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Occurrence and distribution of crown gall disease in Jordan

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Summary. Field inspection indicated that crown gall disease occurs in various fruit-tree-growing areas in Jordan, including Almafraq, Alsalt, Alshobak, Altafila, Alyadoda, Amman, Irbid, Jerash, Jordan Valley and Madaba. Two-hundred tumorigenic *Agrobacterium* isolates were obtained from stone fruit rootstocks (bitter almond, GF677, GF305, Myrobalan, Nemaguard and Mahaleb), pome fruit rootstocks (apple seedling, quince, and clonal rootstock MM106), grapevine, olive, pomegranate, carob, rose and C*ichorium pumilum*. The pathogenicity of these isolates was proved through artificial inoculation on tomato seedlings and *Kalanchöe* plants, and through *tmr* gene detection with PCR. Biochemical and physiological testing of the isolates indicated the occurrence of biovar 1 (60.5%), biovar 2 (23.5%), and biovar 3 (1%), in addition to an intermediate biovar (15%). Seventy-seven percent of isolates were found to be sensitive to agrocin 84; of these, most (66.9%) belonged to biovar 1.

Key words: *tmr* gene, *Agrobacterium*, latent infection.

Introduction

Crown gall induced by *Agrobacterium tumefaciens* (Smith and Townsend) Conn. is a soil-borne disease (Kerr and Brisbane, 1983), characterized by the formation of tumors at wound-sites (Pionnant *et al.*, 1999), systemic infection in the xylem, or it can occur as a latent infection (Marti *et al.*, 1998).

In Jordan, crown gall is one of the most common bacterial diseases attacking fruit trees (Fakhouri and Khalaif, 1995), the disease was noticed to spread very rapidly with the expansion in fruit tree planting and establishing new nurseries without adopting suitable phytosanitary measures. The area planted with fruit trees has expanded during the last few years throughout the country and now comprise about 86,945 hectares (Anonymous, 2000). Jordanian farmers and nurserymen are 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 the early stages.

The most common methods for the detection of *Agrobacterium* strains are by isolation on selective media, identification with biochemical tests and testing for pathogenicity on various indicator plant species (Nesme *et al.,* 1989).

In more recent years Polymerase Chain Reaction (PCR) has been employed to detect *Agrobacterium* from various sources as well as other phytopathogenic bacteria including: *Pseudomonas, Xanthomonas, Clavibacter* and *Erwinia* (Boer *et al.,* 1995; Hass *et al.,* 1995; Karjalainen *et al.,* 1995).

In this paper a PCR diagnostic protocol was used to detect *tmr* gene from tumor samples and latent infection. PCR proved to be more efficient than direct isolation on selective media for detection of *Agrobacterium* biovars from tumors and symptomless plants.

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Materials and methods

Collection and isolation of *Agrobacterium* biovars

Orchards, nurseries of fruit-tree and roses and some herbaceous plants were inspected for crown gall in field trips made to different areas in Jordan.

Samples of newly developed galls from infected plants or seedlings were collected, placed in a refrigerator and taken to the laboratory. Plant samples were washed under running tap water to remove adhering soil particles, surface-sterilized by dipping into 0.5% v:v sodium hypochlorite for 2 min, rinsed 3 times with sterile distilled water (SDW), and blotted dry on sterile filter paper. Small portions were aseptically removed from each sample and placed in few drops of SDW in a mortar and pestle for maceration. The resulting suspension was left to stand for 30 min, then a loopful of it was streaked on the surface of a MG or D1 medium plate (Moore *et al.*, 1988).

Plates inoculated with the suspensions were incubated at 25±2°C until bacterial growth developed. Circular, slimy, white-to-beige colonies on MG medium, or circular olive-green colonies on D1 medium indicated the probable occurrence of *Agrobacterium* colonies. Four colonies with the characters of *Agrobacterium* colonies from each plate were then separately streaked on new MG plates and incubated at 25±2°C until bacterial growth developed. A single white colony from each isolate was restreaked onto a KB medium plate (King *et al.*, 1956) for purification and elimination of fluorescent *Pseudomonas* spp. The pure bacterial isolates suspected to be *Agrobacterium* were grown on MG slants and kept in the refrigerator for further identification (Fahy and Hayward, 1983; Moore *et al.*, 1988).

Identification of biovars

Twenty-four-hour-old cultures of the bacterial isolates were subjected to biochemical and physiological tests for identification according to the methods described by Moore *et al.* (1988). The tests included: urease production, esculine hydrolysis, 3-ketolactose production, sodium chloride tolerance, pigmentation on ferric ammonium citrate, growth on Simmon's citrate medium, acid production from sucrose, alkali production from propionic acid and agrocin sensitivity. All the tests were also run against reference cultures of *A. tumefaciens* C58, B6, and 251/12 (biovar 1) provided by M. Lòpez (Valencia, Spain), and against *A. rhizogenes* 8302 (biovar 2) and *A. vitis* 5858 (biovar 3) to serve as controls.

Pathogenicity tests

Four-week-old seedlings of the two indicator plants, tomato (*Lycopersicum esculentum* Mill cv. GS), and *Kalanchöe daigremontiana* (Kerr and Brisbane, 1983; Moore *et al.*, 1988), were wounded by making a 2–3 mm slit with a sterile scalpel in the crown area, 1 cm above the soil surface. A bacterial mass of each isolate was applied to the wound site with a sterile toothpick. Another set of seedlings was inoculated with the reference cultures C58, B6 and 251/12, and yet another group was wounded only, without inoculation, for comparison purposes. The seedlings were placed on a bench in the greenhouse and checked periodically for tumors.

Detection of *tmr* gene from pure cultures

DNA extraction from bacterial cultures

Twenty-four-hour-old cultures of the identified isolates were subjected to *tmr* gene detection by PCR as described by Ponsonnet and Nesme (1994); Marti *et al.* (1998) and Cubero *et al.* (1999). One ml of a 10^7 cfu ml^{-1} bacterial suspension prepared from 24-h-old cultures of each isolate was placed in a microcentrifuge tube (1.5 ml). Tubes were centrifuged at 13,000 rpm for 5 min, the supernatants were discarded and the pellets were re-suspended in 500 µl filtered SDW and shaken in a vortex, heated to 95°C for 5 min, and centrifuged again at 5,000 rpm for 5 min. Then 500 µl of the resultant supernatant of each isolate was placed in another microcentrifuge tube, centrifuged again at 13,000 rpm for 10 min, after which the supernatant fluid was removed and pellets were re-suspended in 100 µl of nuclease-free water (Promega, Madison, USA), and stored at -20°C for PCR amplification. A positive control (C58) and a negative control, cured Ti plasmid derivative of C58 (C58c1) were also included.

PCR amplification

Primers F49*tmr* (5'-CCATGTTGTTTGCTAGCCAG-3') and F50*tmr* (5'-CCTTCGAATCCGTCGAAAGC-3') were used for the amplification reactions in order to detect the *tmr* gene on the T-DNA of the tested bacterial isolates (Nesme *et al.*, 1989).

PCR reactions were performed in 50 µl containing: 5 µl cell suspension, 1× (5 µl of 10×) PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, pH 9.0 at room temperature), 0.2 mM (1 µl of 10 mM) of each of the four d-NTPs mixtures, (5 µl) formamide 1%, 0.1 µM (5 µl of 1 µM working dilution) of each as F49*tmr* and F50*tmr* primers, and 2U (0.4 µl of 5 U μl^{-1}) of *Taq* DNA polymerase (Promega).

The amplification reaction was performed in a 9700 Perkin-Elmer thermal cycler using the following protocol: initial denaturation at 93°C for 5 min, followed by 40 cycles of 1 min at 93°C for denaturation, 1 min at 60°C for annealing, 2 min at 72°C for extension, and an additional extension at 72°C for 10 min. After the amplification reaction, the samples were stored at 4°C for electrophoresis (Nesme *et al.,* 1989; Marti *et al.*, 1998; and Cubero *et al.*, 1999).

Gel electrophoresis

Three µl of the PCR products, mixed with 7 µl of (6×) bromophenol blue as a loading dye, were separated by horizontal agarose electrophoresis in 1× TBE buffer (Promega), 2% (w:v) LE agarose (Promega, USA) containing 1 µg ml^{-1} ethidium bromide. The marker molecular weight was 100 bp ladder (Promega). The electrophoresis reaction was performed at 75 V for 90 min; the gels were photographed under UV light (302 nm).

Detection of *tmr* gene from tumors

One hundred and twenty tumors from stone fruit rootstocks including bitter almond (*Prunus amygdalus*), GF677 (*P. persica* × *P. amygdalus*), Myrobalan (*P. cerasifera*) and GF305 (*P. persica*), naturally infected with crown gall were used for isolation of *Agrobacterium* and for detection of *tmr* gene from plant tissues with PCR. Isolation, identification and pathogenicity-testing of the isolates were as described above.

PCR detection

DNA extraction from tumors on stone fruit root stocks, tomato plants artificially inoculated with C58 (positive control) and from healthy tomato plants (negative control) was based on the protocol of Edwards *et al.* (1991), modified by Marti *et al.* (1998), Cubero *et al.* (1999) and Llop *et al.* (1999). Briefly, 1 µl of plant extract was placed in a 1.5 ml microcentrifuge tube, centrifuged at 13,000 rpm for 5 min, after which the supernatants were discarded and the pellets were re-suspended in 500 µl filter-sterilized extraction buffer containing: 200 mM Tris HCL, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 2% PVP-15, pH 7.5. Tubes were vortexed and shaken for 1 h at room temperature, heated to 95°C for 5 min for DNA lyses, then centrifuged at 5,000 rpm for 5 min. The supernatant (450 µl) of each extract was placed in another microcentrifuge tube, containing 450 µl of 99% isopropanol. Tubes were mixed gently and left at room temperature for 1 h. The mixture was centrifuged again at 13,000 rpm for 10 min, the supernatant was removed and the pellets were air-dried at room temperature for 1 h by inverting the microcentrifuge tubes on sterile filter paper. The pellets were re-suspended in 100 µl nuclease-free water, and stored at -20°C until use in PCR. PCR reactions were performed in a total volume of 50 µl, as described above, except that 2.5 U of *Taq* DNA polymerase was used, then the PCR products were subjected to electrophoresis as previously described.

Detection of *tmr* gene from symptomless plants

One hundred and twenty bare rooted seedlings of 1-year-old stone fruit rootstocks (100 of GF677, 20 of apricot) were planted in pots (20×20 cm) filled with methyl bromide fumigated soil in July 1999. Before planting the seedlings were checked for tumors and only disease-free seedlings were planted. The stems of these seedlings were wounded with a sterile scalpel at the soil surface and 15 cm above the soil surface. The pots were placed on a greenhouse bench for 1 year. Seedlings were checked periodically for tumor formation on the wound sites, and DNA was extracted from plant sap for detection of the *tmr* gene as described.

The plants were uprooted and samples (crown area and part of the root system) were taken to the laboratory and washed under running tap water to remove adhering soil particles. Isolation and PCR detection was performed as described above, except that 0.5 g of plant tissue was taken from the crown area and macerated in water.

Data analysis

For detection of *Agrobacterium* from tumors and symptomless plants, the proportions were compared using a normal approximation test accord-

ing to the formula:

$$Z = P_1 - P_2 / [\hat{P}\,(1 - \hat{P})\,(1/n_1 + 1/n_2)]^{1/2}$$

where P_i is the estimated proportion of samples, tumorigenic *Agrobacterium* is detected by a given method in n_i samples, and $\hat{P}$ is the weighted average of the proportion with the two methods compared. All pairwise comparisons between proportions were calculated at an overall significance level of 5% (Steel and Torrie, 1980).

Results

Collection and isolation of *Agrobacterium* biovars

Gall samples were collected from diseased trees, growing in various fruit tree growing areas including Almafraq, Alsalt, Alshobak, Altafila, Alyadoda, Amman, Irbid, Jerash, Jordan valley and Madaba (Table 1).

Circular, slimy and white-to-beige *Agrobacterium* colonies grew on the MG plates, and circular, convex, light-blue to dark-olive-green colonies grown on the D1 plates streaked with a suspension of gall samples from naturally infected plants. By contrast, no fluorescent bacterial colonies were detected when cultures of the isolates were streaked onto KB plates and subjected to dark UV light (367 nm).

Pathogenic *Agrobacterium* was isolated from different hosts including stone fruit rootstocks (bitter almond [*Prunus amygdalus*], GF677 [*P. persica* × *P. amygdalus*], Myrobalan [*P. cerasifera*], GF305 [*P. persica*], Nemaguard [*P. persica* × *P. davidiana*] and Mahaleb [*P. mahaleb*]), pome fruit rootstocks (apple seedling [*Malus domestica*], MM106 and quince [*Cydonia oblonga*]), grapevine (*Vitis* spp.), pomegranate (*Punica granatum*), rose (*Rosa* spp.), carob (*Ceratonia siliqua*), olive (*Olea europaea*) and *Cichorium pumilum*.

Identification of biovars

The reactions of the bacterial isolates to the biochemical and pathogenicity tests are presented in Table 1. Two hundred isolates were *Agrobacterium* since they reacted positively to urease and esculine. Based on their reactions to the various tests the isolates could be divided into 4 groups:

1 – Members of this group oxidized lactose to 3-ketolactose, produced pigments on ferric ammonium citrate and acid from sucrose, and grew on nutrient agar supplemented with 5% NaCl. There were variations in the extent to which isolates utilized sodium citrate in Simmon's citrate medium and reduced propionic acid to alkali compound. The reactions of these isolates were identical to those of C58, B6 and 251/12 (biovar 1, *A. tumefaciens* reference culture) in these tests.

2 – Members of this group did not oxidize lactose to 3-ketolactose, or produce pigments from ferric ammonium citrate. They did not oxidize sucrose to acid or reduce propionic acid to alkali compounds. They did not grow on NA supplemented with 5% NaCl; instead, isolates utilized sodium citrate in Simmon's citrate medium. The reactions of these isolates were identical to the reactions of 8301 (biovar 2, *A. rhizogenes* reference culture) in these tests.

3 – Members of this group oxidized lactose to 3-ketolactose, utilized sodium citrate in Simmon's citrate medium, produced acid as a result of sucrose oxidation, and grew on NA supplemented with 5% NaCl. These isolates did not produce pigments from ferric ammonium citrate or reduce propionic acid to alkali. Their reactions were identical to the reactions of 5858 (biovar 3, *A. vitis* reference culture) in these tests.

4 – Members of this group were allocated into an intermediate biovar since their reactions to the tests were unlike those of the other groups.

Most isolates came from stone fruit rootstocks. Seventy-four were from bitter almond: of these, 36, 23, and 15 belonged to biovar 1, 2, and intermediate respectively. Thirty-eight isolates came from GF677: 22 from biovar 1, 7 from biovar 2, and 9 from the intermediate biovar. Twenty-four isolates came from Myrobalan: 12, 6, and 6 from biovar 1, 2, and intermediate respectively. Eight isolates of biovar 1 were isolated from Mahaleb, and 8, also of biovar 1 from GF305. Five isolates from Nemaguard belonged to biovar 2.

On the other hand, fewer isolates came from pome fruit rootstocks. The 4 isolates from quince, and the 2 isolates from apple all belonged to biovar 2. Three isolates belonging to biovar 1 came from MM106, while only 2 isolates of biovar 3 came from grapevine. Eight, 5, 3, 7, and 9 isolates were isolated from rose, olive, pomegranate, carob bean, and *Cichorium pumpilum* respectively, all belonging to biovar 1.

Table 1. Source and results of pathogenicity and agrocin 84 sensitivity tests for *Agrobacterium* biovars isolated from Jordan.

Location	Host	No. of isolates	Biovar[a]	PCR *tmr*	Pathogenicity		Agrocin 84 sensitivity[b]
					Tomato	Kalanchöe	
Almafraq	Rose	6	6 B1	6/6	6/6	0/6	6 S
Almafraq	GF677	17	13 B1, 4 B1/2	17/17	16/17	1/17	13 S, 4 S
Almafraq	Mahaleb	8	8 B1	8/8	8/8	0/8	5 S, 3 R
Almafraq	Quince	4	4 B2	4/4	2/4	2/4	4 S
Sub-total		35 (17.5%)[c]	27 B1, 4 B1/2, 4 B2	35	32	3	32 S, 3R
Alsalt	Apple	2	2 B2	2/2	2/2	0/2	2 S
Sub-total		2 (1%)	2 B2	2	2	0	2 S
Alshobak	Myrobalan	12	6 B2, 6 B1/2	12/12	11/12	1/12	6 S, 5 S, 1 R
Alshobak	GF305	8	8 B1	8/8	7/8	1/8	8 S
Sub-total		20 (10%)	8 B1, 6 B2, 6 B1/2	20	18	2	19 S, 1 R
Altafila	Bitter almond	7	7 B2	7/7	7/7	0/7	7 S
Sub-total		7 (3.5%)	7 B2	7	7	0	7 S
Alyadoda	Bitter almond	34	17 B1, 11 B2, 6 B1/2	34/34	31/34	3/34	15 S, 2 R, 1 S, 10 R, 4 S, 2 R
Alyadoda	MM106	3	3 B1	3/3	3/3	0/3	3 S
Sub-total		37 (18.5%)	20 B1, 11 B2, 6 B1/2	37	34	3	23 S, 14 R
Amman	Rose	2	2 B1	2/2	2/2	0/2	2 S
Amman	*Cichorium pumilum*	9	9 B1	9/9	9/9	0/9	9 S
Amman	Bitter almond	4	4 B1	4/4	4/4	0/4	4 S
Sub-total		15 (7.5%)	15 B1	15	15	0	15 S
Irbid	Olive	5	5 B1	5/5	5/5	0/5	5 S
Sub-total		5 (2.5%)	5 B1	5	5	0	5 S
Jerash	Bitter almond	23	9 B1, 5 B2, 9 B1/2	23/23	23/23	0/23	9 S, 5 S, 4 S, 5 R
Jerash	Grapevine	2	2 B3	2/2	0/2	0/2	2 R
Jerash	Carob	7	7 B1	7/7	7/7	0/7	7 S
Sub-total		32 (16%)	16 B1, 5 B2, 9 B1/2, 2 B3	32	30	0	25 S, 7 R
J. Valley	GF677	7	7 B2	7/7	7/7	0/7	7 S
J. Valley	Pomegranate	3	3 B1	3/3	3/3	0/3	3 S
Sub-total		10 (5%)	3 B1, 7 B2	10	10	0	10S
Madaba	Bitter almond	6	6 B1	6/6	5/6	1/6	5 S, 1 R
Madaba	Nemaguard	5	5 B2	5/5	1/5	4/5	5 R
Madaba	GF677	14	9 B1, 5 B1/2	14/14	3/14	11/14	3 S, 6 R, 2 S, 3 R
Madaba	Myrobalan	12	12 B1	12/12	9/12	2/12	6 S, 6 R
Sub-total		37 (18.5%)	27 B1, 5 B2, 5 B1/2	37	18	18	16 S, 21 R
Total		200	121 B1, 47 B2, 30 B1/2, 2 B3	200/ 200	171/ 200	26/ 200	154 S, 46 R

[a] B1, B2, B3: biovar 1, 2, 3; B1/2, intermediate biovar.
[b] S, sensitive; R, resistant.
[c] In parentheses values in percentage over the total.

Pathogenicity tests

When the bacterial suspension or a mass of bacteria was applied to wounded stems of tomato or *Kalanchöe* seedlings, 171 isolates induced tumors on tomato seedlings after 4 weeks, while 26 isolates after more than 4 weeks induced tumors only on *Kalanchöe* seedlings. Isolates from grapevine did not induce tumors on tomato or *Kalanchöe* seedlings (Table 1). Tumors started at the wound sites as a swelling, then increased in size and became fleshy. The reference cultures of *Agrobacterium* (C58, B6, 251/12 and 8302) also induced tumors on the indicator plants.

Detection of *tmr* gene from pure cultures

Results of *tmr* amplification using template DNA from 200 isolates of *Agrobacterium* showed that 121 isolates of biovar 1, 47 isolates of biovar 2, 30 of the intermediate biovar, and 2 of biovar 3 yielded a band of 172 bp (Fig. 1) identical to that of *tmr*, and similar to that of C58. No band was detected by DNA extraction from C58c1.

Agrocin 84 sensitivity

A clear zone formed around K84 colonies with 77% of isolates when the K84 mass was spotted on MG plates supplemented with 2 μg l^{-1} biotin inoculated with the isolate suspensions. Of these, 66.9% belonged to biovar 1, 20.8% to biovar 2, and 12.3% to the intermediate biovar. A clear zone did not form around 23% of the isolates: 39.1% from biovar 1, 32.6% from biovar 2, 23.9 from the intermediate biovar, and 4.4% from biovar 3.

Detection of *tmr* gene from tumors

Results of isolation and identification of pathogenic *Agrobacterium* with biochemical and pathogenicity tests and with PCR detection of *tmr* from tumor tissues collected from naturally infected rootstocks are listed in Table 2. Twenty-seven isolates were recovered from MG plates, and 9 from D1 plates out of 120 tumor suspensions. When amplified with PCR, *tmr* was detected in 108 DNA samples extracted from the same 120 tumor suspensions.

Detection of the tmr gene from symptomless plants

Results of isolation and identification of *Agrobacterium* with biochemical and pathogenicity tests and with PCR detection of *tmr* from symptomless plants are listed in Table 3. Of 120 plant tissue suspensions streaked on MG and D1 plates, six isolates were recovered from MG plates and one from a D1 plate. When symptomless plant tissue suspensions were amplified with PCR, *tmr* was detected in 31 DNA samples extracted from the same 120 tissue suspensions.

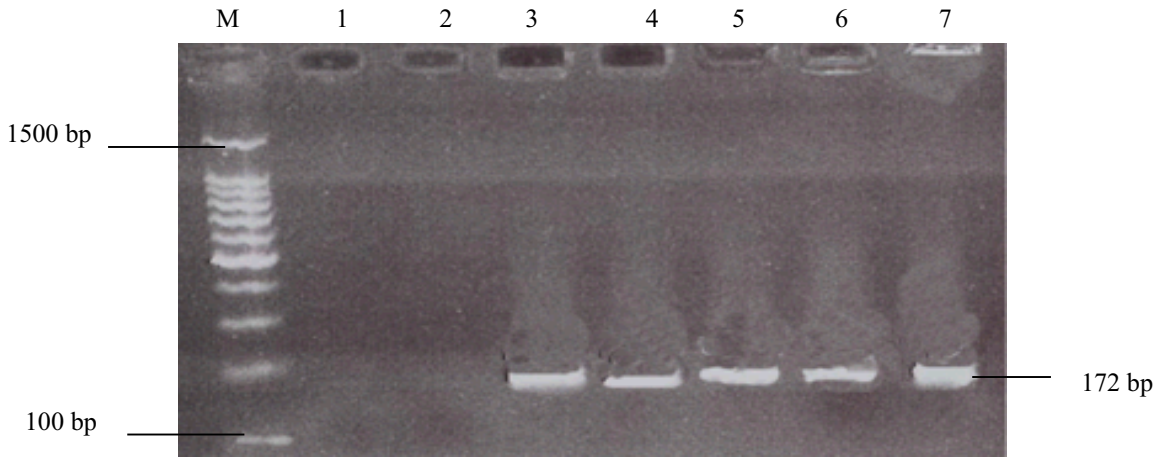

Fig. 1. Agarose gel electrophoresis of PCR *tmr* detection from *Agrobacterium* isolates. Lane M, 100 bp molecular size marker; lane 1, water control; lane 2, DNA from Agrobacterium C58c1; lane 3, DNA from Agrobacterium C58; lanes 4–7, DNA from selected Jordanian isolates.

Table 2. Detection of *Agrobacterium* biovars from tumors of naturally infected plants by isolation on selective media and by PCR *tmr* detection.

Rootstock	Isolation and pathogenicity test[a]		PCR *tmr*
	MG medium	D1 medium	
Almond	9/40[b]	6/40	36/40
GF677	8/20	2/20	19/20
Myrobalan	6/20	1/20	17/20
GF305	4/40	0/40	36/40
Total	27/120	9/120	108/120

[a] Values of the normal approximation test:
Z (*tmr* v. MG) =10.53; Z (*tmr* v. D1) = 12.78; Z (MG v. D1) = 3.25
Zalfa /2 = 1.96, where Alfa=0.05.
[b] No. of positive samples / Total No. of samples tested.

Table 3. Detection of *Agrobacterium* biovars from symptomless plants by isolation on selective media and detection with PCR *tmr*.

Rootstocks	Isolation and pathogenicity test[a]		PCR *tmr*
	MG	D1	
GF677	5/100[b]	0/100	26/100
Apricot	1/20	1/20	5/20
Total	6/120	1/120	31/120

[a] Values of the normal approximation test:
Z (*tmr* v. MG) =4.46, Z (*tmr* v. D1) = 5.96, Z (MG v. D1) = 2.48.
Zalfa/2 = 1.96 where Alfa=0.05.
[b] See Table 2.

Discussion

During the field inspection, crown gall was found in all fruit tree growing areas in Jordan, irrespective of environmental conditions. The highest percentage of *Agrobacterium* came from Madaba and Alyadoda (18.5%) followed by Almafraq (17.5%), Jerash (16%), Alshobak (10%), Amman (7.5%), the Jordan valley (5%), Altafila (3.5%), Irbid (2.5%), and Alsalt (1%). As regards the rootstocks, the highest percentage of *Agrobacterium* isolates (36%) came from bitter almond samples, followed by GF677 (18%), Myrobalan (12%), *Cichorium pumilum* (4.5%), Mahaleb (4%), GF305 (4%), rose (4%), carob (3.5%), nemaguard (2.5%), olive (2.5%), quince (2%), pomegranate (1.5%), MM106 (1.5%), apple (1%), and grapevine (1%). Carob and *Cichorium pumilum* (Compositae) are here first reported as hosts for *Agrobacterium* in Jordan, since the organism was isolated and its *tmr* detected from bacterial DNA extracted from its isolates and produced a band of 172 bp identical to the *tmr* of *Agrobacterium tumefaciens* C58. This finding gives a new dimension to the epidemiology of crown gall in Jordan.

The most common isolates in Jordan were biovar 1 isolates (60.5%), followed by biovar 2 (23.5%), the intermediate biovar (15%), and biovar 3 (1%). These results disagree with Lòpez *et al*. (1987) and Bouzar *et al*. (1991), who reported that biovar 2 was the most common biovar in Spain and Algeria. This could be due to geographical or climatic factors.

The majority of biovar 1 isolates were recovered from stone fruit rootstock: bitter almond, GF677, Myrobalan, Mahaleb, and GF305, except for Nemaguard, in which biovar 2 was found. Biovar 1 was also recovered from carob, olive, pome-

granate and *Cichorium pumilum*. Biovar 2 and the intermediate biovar isolates were recovered from some stone fruit rootstocks, mainly bitter almond, GF677 and Myrobalan. Pome fruit rootstocks, apple and quince yielded biovar 2 isolates, except for MM106, in which biovar 1 was recovered. Biovar 3 isolates were recovered only from grapevine. These results are in general agreement with Lòpez *et al*. (1987), Moore *et al*. (1988) and Bouzar *et al*. (1991), where the biovar of an *Agrobacterium* isolate was not related to its host, except in the case of biovar 3, which was restricted to grapevine only.

Some 85.5% of *Agrobacterium* suspensions induced tumors on tomato seedlings, but only 13% on *Kalanchöe daigremontiana* seedlings. This indicated that tomato plants were more sensitive to *Agrobacterium* than *Kalanchöe*, and that using only one plant species as an indicator host for *Agrobacterium* was not sufficient to demonstrate the pathogenicity of isolates. This was due to difficulties in the inoculation and establishment of infection on *K. daigremontiana,* and the fact that biovar 3 is restricted to grapevine. These results are in agreement with Bouzar *et al*. (1983) and Moore *et al*. (1988).

Using PCR for detection of the *tmr* gene in the T-DNA of the 200 *Agrobacterium* isolates was useful to indirectly determine their pathogenicity since all isolates that induced tumors on indicator plants also produced the expected 172 bp band for the *tmr*. Since the nucleotide sequence of the *tmr* gene may vary between octopine and nopaline Ti plasmid (Heidekamp *et al.,* 1983; Goldberg *et al.,* 1984; Nesme *et al.,* 1989), and since the F49*tmr* and F50*tmr* primers detect the *tmr* gene in the octopine and nopaline Ti plasmid respectively, then the detection of the *tmr* gene could also be useful for distinguishing various *Agrobacterium* strains.

Seventy-seven percent of *Agrobacterium* isolates were sensitive to agrocin 84. However, biovar 1 isolates were more sensitive to agrocin 84 than biovar 2 isolates, while biovar 3 isolates were resistant. These results are in agreement with Kerr (1980), Bouzar *et al*. (1983), Lòpez *et al*. (1987) and Vicedo *et al*. (1993) who reported that *Agrobacterium* isolates of biovar 1 were sensitive while isolates of biovar 3 from grapevine were resistant to agrocin 84. These results suggest that biovar 1 isolates very likely harbor a nopaline type Ti plasmid. And these results are further in agreement with Kerr (1980) and Bouzar *et al*. (1991), who reported that biovar 1 plasmid harbored nopaline Ti plasmid and was sensitive to agrocin 84.

PCR amplification was more effective in detecting the *tmr* gene than was *Agrobacterium* isolation on MG and D1 media, or than identification and pathogenicity tests. Recovery of *Agrobacterium* varied between MG and D1, possibly due to nutritive differences between these media. Successful isolation requires a large number of *Agrobacterium* cells, and, virulence may be lost during routine work in the laboratory, so that latent infections may not be detected. *Agrobacterium* is difficult to detect because of difficulties in isolation but was easily detected by PCR, since the *tmr* was detected in 90% of the DNA extracted from naturally infected plants, while pathogenic *Agrobacterium* was recovered from only 22.5% and 7.5% of plants when tumor suspensions were streaked on MG and D1 media respectively. In general these results are in agreement with Moore (1976), Nesme *et al*. (1989), Hass *et al*. (1995), Sachadyn and Kur (1997), Marti *et al*. (1998), Cubero *et al*. (1999), Pionnant *et al*. (1999) and Teyssier-Cuvelle *et al*. (1999), all reporting the effectiveness of PCR for *Agrobacterium* detection from plant material.

Latent infection of *Agrobacterium* is difficult to detect because of the lack of clear symptoms and difficulties in isolation but was easily detected by PCR. Detection of the *tmr* gene from symptomless stone fruit rootstock indicated latent infection in 25.5% of the seedlings, while *Agrobacterium* was found in 5% and 0.83% of samples when tissue suspensions were streaked on MG and D1 media respectively. The existence of latent infection was also reported by Marti *et al*. (1998) in rose. This finding may be useful for seedling certification programs.

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