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Occurrence of Crown Gall Disease on Fruit Trees in Jordan

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THE OCCURRENCE of the crown gall bacterium was investigated in samples of vine, peach, pear, olive, apple and cherry and in soils collected from various locations in Jordan. Two hundred and thirty one agrobacterial strains were isolated from these samples and identified by biochemical tests.

Pathogenicity tests gave positive results with 52 of these strains. Twelve were isolated from soils and 29,11 from vine and other fruit trees gall, respectively. Based on differential biochemical and cultural tests forty five of the pathogenic strains were divided into three biovars, biovar 1 (19), biovar 2 (10) and biovar 3 (16). Fifteen of the later were isolated from vines.

Sensitivity tests showed that the three biovar strains were fully sensitive to netilmicin and polymyxin (100%), but showed variable sensitivity to the other antimicrobial drugs. Amikacin and oxacillin could be used to differentiate between the three biovars of **Agrobacterium.**

Key word : Crown gall in Jordan, **Agrobacterium tumefaciens** biovars, Isolation from soils and trees.

Crown gall bacteria occur in almost all regions in soils and especially in the rhizosphere of plants. They can be isolated from young galls and tumorous outgrowths on different parts of diseased plants. Crown gall disease does not necessarily kill the plants, but their growth is often impaired and stunted. Crown gall, caused by *Agrobacterium tumefaciens* is a common disease of grapes in many regions, *e.g.,* Australia and Eastern Europe (Panagopoulos and Psallidas, 1973), South Africa (Loubser, 1978) and the United States (Burr, 1978). In Jordan gall formation on fruit trees, *e.g.* olive, apple- peach and vine has been observed for many years but has never been studies (Qasem, 1970). The extension of orchards and vineyards throughout the country during recent years has aggravated this problem particularly with respect to stone fruit, olive, apple and vine. In recent years failure in grafting vine in Jordan increased tremendously (pers. comm. of the Ministry of

Agriculture). As investigation on the causative agent of the disease and its effects on different plants has not been done in Jordan, the present study was undertaken to demonstrate the occurrence of the crown gall bacterium on fruit trees and in soils collected from regions where galled plants have often been observed.

Material and Methods

Collection of samples

Plants with galls were randomly collected from different sites in Jordan without taking into consideration the age of galls ; however most plant samples were younger than three years. Samples were distributed as follows : 28, 33, 20, 14, 10, 10 and 13 vine samples from Al-Rayyan, Deir Alla, Al-Baqureh, Al-Jerm, Jerash, Maan and Ajlun respectively; 15 apple samples from Irbid, 10 pear samples from Jerash, 15 olive samples from Madaba, 8 peach samples from Maan and 8 cherry samples from Irbid. Soil samples were collected as follows : 33, 22, 22 and 10 soil samples from Deir Alla, Al-Baqureh, Al-Rayyan and Madaba, respectively.

Culture media

The medium of Kado & Heskett (1970) as well as nutrient agar medium (Difco) were used to isolate the organisms from the soil and plant samples. Nutrient agar supplemented with Yeast extract (1%) was used to cultivate the organisms for the inoculation of the test plants. To determine the biovars of the pathogenic agrobacteria, the media of New & Kerr (1971), Brisbane & Kerr (1983) and Schroth *et al.* (1965) were used.

Methods

Galls were washed with tap water, surface sterilized with 10% sodium hypochlorite for 10 min, then washed 2-3 times with sterile distilled water. They were then cut into small pieces and ground in a mortar or in an electric blender. Tenfold dilutions were made of gall extracts and 0.1 ml volumes of these dilutions were spread on culture plates in triplicate with an L-shaped glass rod and incubated at 26° for seven days. Soil suspensions were generally prepared at a dilution of 10 g soil/100 ml sterile distilled water. The suspensions were shaken mechanically for 30 min then allowed

to settle for 5 min. Serial dilutions were made and 0.1 ml of each dilutions were spread as above.

Biochemical tests

Suspected agrobacteria on the basis of colony morphology were purified by streaking on nutrient agar and maintained on the same medium. They were then characterized biochemically and identified using the procedure of Cowan & Steel (1974). Gram reaction, hydrolysis of gelatin and starch, ureas and indole tests, H2S production and fluorescence on King, s B lysine medium were examined. In order to determine the biovars of the Agrobacterium strains, the following biochemical and physiological tests of Keane *et al.* (1970), Sule (1978), Kersters *et al.* (1973), and Brisbane & Kerr (1983) were carried out : 3-ketolactose production (Bernaerts & De Ley, 1963), acid production from m-erythrital and melezitose and alkali formation from sodium malonate, sodium tartrate and sodium propionate.

Pathogenicity tests

All Gram-negative strains with negative results in starch, gelatin, indole and H2S tests and positive in the urease test were tested for pathogenicity in triplicate to *Lycopersicon esculentum, Nicotiana tobaccum* and *Kalanchoe diagremontiana.* Cultures were grown on nutrient agar slants supplemented with yeast extract at 26°C for 24-48 hr. Four weeks old test plants were inoculated with a loopful of bacterial cells by puncturing the stem with a needle or scalpel. The plants were kept in a greenhouse at 27°C and results were recorded after one, two and three months. Since the greenhouse was not supplied with a humidifier it was kept relatively humid by spraying water in it or leaving water under the benches.

Reference strains

The following three strains were kindly received from Dr. Hanan Malkawi, Washington State University, Dept. of Bacteriology and Public health U.S.A. : 1.A 136 *Agrobacterium tumefaciens,* from which the plasmid was removed, so it is avirulent to most kinds of plants. It was used as a negative control. 2.A 136 Ab *Agrobacterium tumefaciens* which is a wide host bacterium. 3.A 136 Pti 854 *Agrobacterium tumefaciens* which is a limited host bacte-

rium. In the sensitivity tests *Pseudomonas aeruginosa* (ATCC) (# 27853) and *Escherichia coli* (ATCC) (# 25922) were used.

Sensitivity tests

Bacterial cultures were grown in trypticase soy broth to a density of 10^7 per ml and spread with swaps on MacConky agar plates. After 10 min the following antibiotic disks (Mast) were placed on the plates and incubated at 26° for 24-48 hr : Amikacin (10 mcg) Ampicillin (10 mcg), Polymyxin B (300 U), Ceftazidime (30 mcg), Netilmicin (30 mcg) and Oxacillin (1 mcg).

All tests in this report were carried out in triplicate.

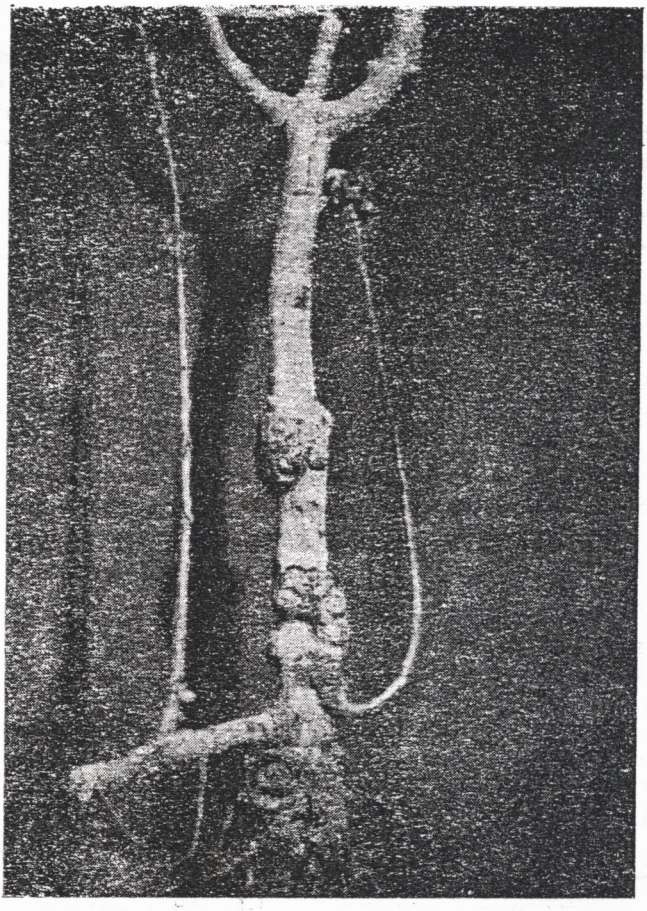
Results and Discussion

Figure 1 shows typical gall formation on naturally infected apple and vine plants.

A total of two hundred and thirty one bacterial strains were isolated from galled plants of apple, peach, cherry, olive and vine and from soils and were purified and confirmed as agrobacteria. These were distributed as follows : 24, 18, 50 and 20 from Deir Alla. Al-Baqureh, Al-Rayyan and Madaba soil, respectively : 15, 15, 12, 4, 7, 2 and 10 from vines in Al-Rayyan, Deir Alla, Al-Baqureh, Al Jerm, Jerash, Maan and Ajlun, respectively; and 23, 18, 12, 7 and 4 from apple, pear, olive, peach and cherry, respectively (Table 1). On Kado & Heskett medium colonies were visible after 3 days and were circular, glistening and light blue in colour but later became dark olive green. On nutrient agar colonies were small white, circular, mucoid, glistening and with entire margin. The biochemical characteristics of these isolates resembled those reported by other authors (Table 2).

Of 231 Agrobacterium strains isolated 52 were pathogenic on one or more of the test plants. Twelve were isolated from soils : 2 from Deir Alla, 2,5 and 3 from Al-Baqureh, Al-Rayyan and Madaba soil, respectively; 6, 2 and 3 from apple, pear and peach, respectively; and 5, 7, 7, 3, 1 and 6 from vines in Al-Rayyan, Deir Alla, Al-Baqureh, Al-Jerm, Jerash, Maan and Ajlun, respectively. The pathogenicity tests and some biochemical characteristics of these

(A)



(B)

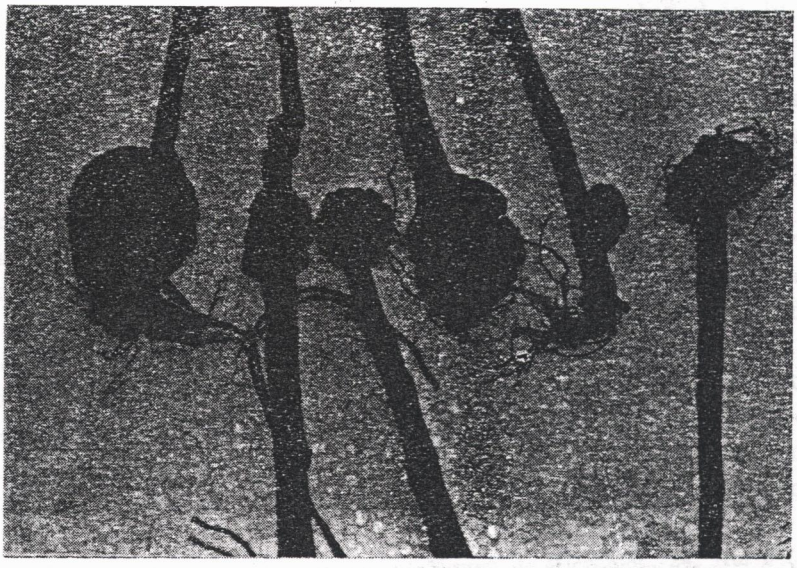


Fig. 1. Gall formation on naturally infected plant. (A: Apple; B : Vine).

isolates have been briefly discussed in a previous work (Abussaud & Almomani, 1987). They induced galls with variable size, the most virulent strains were from peach, which gave large galls on all three test plants after three weeks (Fig. 2). Large galls were also formed on tobacco by strains from apple and pear after one month. Most strains from vine induced small galls on tobacco and tomato, few induced large galls on tobacco after two months. The overall frequency of pathogenic strains was 12%, 45% and 17% for soil, vine and the other fruit trees, respectively, 45 of the 52 pathogenic strains (the rest died in culture) were further studied. Their ability to utilize erythritoal, propionate and to grow on the three selective media for the three biovars of *Agrobacterium* as well as their in vitro susceptibility test to different antibiotics was examined. Based on these tests they were divided into the three biovars of Keane *et al.* (1970) and Panago-

TABLE 1. Distribution of samples and strains confirmed as pathogenic and non-pathogenic agrobacteria.

Samples	Sites	No. of samples	Agrobacteria	
			Total no.	No. of pathogenic
Soil	Deir Alla	33	24	2
Soil	Al Baqureh	22	18	2
Soil	Al Rayyan	22	40	5
Soil	Madaba	10	20	3
Vine	Al Rayyan	28	15	5
Vine	Deir Alla	33	15	7
Vine	Al Baqureh	20	12	7
Vine	Al Jerm	14	4	3
Vine	Jerash	10	7	0
Vine	Maan	10	2	1
Vine	Ajlun	13	10	6
Apple	Irbid	15	23	6
Pear	Jerash	10	18	2
Olive	Madaba	15	12	0
Peach	Maan	8	7	3
Cherry	Irbid	8	4	0
Total		271	231	52

TABLE 2. Biochemical and physiological properties of the Jordanian isolates and reference strains in comparison with data reported by other authors (Keane *et al*, Panagopoulos, 1973 and 1978; and S. Sule, 1978).

Biochemical tests.	Kean *et.al* (1970)	Panagopoulos (1973)	Sule 1978)	Panagopoulos (1978)	Reference strains	Jordanian isolates
Gram reaction	–	–	–	–	–	–
3-ketolactose production	+/–	+/–	+/–	+/–	+	+/–
H2S production	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Urease	+	+	+	+	+	+
Oxidase	–/+	+	–/+	–/+	+	–/+
Utilization of Citrate	–/+	+	–/+	–/+	+	–/+
Starch =	–	–	–	–	–	–
Gelatin =	–	–	–	–	–	–
Glucose =	+	+	+	+	+	+
Arabinose =	+	+	+	+	+	+
Mannitol =	+	+	+	+	+	+
Sucrose =	+	+	+	+	+	+
Indol formation	–	–	–	–	–	–

poulos & Psallidas (1973). 19, 10 and 16 were included in biovar 1, 2 and 3, respectively.

Members of biovar 1 were all 3-ketolactose and propionate positive and erythritol and malonate negative. Those from biovar 2 were erythritol, malonate and tartrate positive, but melezitose, propionate and 3-ketolactose negative. Those from biovar 3 were malonate and tartrate positive (Table 3). Nine of biovar 1 strains were isolated from vine and the rest from apple (4), soil (3), peach (2) and pear (1). Biovar 3 was composed of 16 strains, 15 from vine and 1 from peach. Biovar 2 contained 10 strains, 6 from soil and the rest from apple (2), pear (1) and vine (1). On Schroth's medium all biovar 1 strains grew well, while only two of biovar 2 and 7 of biovar 3 showed weak growth. On New & Kerr's medium all biovar 2 strains grew well while 2 of biovar 3 strains showed

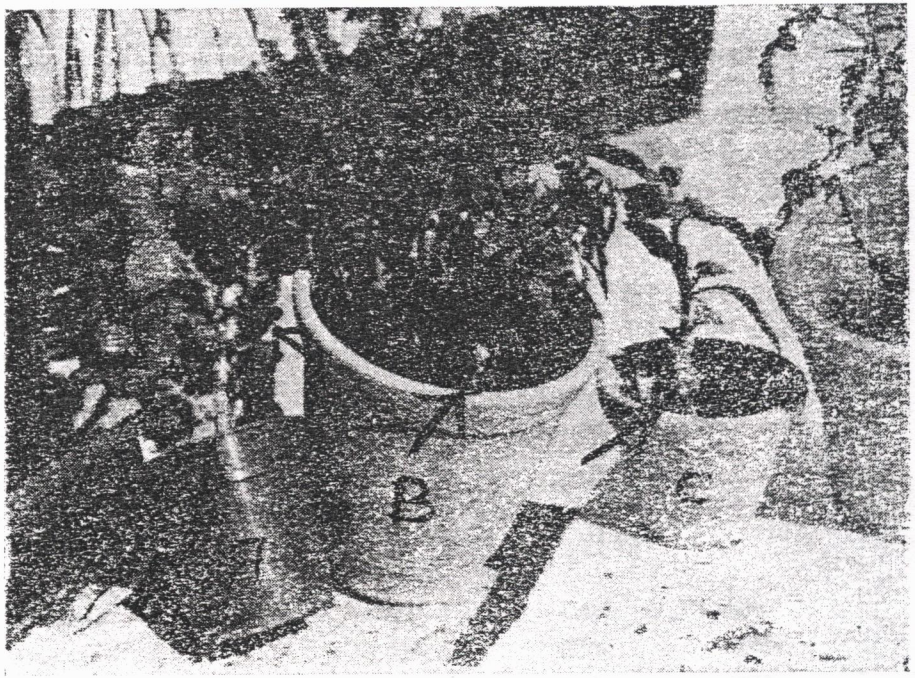


Fig. 2. Gall formation on test plant after inoculation with a strain of "Agrobacterium" from peach. (Kalanchoe (A); Tomato (B); Tobacco (C).

weak growth and none of biovar 1 grew. On Brisbane & Kerr's medium all biovar 3 strains grew well but one and two strains of biovars 2 and 1, respectively, showed weak growth.

Many selective media for agrobacteria have been described *e.g.* Patel (1026), Schroth *et al.* (1965), Clark (1969), New & Kerr (1971), Kado & Heskett (1970) and Brisbane & Kerr (1983), compared the selectivity of different media for agrobacteria and found that of 20 biovar 1 strains only 18, 19 and 4 strains grew on the media of Kado & Heskett, Schroth *et al.* and Brisbane & Kerr, respectively. None grew on New & Kerr's medium; and of 10 biovar 3 strains 9 and 10 grew on the media of Kado & Heskett and Brisbane & Kerr, respectively. None grew on the others; and of 18 biovar 2 strains all grew well only on New & Kerr's medium.

The use of the three media Schroth, New & Kerr and Brisbane & Kerr is fairly commonplace now in the determination of the three biovars of *Agrobacterium*. Our results support this finding.

It is well documented that *Agrobacterium* spp. survive in soil (Spiers, 1979, Burr & Katz, 1983 and Kerr & Hitay, 1974). However the ratio of pathogenic to non pathogenic agrobacterial strains in soil in this report is higher than that reported by Burr & Katz (1983) but lower than that found by Kerr & Hitay (1974). This Difference might be due to the type of soil and to the type of test plants used in the pathogenicity tests, and to the media used in the isolation of strains and to the host plants growing previously in those soils. Non pathogenic agrobacteria have been isolated from plant galls but their frequency in soil is much higher. In *Agrobacterium,* there appears to be considerable host specificity, particularly in biovar 2 and 3. Biovar 3 strains have been isolated predominatly from grapevine (Kerr & Panagopoulos, 1977, Perry & Kado, 1982, Burr and Katz, 1983, Tarbah & Goodman, 1986). This was also found to be true in Jordan. Since 15 of 16 biovar 3 strains were isolated from grapevine. Biovar 2 strains are rarely isolated from grapevine, but are commonly found on stone fruit (Kerr, 1969). We found that only 1 of 10 biovar 2 strains was isolated from grapevine. Panagopoulos *et al.* (1978) found a few grapevine isolates that would infect tomato, but still concluded that biovar 3 strains have a much narrower host range than biovar 1 and 2 Süle (1978), however, found grapevine strains from Hungary that would initiate gall on sunflower and tomato. We found that most of our grapevine strains induced galls on tomato and tobacco.

The results indicate that galls on plants in Jordan are most probably due to the widespread occurrence of agrobacteria in soils and on a range of hosts and that all three biovars of the pathogen are present. The high frequency of biovar 3 strains in grapevine indicates also that failure in grafting grapevine in Jordan might be due to this biovar. This has been also found by Burr & Katz (1983).

The susceptibility tests showed (Table 3) that the three biovar strains are sensitive to netilmicin and polymyxin (100%) but have variable sensitivity to the other antibiotics. Amikacin could be added to the tests which are used to differentiate between the three biovar strains.

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TABLE 3. The distribution of 45 pathogenic Agrobacterium strains among the three biovars (A) and their in vitro susceptibility tests to different antibiotics (B).

Tests	Biovar 1 (19)	Biovar 2 (10)	Biovar 3 (16)
3-ketolactose production	19	0	0
Erythritol utilization	1	9	0
Melezitose =	13	1	6
Malonate =	0	8	16
Tartrate =	4	9	15
Propionate =	19	0	4
Growth on media of :			
Schroth *et al.*	19	2 weak	7 weak
New & Kerr	0	10	2 weak
Brisbane & Kerr	2 weak	1 weak	16
	B		
Amikacin	85%	43%	60%
Ampicillin	85%	100%	100%
Polymyxin	100%	100%	100%
Ceftazidime	61%	71%	90%
Netilmicin	100%	100%	100%
Oxacillin	61%	14%	10%

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