two media differ in their composition, including their iron concentration.

Effect of the antagonists on herbaceous plants

Root and stem inoculations

Table 3 and 4 show the effect of the antagonistic strains K84 and K 1026 in protecting tomato and tagetes plants from tumour formation after inoculation of pathogenic agrobacteria. We noted the effectiveness of the antagonist K84 against the local Tunisian isolates (MS) and against the reference strain C58. However, this antagonist was ineffective against strain B6, confirming the resistance of this strain to agrocin 84. Similar results were obtained on the roots (Table 3) and stems (Table 4) of these plants. Interestingly, strain K1026 was highly effective

Table 5. Effect of the antagonistic *Agrobacterium radiobacter* strains K84 and K1026 on gall formation in potted rootstocks.

Rootstock	Strain/isolate	Treatment	No. of plants analysed	No. of plants with galls (%)	No. of galls	Index of biocontrol efficiency[a]
Bitter almond	Local (MS)	Control	45	95.5	101	
		K84	42	7	3	92.6
		K1026	50	4	1	95.8
	C58	Control	45	80	79	
		K84	45	20	15	75
		K1026	57	3.5	1	95.6
GF 677	Local (MS)	Control	43	72	20	
		K84	44	0	0	100
		K1026	44	0	0	100
	C58	Control	51	59	22	
		K84	49	0	0	100
		K1026	48	0	0	100
BA29	Local (MS)	Control	50	36	13	
		K84	55	1.8	1	95
		K1026	49	0	0	100
	C58	Control	54	12.9	4	
		K84	47	0	0	100
		K1026	45	0	0	100

[a] See Table 3.

Table 6. Effect of the antagonistic *Agrobacterium radiobacter* strains K84 and K1026 on gall formation in rootstocks under field conditions.

Rootstock	Treatment	No. of plants analysed	No. of plants with galls (%)	Index of biocontrol efficiency[a]
Bitter almond	Untreated	47	100	
	K84	59	3	97
	K1026	117	3	97
Peach × almond GF677	Untreated	229	3.9	
	K84	220	0.5	87.2
	K1026	281	0	100
Quince BA29	Untreated	201	1.3	
	K84	227	0.5	61.5
	K1026	219	0	100

[a] See Table 3.

against all the tested isolates, including the reference strain B6.

Effect of antagonistic strains K84 and K1026 on woody rootstocks

The comparative efficacy of strain K84 and its GEM K1026 against local isolates and the reference strains of *A. tumefaciens* is summarised in Table 5. Both antagonists significantly reduced the percentage of infected plants as well as the number of galls regardless of the pathogenic *Agrobacterium* strain used for inoculation. It appears that strain K1026 was as effective as strain K84, as revealed by their index of biocontrol efficiency.

Both antagonists were effective in preventing gall formation in the field (Table 6). Their effectiveness was greater with the bitter almond rootstock than with the two others rootstocks, quince BA29 and peach×almond GF677. There was a significant reduction in the number of galled plants and in the number of galls per plant.

Although the test soil was heavily contaminated, the quince BA29 and peach×almond GF677 rootstocks showed a very low percentage of infected plants and few galls.

Discussion

During field inspection, crown gall was found in all fruit-tree growing areas in Tunisia, particularly in stone fruit rootstocks. The highest percentage of *Agrobacterium* isolates (50%) was in bitter almond and peach×almond rootstocks, followed by peach (17%), Myrobolan (11%), BA29 (7%), MM106 (3%), soil (5%) and olive (5%). The most common isolates in Tunisia were biovar 1 isolates (64.64%), followed by the intermediate biovar (23%) and biovar 2 (10%).

The majority of isolates of biovar 1 (94%) induced galls on tomato seedlings, but only 73% of isolates caused tumours on kalanchoe seedlings. This indicated that tomato seedlings were more sensitive to *A. tumefaciens* than were *Kalanchoe daigremontiana* seedlings, and that a single plant might be insufficient as an indicator plant of a pathogenic isolate.

In both *in vitro* and *in planta* experiments, strains K84 and GEM K1026 were clearly antagonistic to a number of virulent *Agrobacterium* strains of different origins, including strains from Tunisia.

Comparison of strains K84 and K1026 *in vitro* showed that the most sensitive isolates belonged to biovar 1. These results are consistent with López *et al*. (1987) and Vicedo *et al*. (1993), who reported that biovar 1 was more sensitive to agrocine 84 than biovar 2.

In vitro antagonism tests showed that the inhibition zones were wider on a Mannitol-glutamate medium than on Stonier's Medium**.** These results were consistent with Peñalver *et al*. (2001),

who reported that in an iron-deficient medium, the crown gall biocontrol agent K84 produced a hydroxamate iron chelator and also an antibiotic-like substance (ALS). These authors found a correlation between levels of a hydroxamate siderophore and ALS K84 in strain K84, and they concluded that the two compounds had a biosynthetic route in common and might be the same. These findings, warrant the conclusion that both ALS 84 and siderophores contribute to the biological control of crown gall in soils with an iron deficiency.

Experiments *in planta*, showed the effectiveness of K84 and K1026 as biological control agents of crown gall, especially on bitter almond rootstock. This degree of antagonism was not observed on the two other rootstocks because of the low percentage of untreated plants that were infected. This can be explained by the soil selection of virulent strains of *Agrobacterium* adapted to bitter almond rootstock.

Our results showed that GEM strain K1026 is of potential interest for the biocontrol of crown gall of stone fruit-trees in Tunisia. To our knowledge, this is the first report on the effectiveness of this strain under North African conditions.

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