Biotypes of Agrobacterium tumefaciens in the coastal region of Libya[1]

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ABSTRACT

The crown gall disease caused by *Agrobacterium tumefaciens* (Smith and Townsend) Conn. is commonly found in the coastal region of Libya on stone fruits, pome fruits, henna *(Lawsonia inermis)* and adhatoda *(Adhatoda vasica)*. The pathogen was isolated from tumors and rhizosphere soil of infected peach, apple and adhatoda. Henna and rose isolates were recovered from the soil around galled plants. According to their physiological and biochemical characteristics, eleven isolates of *A. tumefaciens* were separated into two distinct groups: biotype 1 and biotype 2. Isolates LA-2, LA-8, and LA-11 belong to biotype 1, while LA-1, LA-3, LA-4, LA-5, LA-6, LA-7, LA-10, and LA-12 isolates are of biotype 2. As to their prevalence, biotype 2 was found to be more common. Two isolates (LA-1 and LA-10) out of eleven were sensitive to agrocin-84 when tested in vitro. Pathogenicity tests indicated that all isolates were tumorigenic on carrot disks.

INTRODUCTION

Crown gall is an economically important disease that occurs throughout the world. The causative agent *Agrobacterium tumeficiens* (Smith and Townsend) Conn. attacks mainly dicotyledonous and to a lesser extent monocotyledonous plants (6, 7).

The existence of biotypes 1 and 2 in many countries of the world and their ability to infect a wide host range were studied by many workers (3, 12, 19, 22). A new group isolated from grapevine and designated as biotype 3 was found to be host specific (4, 5, 14).

In Libya, crown gall disease is commonly found on stone fruits, pome fruits and roses. The disease is probably more responsible than any other malady for the loss of many nursery trees. Its incidence has increased steadily in many nurseries and orchards as a result of introducing infected root-stocks to new cultivated areas free from the pathogen.

The economic losses sustained after planting are difficult to evaluate. Under certain situations, an estimated value of about 100% loss in the total number of plants of different major crops was reported (1).

The objective of this study was to identify the biotypes of *A. tumefaciens* local isolates using standard biochemical and physiological tests, and to test sensitivity of these biotypes to agrocin produced by strain K84 in vitro.

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MATERIALS AND METHODS

Galls of different size and shape appearing on the crown and roots of various infected plants, and samples of infested soils were used for isolation of the crown gall bacteria. Known biotypes of *A. tumefaciens* and *A. radiobacter* were used as standards for comparison purposes in the different tests (Table 1).

Table 1 – Isolates of Agrobacteria, their source of isolation, location and suppliers.

Isolate	Source of Isolation	Locality of origin	Suppliers*
Ag 20	Almond	Amalias, Ilia	C. G. Panagopoulos
C58	Wild type from cherry gall	Sodus Co., NY	S. Sule
FACH1	Grape cutting	USA	F.A.Tarbah
K27	Peach gall	South Australia	S. Sule
K84	Soil around peach gall	S. Australia	C.G. Panagopoulos
**69	–	–	S. Sule
LA1	Soil around adhatoda gall	College farm Tripoli	
LA2	Adhatoda gall	College farm Tripoli	
LA3	Soil around henna gall	Souk El-Juma Tripoli	
LA4	Soil around henna gall	Souk El-Juma Tripoli	
LA5	Soil around peach gall	Shahhat	
LA6	Soil around apple gall	Tarhuna	
LA7	Peach gall	Shahhat	
LA8	Soil around rose gall	College farm Tripoli	
LA10	Apple gall	Misurata	
LA11	Apple gall	Misurata	
LA12	Soil around henna gall	El-Garabulli	

*Strains LA1-LA8, LA10-LA12 were isolated in plant protection laboratory, faculty of agriculture.

**Source of isolation, locality of origin were not given by the supplier.

Ag 20, C58, FACH1 are strains of biotype 1.

K84, **69 are strains of biotype 2.

Young galls on adhatoda *(Adhatoda vasica)* peach *(Prunsus persica)* and apple (*Malus* spp) were selected and washed with running tap water. The outer gall surface was removed using a sterile razorblade and the remaining tissue was cut into small pieces about $2mm^2$. After rinsing twice with sterile distilled water, pieces of gall tissue were plated on D_1-agar medium, and the plates were incubated at 28 C for 3-4 days.

Five grams of soil samples from the rhizosphere of infected peach, apple, rose and henna were shaken mechanically with 100 ml of 1:10 diluted phosphate buffer solution (pH 7.2) for 10 minutes at 25 C, then 0.5 ml suspension was transferred to D_1-agar plate and spread homogeneously using a sterile L-shaped glass rod (8). All plates were incubated for 3-4 days at 28 C. Pure single colonies of all *Agrobacterium tumefaciens* isolates were maintained on Peptone Glucose Yeast Extract Agar (PGYA) and Nutrient Agar (NA).

Physiological and biochemical tests were done according to Moore et al. (17). Pathogenicity was tested by inoculating fresh sterile root carrot disks (2, 13) with all bacterial isolates obtained from galls and rhizosphere soil and grown on NA-slants for 48 hr. Three replicates were used for each isolate. Control carrot disks were similarly treated with sterilized water.

A. radiobacter strain KJ84 was tested for its ability to inhibit various isolates of *A. tumefaciens* through production of agrocin in vitro. Stonier's method (21) was used. This procedure was also applied using sucrose nutrient agar medium (SNA) (18).

RESULTS AND DISCUSSION

According to the results obtained from physiological and biochemical tests presented in table 3, the eleven isolates of *A. tumefaciens* were separated into two distinct groups: biotype 1 and biotype 2. Isolates designated as LA-2, LA-8 and LA-11 produced typical colonies on Schroth's medium containing mannitol as a sole carbon source and known to be selective for biotype 1. Because of this and due to failure of these isolates to grow on New and Kerr's medium, isolates LA-2, LA-8 and LA-11 were assigned to biotype 1.

Table 2 – Biotypes of 11 isolates of *Agrobacterium tumefaciens*, their pathogenicity and sensitivity to agrocin 84.

Isolate Number	Biotype	Pathogenicity	Sensitivity to K 84
La 1	2	+	+
LA 2	1	+	–
LA 3	2	+	–
LA 4	2	+	–
LA 5	2	+	–
LA 6	2	+	–
LA 7	2	+	–
LA 8	1	+	–
LA10	2	+	+
LA11	1	+	–
LA12	2	+	–

+ = Positive – = Negative

Isolates LA-1, LA-3, LA-4, LA-5, LA-6, LA-7, LA-10, and LA-12 grew well on New and Kerr's medium selective for biotype 2, utilizing erythritol as a sole source of carbon while failing to grow on Schroth's medium. Except for isolates LA2 and LA6, the results of growth of other isolates on ferric ammonium citrate were in agreement with those of Keane et al. (9). Variation in the reaction of those two isolates may be due to the instability of the test making it unsuitable for differentiation between biotypes (22).

In the oxidase test isolates LA1, LA4 and LA6 of biotype 2 gave a positive reaction, a result which contradicts the findings of Keane et al. (9) but is in agreement with the results reported by Panagopoulos and Psallidas (19). Variation in results had been ascribed by some investigators to the use of glucose in the medium which may lead to negative reactions (19). Incorporation of glucose in the medium during this study resulted in both positive and negative reactions for the same biotype leading us to believe that variations may be due to other reasons.

In the citrate test there was only one variation in the results with isolate LA8 giving a positive citrate reaction. The fact that some isolates of biotype 1 may give a positive result was reported by Panagopoulos and Psallidas (19).

Table 3 – Differential characteristics of the eleven isolates of *A. tumefaciens* obtained.

Diagnostic test*	**Biotypes**			**Isolates**										
	1	2	3	LA-1	LA-2	LA-3	LA-4	LA-5	LA-6	LA-7	LA-8	LA-10	LA-11	LA-12
3-Ketoglycoside	+	–	–	–	+	–	–	–	–	–	+	–	+	–
Growth on:														
Schroth et al. medium	+	–	–	–	+	–	–	–	–	–	+	–	+	–
New and Kerr medium	–	+	–	+	–	+	+	+	+	+	–	+	–	+
Tolerance to:														
2% NaCl	+	V	+	–	+	+	+	+	+	+	+	+	+	+
3% NaCl	+	–	ND	–	+	+	+	+	+	+	+	+	+	+
4% NaCl	+	–	ND	–	+	+	+	+	+	+	–	+	+	+
5% NaCl	–	–	ND	–	–	–	–	–	–	WK	–	–	–	–
Citrate utilization	–	+	ND	+	–	+	+	+	+	+	+	+	–	+
Ferric Ammonium citrate	+	–	ND	–	+	–	–	–	+	–	+	–	+	–
Utilization of L-tyrosine	+	–	ND	+	–	–	+	–	+	–	+	–	+	–
Acid from:														
Erythritol	–	+	–	+	–	+	+	+	+	+	–	+	–	+
Melezitose	+	–	–	–	+	–	–	–	–	–	+	–	+	–

+ = Positive, – = Negative, V = Variable, WK = Week: Slight growth, ND = Not determined

*According to Moore et al. (14).

In regard to the production of acid from erythritol, melezitose and the ability to utilize L-tyrosine all isolates tested gave typical results which were in close agreement with the finding of several investigators (9, 20).

The agrocin sensitivity test (Table 3) showed that the isolates LA-1 and LA-10 were inhibited on both Stonier's defined medium and sucrose nutrient agar madium, a result that supports studies of several other investigators (11, 18, 21). It has been ascertained that pathogenic isolates sensitive to agrocin 84 in vitro behave similarly under field conditions. Although increase in the time period of incubation led to the formation of colonies resistant to agrocin 84 within the zone of inhibition of the sensitive strain, an observation similarly reported by others (10, 11, 18, 21), such resistant colonies were usually non-pathogenic (11).

All *A. tumefaciens* isolates locally obtained were pathogenic to carrot (table 3). These results were in agreement with several investigators (2, 13, 15, 16) who stated the validity of this technique as a specific test for pathogenicity of *A. tumefaciens*. As to their prevalence biotype 2 of *A. tumefaciens* was found to be more common than biotype 1 in the coastal region of Libya.

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الطرز الحيوية لمسبب مرض التدرن التاجي
Agrobacterium tumefaciens (Smith and Townsend) Conn.
بالمنطقة الساحلية من ليبيا

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المستخلص

ينتشر مرض التدرن التاجي المتسبب عن *Agrobacterium tumefaciens* (Smith and Townsend) Conn. بالمنطقة الساحلية من ليبيا على عدة عوائل: أشجار اللوزيات، التفاحيات، الحناء *Lawsonia inermis* والادهاتودا *Adhatoda vasica*، وقد عزل المسبب من التدرنات والتربة المحيطة بالجذور المصابة لاشجار الخوخ، التفاح، الادهاتودا. أما بالنسبة لعزلات الحناء والورد فقد تم الحصول عليها من التربة حول النباتات المصابة. من خلال دراسة الخصائص الفسيولوجية والبيوكيميائية للعزلات المتحصل عليها، أمكن تصنيف 11 عزلة من *A. tumefaciens* إلى مجموعتين: الطراز الحيوي 1 «biotype 1» والطراز الحيوي 2 «biotype 2». العزلات LA-8 وLA-6 تمثل الطراز الحيوي 1 بينما العزلات LA-3، LA-4، LA-5، LA-6، LA-7، LA-10، LA-12، تتبع الطراز الحيوي 2، ويعتبر الطراز الحيوي 2 أكثر انتشاراً.

عند اختبار حساسية العزلات المتحصل عليها للمضاد الحيوي «Agrocin 84» في المختبر وجد أن العزلتين «LA-1، LA-10» ذات حساسية واضحة لهذا المضاد. كما اختبرت القدرة الأمراضية لهذه العزلات ودلت النتائج على أن جميع العزلات لها القدرة على إحداث تدرنات على شرائح الجزر.