Arab Gulf J. Scient. Res., Agric. Biol. Sci., **B6** (1), pp. 91-102 (1988)

Characteristics of Jordanian Isolates of the Genus *Agrobacterium**

M.J.I. Abussaud and F.A. Almomani

Department of Biological Sciences, Yarmouk University, Irbid, Jordan

ABSTRACT. Jordanian isolates of *Agrobacterium* were separated into three biotypes on the basis of physiological and biochemical characteristics. Of 203 *Agrobacterium* isolates 52 proved to be pathogenic. 29 were isolated from grapevines, 12 from soil and 6,3,2 from apples peach and pear, respectively. None of the grapevine isolates was included in biotype 2. With 22 isolates biotype 1 was more common than the others. Tomato seedlings seem to be not a suitable host for testing the pathogenicity of grapevine isolates in Jordan. Peach isolates formed the largest gall on tomato, tobacco and klanchoe.

Crown gall disease on fruit trees and on other plants of various families has been observed in Jordan for many years (Qasem 1970). The disease causes severe damage in nurseries and in stone fruit orchards. The extension of orchards and vineyards throughout the country during the last years has aggravated this problem particularly with respect to stone fruit, olive, apple and grape plants. As investigation on the causative agent of the disease and its effect on different plants has not been done in Jordan, a study was undertaken using various infected plants and soil samples to demonstrate whether the gall formation is due to the presence of *Agrobacterium tumefaciens* or other cause. Two hundred and three *Agrobacterium* isolates were isolated and purified. The objective of this study is to identify the biotypes of Jordanian isolates by using biochemical and physiological tests and comparing the results with those from literature.

* This work has been funded from the Centre for Jordan Studies at Yarmouk University, Irbid, Jordan.

Materials and Methods

Bacterial Culture

Two hundred and three cultures were isolated in a previous study (unpublished work) from soil samples and naturally infected plants which have been collected from different locations in Jordan where crown gall have been observed using the media of: Kado and Heskett (1970), New and Kerr (1971), Schroth *et al.* (1965), Clark (1969) and nutrient agar (Difco U.S.A.). The methods of Kerr (1969) was followed for the isolation of these cultures. Three cultures (*Agrobacterium tumefaciens*) were kindly received from Hanan Malkawy, Washington State University, Dept. of Bacteriology and Public Health U.S.A. and used for comparison.

Pathogenicity Tests

All isolates were tested for pathogenicity to one or more of the following test plant seedlings (4 weeks old): *Lycopersicon esculentum, Nicotina tobacum* and *Kalanchoe diagremontiana* and Grape (one year old), by puncturing the stems with a needle or scalple and introducing a heavy suspension of the bacterium according to the method of Kerr (1969). Plants were kept in a green house and the results were recorded after 1,2 and 3 months.

Media

The isolates were grown on nutrient agar slants supplemented with 0.1%(w/v) yeast extract at 26°C for 48hr before being used in the pathogenicity tests. For maintaining the cultures the medium used by Lippincott *et al.* (1981) was used.

In the biochemical tests the following basal medium was used: (g/l): $(NH_4)SO_4$, 1; K_2HPO_4, 0.6; KH_2PO_4, 0.4: NaCl, 2: $MgSO_4$, $7H_2O$, 0.2. The pH was adjusted to 7.0 with KOH solution. Most of the biochemical tests were conducted using the procedures of Cowan and Steel (1965) except the following tests which were carried out as mentioned in the appropriate places:

1) Acid production from carbohydrates (glucose, lactose, xylose,arabinose, sucrose and mannitol), gelatine hydrolysis, NaCl test and utilization of malonate and citrate were used as described by Süle (1978).
2) 3-Ketolactose production according to Bernaert and De Ley (1963).
3) Pellicle formation in ferric ammonium citrate (FAC) according to Kean *et al.* (1970).

Results

In the present investigation, the colony morphology of the isolates on the medium of Kado and Heskett (1970) after 3-5 days incubation at 26°C was studied, the results showed that they were smooth, glistening, translucent, convex, circular with entire margin, light blue in colour but change into olive green with time. On nutrient agar they formed small white colony. On the medium of Bernaert and De Ley (1963), containing glucose, Difco yeast extract. $CaCO_3$ and agar, they form either white or beige colony, quick growth and large colonies were noticed on this medium. All cultures were Gram negative, motile and produced acid from the tested sugars. All produced H_2S, catalase and urease; failed to hydrolyse gelatine, casein and starch. All failed to produce indol, to grow at 4°C and to produce pigment on King B medium (King *et al.* 1954).

Table 1 shows the distribution of the isolates, origins, biotypes, and percentage of the pathogenic one. Most of them were from soil and grapevines 77 and 65, respectively. On the basis of biochemical and physiological tests irrespective of origin and pathogenicity to the tested plants the 203 isolates could be divided into three groups that correspond nicely to the three biotypes of Süle (1978). Group 1,2 and 3 include 72,70 and 61 isolates, respectively. All isolates were tested for pathogenicity to one or more of the test plants mentioned in materials and methods. Out of 203 isolates only 52 proved to be pathogenic to one or more of the test plants. The distribution of these pathogenic isolates and their origin is shown in Table 1 and 2. The highest frequency of pathogenicity was among the grapevine isolates (44.6) followed by those from peach, apple and soil 43%,

Table 1. The sources and numbers of isolates, biotypes, number of pathogenic isolates, and the frequency of pathogenicity

Sample	No. of isolates	Biotype			Patho-genicity %
		1	2	3	
Soil	77(12)	25(2)	19(8)	33(2)	15.6
Grape	65(29)	24(13)	13(0)	28(16)	44.6
Apple	23(6)	8(4)	15(2)	0	26
Pear	18(2)	6(1)	12(1)	0	11
Olive	12(0)	4(0)	8(0)	0	0
Peach	7(3)	5(2)	2(1)	0	43
Chery	1(0)	0	1(0)	0	0
Total	203(52)	72(22)	70(12)	61(18)	26

Numbers in parenthesis represent number of pathogenic isolates in each sample.

Table 2. The isolates that can induce tumor on one or more of the tested hosts and their biotyping, biochemical and physiological activity, and origin

Isolate number	Origin	Citrate	Tartarate	Malonate	3-Ketolactose	Growth at 38°C	Oxidase	Pellicl formation on FAC	Melezetoze	Rafinose	Litimus milk	Selenate	5% ethanol	3% NacL	Biotype	Patbogenicity on			
																tomato	tobacco	kalonchoa	grape
C12	**Rg7**	+	+	+	–	w	–	–	–	+	A	w	w	w	2	+	+	?	?
C31	**Bg**	+	+	+	–	+	–	–	–	–	B	w	w	w	2	+	+	?	+
C56	**Rg**	+	+	+	–	+	–	–	–	–	B	w	w	w	2	+	+++	?	+
C59	**Rg**	+	–	+	–	w	–	–	+	+	B	w	w	w	3	+	+	?	+
C61	**Rg**	+	+	+	+	w	+	+	+	+	B	w	w	+	3	++	++	?	++
C87	**Bc**	–	+	+	–	w	–	–	–	–	A	w	w	+	2	++	++	?	+
D32	**Dgg**	–	+	–	+	+	+	+	+	+	B	+	w	w	1	+	++	?	++
D52	**Pear**	+	+	+	–	w	+	–	–	–	B	w	+	+	2	++	+++	+++	++
D65	**Bgg**	+	+	–	+	+	+	–	+	+	B	+	w	+	1	++	++	?	+
D70	**Bgg**	–	+	–	+	+	+	–	+	+	B	+	+	+	1	+	+++	?	++
D79	**Rgg**	+	+	–	+	+	+	+	+	+	B	+	+	+	1	+	+	?	+
E22	**Dc**	+	+	+	–	w	–	–	–	+	A	w	+	+	2	+	+	?	+

E32	**Pear**	−	−	−	+	+	+	−	+	−	B	+	w	+	1	+	+	?	??
E77	**Bgg**	−	−	+	+	+	+	+	+	+	B	+	+	+	1	++	++	+++	+++
E78	**Bgg**	−	+	−	+	+	+	−	+	+	B	+	+	+	1	+	+	?	+
A3	**Apple**	+	+	+	−	w	−	−	−	+	B	w	w	+	2	+	+++	?	+
A15	**Jgg**	+	+	+	−	+	+	−	+	−	B	w	w	+	3	+	+	?	+
A17	**Jgg**	+	+	+	−	+	+	−	+	−	B	w	w	+	3	+	+	?	+
A18	**Jgg**	+	+	+	−	+	+	−	+	−	B	w	w	w	3	−	?	?	+
B57	**D2**	+	+	+	+	−	−	−	−	−	B	w	w	+	2	?	2	++	+
S50	**Dgg**	+	+	−	+	+	+	+	+	+	B	+	+	w	1	++	?	?	++
S59	**Rg**	+	+	+	−	w	+	−	−	−	B	w	w	+	2	+	+	?	−
n45	**w**	+	+	−	+	+	+	+	+	+	B	w	w	+	1	+	++	?	?
n48	**w**	+	+	+	−	w	+	−	−	−	B	w	w	+	2	+	+	?	?
n49	**W**	−	+	+	+	+	+	+	+	+	B	w	w	+	1	+	+	?	?
M4	**Apple**	−	+	+	−	w	−	−	−	−	A	+	+	w	2	+	++	?	+
M8	**Apple**	+	−	−	+	+	+	+	+	+	B	+	+	+	1	+	++	?	+
M11	**Apple**	+	−	−	+	+	+	+	+	+	B	+	+	+	1	+	++	?	+
M16	**Apple**	+	−	−	+	+	+	+	+	+	B	w	w	+	1	+	+	?	+
g5	**Dgg**	+	+	−	−	−	−	−	+	−	B	+	+	w	3	+	+	?	+
g16	**Dgg**	−	−	−	+	+	+	+	+	+	B	w	w	+	1	+	++++	?	+++

Isolate number	Origin	Citrate	Tartarate	Malonate	3-Ketolactose	Growth at 38°C	Oxidase	Pellicl formation on FAC	Melezetoze	Raffinose	Litimus milk	Selenate	5% ethanol	3% NacL	Biotype	Patbogenicity on			
																tomato	tobacco	kalonchoa	grape
g42	**Bgg**	+	+	+	–	+	+	–	+	–	B	–	–	+	3	+	+++	?	++
g43	**Dgg**	+	+	+	–	+	+	–	+	–	B	w	w	w	3	+	+++	?	++
g51	**Bgg**	–	+	+	–	w	+	–	+	–	B	w	w	w	3	+	++	?	?
g52	**Bgg**	+	+	+	–	+	–	–	+	–	B	+	+	w	3	+	+	?	+
g55	**Rgg**	–	–	–	+	+	+	+	+	+	B	w	w	+	1	+	++	?	+
g56	**Rgg**	+	+	+	–	+	–	–	+	–	B	+	+	+	3	++	+++	?	?
g59	**Rgg**	–	–	–	+	+	+	+	–	+	B	+	+	+	1	++	+++	?	+
g64	**Dgg**	–	+	–	+	+	+	+	+	+	B	w	+	+	1	++	+++	?	?
g66	**Mgg**	+	+	+	–	+	–	–	+	+	B	w	+	+	3	+	+	?	+
g68	**Dgg**	+	+	+	+	+	–	–	+	+	B	+	+	+	3	+	+	?	+
g70	**Rgg**	–	+	–	+	+	+	+	+	+	B	+	+	+	1	++	+++	?	++++
g72	**Peach**	–	–	–	+	+	+	+	+	+	B	+	+	+	1	++++	+++	+++	++++

g73	**Peach**	–	–	–	+	+	+	+	+	+	B	+	+	+	1	++++	++++	+++	++++
g76	**Apple**	–	–	–	+	+	–	+	+	+	B	w	+	+	1	++	++++	++++	
Eb2	**grape**	+	+	+	–	w	–	–	+	–	B	w	w	+	3	+	+	?	++
Eb3	**grape**	–	–	+	–	w	–	–	+	+	B	w	w	+	3	+	+	?	++
Eb5	**grape**	+	+	+	–	–	–	–	+	+	B	w	w	+	3	+	+	?	++
Eb7	**grape**	–	–	–	+	+	+	+	+	+	B	+	w	+	1	+	+++	+++	+++
Eb11	**grape**	+	+	+	–	–	–	–	+	+	B	w	+	+	3	+	+	?	++
Eb13	**grape**	+	+	+	–	–	–	–	+	–	B	w	w	+	3	+	+	?	++
g80	**Peach**	+	–	–	–	w	–	–	–	–	B	w	w	w	2	++++	++++	+++	?

Notes:

+ doubtful gall.
– negative.
w weak growth.
A Acid.
B Base.
? nontested.
++ small gall.
+++ large gall can be photo.
++++ very large gall.

Dgg Deiralla grape gall.
Bgg Baqura grape gall.
Rgg Rayyan grape gall.
Jgg Jerm grape gall.
Mgg Maa'n grape gall.

26%, 15.6%, respectively. The highly and widely virulent isolates to the tested plants were from peach which gave large gall on tomato, tobacco, kalanchoe and grape. Isolates from apple and pear formed also gall on tomato and tobacco (Table 2).

Most grapevine isolates did not form clear gall on grape seedlings, however few grapevine isolates showed gall on tobacco after two months. Therefore, tobacco seemed to be a better host than tomato for the jordanian grapevine isolates. Of the biotypes causing crown gall disease in Jordan biotypes 1 and 3 seem to be more common than biotype 2.

Biotype 1

Out of 72 *Agrobacterium* isolates, which have been assigned biotype 1, only 22 isolates proved to be pathogenic (31%). They were all 3-ketolactose positive, grow at 38°C, gave basic reaction in litmus milk. Most of them produced oxidase; utilized raffinose and melezitose and tolerate 3% NaCl. 18 of 22 formed pellicle on ferric ammonium citrate and 14 grow in the presence of selenate in the medium. Only 2 of 22 utilized malonate.

Biotype 2

Twelve pathogenic isolates were included in this biotype. All of them except 1 utilized tartrate and malonate and 10 utilized citrate. None utilized melezitose nor produced pellicle on FAC and very few grew at 38°C, produced oxidase or utilized raffinose. All except one do not produced 3-ketolactose.

Biotype 3

Eighteen isolates were included in this biotype. Almost all isolates utilized melezitose, malonate, tartrate and citrate. Most of them failed to produce 3-ketolactose, pellicle on FAC and to tolerate selenate in the growth medium (Table 3).

Discussion

It is generally agreed that the causal agent of crown gall disease consists of at least three kinds of organisms which are morphologically similar but differing in their pathogenicity, nutritional and biochemical properties (Wormald 1945). The basis for grouping the genus *Agrobacterium* has been always controversial, Kean *et al.* (1970) and Kersters *et al.* (1973) separated the species into two large groups. Panagopoulos and Psallidas (1973) confirmed these finding and suggested the existence of a third group. Panagopoulos *et al.* (1978) and Süle (1978) confirmed the existence of a third group. In the present investigation Jordanian isolates were separated into three biotypes based on biochemical and physiological characters.

Table 3. Tests for distinguishing biotypes. Number of positive isolates/number of tested in each biotype

Test	Biotype 1	Biotype 2	Biotype 3
Citrate	7/22	10/12	16/18
Tartarate	10/22	11/12	16/18
Malonate	2/22	11/12	17/18
3-Ketolactose	22/22	1/12	2/18
Growth at 38°C	22/22	2/12	9/18
Oxidase	21/22	3/12	7/18
Pellicle on ferric Ammonium citrate	18/22	0/12	1/18
Melezitose	21/22	0/12	18/18
Raffinose	21/22	3/12	7/18
Alkaline reaction in Litmus milk	22/22	8/12	18/18
Tolerence of selenite	14/22	1/12	4/18
Tolerence of ethanol	13/22	3/12	6/18
Tolerence of NaCl 3%	20/22	7/12	12/18

Seventy two, seventy, and sixty one isolates have been included in biotype 1,2 and 3 respectively. Of these, 22, 12 and 18 proved to be pathogenic, respectively. No pathogenic biotype 2 was isolated from grapevine. This was also found by panagopoulos and Psallidas (1973). However not all isolates from grapevines belong to biotype 3. Out of 29 isolates from grapevines 13 were included in biotype 1 and 16 in biotype 3. Most isolates from soil belong to biotype 2 (8 of 12) and none of apple,pear, olive, peach and chery isolates was included in biotype 3. These finding strengthen the observation that grapevine isolates have high frequency of biotype 3. Our isolates of biotype 1 and 2 gave the same reactions with those of Süle (1978) except the erythritol test which unfortunately could not be done because of the lack of this sugar. Biotype 3 differs from that of Süle in the melezitose test and 3-ketolactose test too. While all our isolates utilized melezitose only 10 of 27 isolates of Süle utilized melezitose. 17 of 27 of Süle isolates did not produce 3-ketolactose while 16 of 18 of our isolates could not produce 3-ketolactose. In general we can say that our isolates fit to a large extent the characteristics of the biotypes 1, 2 and 3 of the Hungarian isolates (Süle 1978) and the Greek isolates (Panagopoulos and Psallidas 1973).

In Yugoslavia Arsenijevic *et al.* (1974) noted that their isolates from grapevines were not pathogenic to sunflower. In Hungary Süle 1978) reported that all the Hungarian isolates from grapevines were pathogenic to sunflower unfortunately our isolates were not tested for pathogenicity to sunflower but tested

for pathogenicity to tomato, tobacco, kalanchoe and grapevine. It has been found that the susceptibility of tobacco was much higher than tomato to the majority of the grapevine isolates. In contrast peach isolates formed large gall on all tested plants and seem to be highly virulent (Table 2).

Buchanan and Gibbons (1974) indicated that the parasitic activity of this bacterium is not highly specialized. Wormald (1945) showed that in Britain there were physiological races with different host relationships. None of our olive isolates was found to be pathogenic to any of the tested plants in this study. However, the results of tests on plants commonly used for the detection of pathogenicity are not always conclusive and isolates giving negative results should be tested on their original host plants.

References

Abussaud, M.J. and **Khudeir, M.** (1986) *Prevalence of Crown Gall Disease in Jordan*, Report to the Deanship of Scientific Research and Higher Studies at Yarmouk University in Irbid, Jordan.

Arsenijevic, M., Stojic, J. and **Panic, M.** (1974) Contribution to the study of bacterial canker of the grapevine (*Agrobacterium tumefaciens*), *Zastita bilija* **25:** 257-264.

Bernaert, M.J., and **De Ley, J.** (1963) A biochemical test for crown gall bacterium, *Nature* (London) **197:** 406-407.

Buchanan, R.E., and **Gibbons, N.E.** (1974) *Bergeys Manual of Determinative Bacteriology*, 8th ed. Williams and Wilkins, Baltimore.

Clark, A.G. (1969) A selective medium for the isolation of *Agrobacterium* species, *J. Appl. Bacteriol.* **32:** 348-351.

Cowan, S.T., and **Steel, K.J.,** (1965) *Manual for the Identification of Medical Bacteria*, Cambridge Univ. Press.

Kado, G.I. and **Heskett, M.G.** (1970) Selective media for the isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*, *Phytopathol.* **60:** 969-976.

Kean, P.J., Kerr, A. and **New, P.B.** (1970) Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates, *Australian J. Biol. Sci.* **32:** 585-595.

Kerr, A. (1969) Crown gall of stone fruit. I: Isolation of *Agrobacterium tumefaciens* and related species, *Austr. J. Biol. Sci.* **22:** 111-116.

Kersters, K., De Ley, J., Sneath, P.H.A. and **Sackin, M.** (1973) Numerical taxonomic analysis of *Agrobacterium*, *J. Gen. Microbiol.* **73:** 227-239.

King, E.O., Ward, M.K., and **Raney, D.E.** (1954) Two simple media for the Demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* **44:** 301-307.

Lippincott, J.A., Lippincott, B.B., and **Starr, M.P.** (1981) The genus *Agrobacterium*, pp. 842-855, Selection from *Prokaryotes. A Handbook on Habitat Isolation and Identification of Bacteria*, Springer Verlag, New York.

New, P.B., and **Kerr, A.** (1971) A selective medium for *Agrobacterium radiobacter* biotype 2, *J. Appl. Bacteriol.* **34:** 233-236.

Panagopoulos, C.G. and **Psallidas, P.G.** (1973) Characteristic of Greek isolates of *Agrobacterium tumefaciens* **(Smith, E.F.** and **Townsend)** (eds.) *Conn. J. Appl. Bacteriol.* **36:** 233-240.

Panagopoulos, C.G., Psallidas, P.G., and **Allivzatos, A.S.** (1978) Studies on biotype 3 of *Agrobacterium radiobacter* var. *tumeaciens*, *Proc. 4th Intern. Conf. Plant Pathogenic Bacteria*, Angers ed. Station de Pathologie végétale et Phytobactériologie, pp. 221-243. Angers: I.N.R.A.

Qasem, S. (1970) Occurrence and distribution of plant Disease in Jordan, *Research Bulletin 1*, Jordanian Scientific Research Council, p. 6.

Schroth, M.N., Thompson, J.P. and **Hildebrand, D.C.** (1965) Isolation of *Agrobacterium tumefaciens* A. radiobacter group from soil, *Phytopathol.* **55:** 645-647.

Süle, S. (1978) Biotype of *Agrobacerium tumefaciens* in Hungary, *J. Appl. Bacteriol.* **44:** 207-213.

Wormald, H. (1945) Physiologic races of the crown gall organism in Britain, *Tr. Br. Myc. Soc.* **28:** 134-146.

(Received 18/04/1987;
in revised form 14/11/1987)

المواصفات البيوكيماوية لعزلات بكتيريا الأجروبكتريوم من الأردن

محمود جدوع أبو السعود و وفؤاد عبدالعزيز المومني

دائرة العلوم الحياتية ـ جامعة اليرموك ـ اربد ـ الأردن

لقد درست المواصفات البيوكيماوية والفسيولوجية وكذلك القدرة المرضية ل ٢٠٣ عزلات بكتيرية عزلت من نباتات مصابة بمرض التدرن التاجي ومن عينات ترابية جمعت من مناطق مختلفة ظهرت فيها أعراض المرض.

وزعت هذه العزلات بناء على الصفات المدروسة إلى ثلاثة مجموعات مماثلة لتلك التي عزلت في اليونان وهنجاريا ١٩٧٨، حيث احتوت المجموعة الأولى على ٧٢ عزلة بينما احتوت المجموعات الثانية والثالثة على ٧٠ و ٦١ عزلة على الترتيب.

درست قدرة هذه العزلات على إحداث اورام سرطانية في اشتال العنب والبندورة والدخان والكلنشوه حيث حقنت هذه الأشتال (٣ ـ ٤ اسابيع) بالعزلات البكتيرية ووضعت داخل البيت الزجاجي لمدة ٢ ـ ٣ اشهر: عند ظهور الأورام أخذت هذه واستعملت لعزل نفس النوع من العزلات المحقونة في النباتات للتأكد من مَرَضيتها. بينت هذه الفحوصات ان هناك ٥٢ عزلة من ال ٢٠٣ كانت قادرة على اصابة واحد أو أكثر من نباتات التجربة المستعملة بمرض التدرن التاجي وأنها كانت موزعة كما يلي: ٢٩ عزلة كانت معزولة من نباتات عنب مصابة، ٦ من تفاح، ٣ من دراق و ٢ من خوخ و ١٢ كانت معزولة من عينات التربة المختلفة، كما بينت الدراسة ان معظم عزلات العنب كانت أقل إصابة لنباتات الدخان والبندورة، بينما عزلات الدراق كانت أخطر العزلات حيث كوّنت أورام على جميع نباتات التجربة المستعملة وفي أقصر وقت كذلك. كما تبين ان أشتال البندورة كانت مناسبة بدرجة أقل من أشتال الدخان لإثبات القدرة على احداث المرض في عزلات العنب.

كان توزيع هذه العزلات الممرضة في المجموعات الثلاثة المذكورة سابقا كما يلي: المجموعة الأولى (٢٢) المجموعة الثانية (١٢) والمجموعة الثالثة (١٨) مما يدل على ان المجموعة الأولى هي الأكثر انتشارا في الأردن.