J.H. Haas *et al.* (1991) *Phytoparasitica* 19(4):311-318

THE PRESENCE OF CROWN GALL OF GRAPE INCITED BY *AGROBACTERIUM TUMEFACIENS* BIOVAR 3 IN ISRAEL

JERRY H. HAAS[1], AIDA ZVEIBIL[1], D. ZUTRA[1], EDNA TANNE[2] and SHULAMIT MANULIS[1]

Crown gall was previously reported on grape in Israel but the pathogen was not isolated and characterized. The three recognized biovars of *Agrobacterium tumefaciens* can be tumorigenic on grape, but biovar 3 is the most important world wide. A single occurrence of tumors in a vineyard yielded bacteria which incited galls on grape, *Nicotiana glauca* and tomato, but not on bryophyllum. The bacteria were confirmed as *A. tumefaciens* because they contained DNA which hybridized with T-DNA from a Ti plasmid. Biochemical and physiological tests, octopine production and utilization, and agrocin 84 insensitivity conformed with those of bv. 3. Subsequent occurrences of the grape disease have not been found, but the presence of *A. tumefaciens* bv. 3 in Israel is a potential threat to nurseries and vineyards.
KEY WORDS: Crown gall; grape; *Vitis vinifera*; *Agrobacterium tumefaciens*; biovar; Israel.

INTRODUCTION

Agrobacterium tumefaciens (Smith and Townsend) Conn is tumorigenic on a large number of plant species (8). Although the pathogen has a wide host range, individual strains have restricted host ranges (1). Of the many susceptible crops grown in Israel, gall symptoms are common only on some perennial Rosaceae, *e.g.* apple, apricot, peach and rose (25).

The taxonomic status of *Agrobacterium* spp. is still not resolved. The latest edition of Bergey's Manual (13) accepts *A. tumefaciens* for almost all gall-forming agrobacteria and subdivides the species into three biovars (bv. 1, bv. 2 and bv. 3) based upon

Contribution from the Agricultural Research Organization. No. 3289-E, 1991 series. Received May 23, 1991; received in final form Aug. 19, 1991.
[1] Dept. of Plant Pathology and [2]Dept. of Virology, ARO, The Volcani Center, Bet Dagan 50250, Israel.

physiological characteristics and DNA relatedness. The Manual suggests that biovars are different enough to be recognized as species and that these species should include both tumorigenic and nontumorigenic strains (presently named *A. tumefaciens* and *A. radiobacter*, respectively). Ophel and Kerr (17) have recently proposed that the bv. 3 strains be recognized as *A. vitis*.

A host species may form galls in response to inoculation with *A. tumefaciens* of any of the three biovars. An example of this phenomenon is grape, *Vitis vinifera* L., which often is affected by *A. tumefaciens* bv. 3 (2,4,6,20,22,23) or its synonym *A. vitis*. Some strains of bvs. 1 and 2 also are tumorigenic on grape (19,26) and the gall symptoms are identical to those incited by bv. 3. Grape crown gall has been reported from Israel (24), but the pathogen was not identified. The most prevalent strain on Rosaceae is bv. 1 (25), and successful biocontrol has been achieved with *A. radiobacter* strain K84 (10). A single occurrence of bv. 2 has been reported on cotton (28).

The grape disease is important in many countries (6,9,18,22,23). It is significant because, in addition to galls, some strains of bv. 3 incite root lesions on grape (3). The most damaging symptoms follow early spring frost injury, when galls develop on internally injured canes. Wounding and tumor formation are followed by stem dieback distal from the tumor (15). Control of the disease is at present not possible. Biovar 3 is not inhibited by the standard biocontrol agent *A. radiobacter* strain K84 or its non-plasmid-transferable derivative K1026 (11). In most plants the bacteria are not systemic but they may be in grape (5,14,23). Thus, clean nursery stock programs are required to prevent the spread of the pathogen.

In January 1990, for the first time in several years, a few plants with root galls were detected during planting of a new vineyard at Biqat Arad, in the Negev region of Israel. The origin of the plants was a nursery in Zikhron Ya'aqov, in the coastal plain. The rootstock of the plants with tumors is not known, but those used in Israel are rootstocks of U.S.A. selections resistant to root-knot nematode (*Meloidogyne* spp.).

The present study was undertaken to identify the pathogen involved, because of the potential importance of *A. tumefaciens* on grape.

MATERIALS AND METHODS

Isolations from plant tumors, bacteria culture and maintenance

Galls from the vineyard and from artificially inoculated plants were surface sterilized in 0.5% sodium hypochlorite, washed twice with sterile distilled water, and crushed. Aliquots were plated on modified Roy-Sasser medium (MRS) (16) and incubated at 28°C.

The MRS is semi-selective for agrobacteria and is modified by reducing the quantity of boric acid to 0.5 g l^{-1}; we have found that this allows growth of all biovars of *A. tumefaciens*. Even with the normal concentration of boric acid, many nontumorigenic strains of bv. 1 grow on the medium (6). Simultaneously, pure cultures of confirmed

isolates of bvs. 1, 2 and 3 (Table 1) were plated on MRS. Cultures were maintained on Difco Nutrient Agar (NA).

TABLE 1
PATHOGENICITY AND AGROCIN SENSITIVITY OF NEW *AGROBACTERIUM* SP. STRAINS FROM GRAPE IN ISRAEL AND KNOWN STRAINS OF BIOVARS 1, 2 AND 3

Agrobacterium strain	*Source*	*No. of isolates*	*Gall induction*				*Agrocin sensitivity*
			Grape	*Bryophyllum*	*N. glauca*	*Tomato*	
G114	Grape (Israel)	4	+	-	+	+	-
Ag57,Ag63/85 (bv. 3)	Grape (Greece)	2	+	-	+	-	-
13B (bv. 1)	Rose (Israel)	1	-	+	+	+	+
K84 (bv. 2)	Soil (Australia)	1	-	-	-	-	-

+, - Positive and negative reaction, respectively.

Bacterial strains

Known strains of each of the three biovars were used as control cultures in all inoculation and diagnostic tests (Tables 1 and 2). *Agrobacterium tumefaciens* bv. 1 strain 13B is a highly virulent isolate from our collection (J.H. Haas). For biochemical tests and as a nontumorigenic control, bv. 2 strain K84 of *A. radiobacter* from A. Kerr was used. Tumorigenic bv. 3 strains Ag57 and Ag63/85 from grape (12) were received from C.G. Panagopoulos.

Tumorigenicity tests

Crushed gall tissue (16) or suspensions of bacteria from NA medium (1×10^9 cells ml^{-1}) were used as inoculum. The test plants were grape cv. Cabarnet Franc, bryophyllum (*Kalanchoe pinnata* Pers.), tobacco (*Nicotiana glauca* Graham) and tomato (*Lycopersicon esculentum* Mill. cv. Marmand). Leaves and stems were punctured with an insect-mounting needle and the wounds were wetted with inoculum absorbed on a cotton swab. The test-plants were maintained in a greenhouse chamber (15 to 30°C) for a maximum of 6 wk before tumorigenicity was evaluated.

Biochemical tests

Diagnostic tests for biovar determination (Table 2) were conducted according to Moore *et al.* (16).

Agrocin sensitivity was tested according to the protocol of Stonier (21) using *A. radiobacter* strain K84 as the bacteriocin producer.

The ability of bacteria to catabolize octopine was tested on solid and liquid media (16). The presence of nopaline and octopine in galls was confirmed by paper

TABLE 2

DIFFERENTIAL DIAGNOSTIC REACTIONS FOR BIOVAR IDENTITY OF FOUR *AGROBACTERIUM TUMEFACIENS* STRAINS FROM GRAPE IN ISRAEL (G114) AND KNOWN STRAINS OF BIOVARS 1, 2 AND 3

Diagnostic test	*Strain (biovar)*			
	13B (1)	*K84 (2)*	*Ag57 (3)*	*G114*
3-Ketolactose production	+	-	-	-
Growth in 2% NaCl	+	-	+	+
Growth at 35°C	+	-	+	+
Growth at 37°C	+	-	-	-
Action on litmus milk	Alk.	Acid	Alk.	Alk.
Acid from:				
Sucrose	+	+	+	+
Erythritol	-	+	-	-
Melezitose	+	-	-	-
Alkali from:				
Malonic acid	-	+	+	+
L-tartaric acid	-	+	+	+
Growth in ferric ammonium citrate	+	-	-	-
L-tyrosine utilization	-	+	-	-
Oxidase production	+	-	+	+
Octopine production/utilization	-	-	+	+

+, - Positive and negative reaction, respectively.

electrophoresis of gall-tissue extracts (27). Pure nopaline and octopine (Sigma) were run separately and co-chromatographed with the extracts.

Colony hybridization

Bacteria were grown on a Hybond N nylon membrane (Amersham Inc., Arlington Hts., IL, USA) overlaid on a NA plate, and incubated for 48 h at 28°C. Colonies were lysed and filters were hybridized according to the manufacturer's recommendations. Hybridization and stringency washes were done at 65°C. The DNA probe was obtained from L.W. Moore and radiolabeled with ^{32}P. It contained a 25 kb fragment of the entire T-DNA of plasmid pTiB6-806.

RESULTS AND DISCUSSION

Bacteria isolation and host range

Tumors were collected from the roots of plants culled from the planting material. Fluid from crushed tissues was plated on MRS as were the known strains of bvs. 1, 2

and 3. Colonies of *A. tumefaciens* bv. 3 on Roy-Sasser medium are reported to be typically white with red centers (16), but after 4 days, cultures of *A. tumefaciens* had mixtures of red-centered and all-white colonies. The color reaction (triphenyltetrazolium chloride reduction) was not related to the colony density on the agar and was not consistent. Red-centered colonies could produce white ones upon subsequent transfer to MRS and *vice versa*. Both red and white colonies isolated from grape galls were examined for tumorigenicity on bryophyllum and *N. glauca*; 32 were tumorigenic on *N. glauca* but not on bryophyllum. Five strains originating from white and 27 from red-centered colonies were pathogenic. Gall tissue fluid also was inoculated on these hosts and was tumorigenic only on tobacco.

Four exemplars of the new grape isolates, designated G114 A to D, were inoculated into grape and tomato and induced galls on both (Table 1); a photograph of a tumor on grape is presented in Fig. 1. This contrasted with the host range of Ag57 (bv. 3 from

Fig. 1. Tumor on grape 10 weeks after inoculation with *Agrobacterium tumefaciens* bv. 3, strain G114.

grape) and 13B (bv. 1 from rose) (Table 1). Koch's postulates were completed with the G114 strains by isolating *A. tumefaciens*-type colonies from the grape galls. There was no correlation between the color reaction on MRS of cultures from the original tumors

and the re-isolates from artificially inoculated tumors.

None of the 32 G114 isolates of the confirmed biovar 3 strains was inhibited by agrocin 84 (Table 1).

Biochemical and molecular characteristics of G114

Because of the identical pathogenic and agrocin sensitivity reactions of all the new grape isolates, the following tests were conducted with strains G114 A–D. The presence of T-DNA in the strains from grape galls was confirmed by colony hybridization (7) with a DNA probe. All the tumorigenic strains reacted with the probe, whereas the nontumorigenic strain K84 did not react (Table 2).

Grape and *N. glauca* galls incited by G114 and Ag57 contained octopine. This opine supported the growth of G114 strains on solid and liquid media when it was the only N and C source in the medium (Table 2).

The galls on grape plants collected in Israel contained bacteria typical of *A. tumefaciens* bv. 3 as characterized by biochemical properties and pathogenicity tests (4,9,12,13,16,18) and correspond with the description of the new species, *A. vitis*, proposed by Ophel and Kerr (17).

Although bv. 1 and bv. 2 strains may be tumorigenic on grape (19,26), all 32 tested isolates from grape in Israel were non-pathogenic to bryophyllum but incited galls on *N. glauca*. This is presumptive evidence that the isolates were not bv. 1 or bv. 2. In all our previous (unpublished) tests with tumorigenic isolates from Israel, bv. 1 and bv. 2 always incited galls on bryophyllum. Prior occurrences of grape crown gall (25) may also have been incited by this biovar.

Plant decline symptoms or galls have not been found in the vineyard where the original tumors were collected and no reports have been received from nurseries or vineyards of additional occurrences of grape crown gall. Crown gall can cause significant losses in grape nurseries and vineyards but at present its distribution in the country is limited. It is likely that the pathogen has survived in the plants but that the tumor development did not occur in the hot and dry climate of the Negev region. However, spring-time frosts are sometimes experienced and in subsequent years the cane gall, stem dieback syndrome may develop (5,15).

Strain G114 has been deposited (ATCC No. 49744) for preservation and distribution.

ACKNOWLEDGMENTS

A portion of this research was funded by the Israel Grape Marketing Board. A. Kerr and C.G. Panagopoulos kindly donated confirmed strains of biovars 2 and 3, respectively. We thank L.W. Moore for supplying the DNA probe.

REFERENCES

1. Anderson, A.R. and Moore, L.W. (1979) Host specificity in the genus *Agrobacterium*. *Phytopathology* 69:320–323.
2. Bien, E., Lorenz, D., Eichhorn, K. and Plapp, R. (1990) Isolation and characterization of *Agrobacterium tumefaciens* from the German vine region Rheinpfalz. *Z. Pflanzenkr. Pflanzenschutz* 97:313–322.
3. Burr, T.J., Bishop, A.L., Katz, B.H., Blanchard, L.M. and Bazzi, C. (1987) A root-specific decay of grapevine caused by *Agrobacterium tumefaciens* and *A. radiobacter* biovar 3. *Phytopathology* 77:1424–1427.
4. Burr, T.J. and Katz, B.H. (1983) Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap, and from vineyard soil. *Phytopathology* 73:163–165.
5. Burr, T.J. and Katz, B.H. (1984) Grapevine cuttings as potential sites of survival and means of dissemination of *Agrobacterium tumefaciens*. *Plant Dis.* 68:976–978.
6. Burr, T.J., Katz, B.H. and Bishop, A.L. (1987) Populations of *Agrobacterium* in vineyard and nonvineyard soils and grape roots in vineyards and nurseries. *Plant Dis.* 71:617–620.
7. Burr, T.J., Norelli, J.L., Katz, B.H. and Bishop, A.L. (1990) Use of Ti plasmid DNA probes for determining tumorigenicity of *Agrobacterium* strains. *Appl. Environ. Microbiol.* 56:1782–1785.
8. De Cleene, M. and De Ley, J. (1976) The host range of crown gall. *Bot. Rev.* 42:389–466.
9. Dhanvantari, B.N. (1983) Etiology of crown gall in Ontario. *Can. J. Bot.* 61:2641–2646.
10. Farkas, E. and Haas, J.H. (1985) Biological control of crown gall in rose nursery stock. *Phytoparasitica* 13:121–127.
11. Jones, D.A. and Kerr, A. (1989) *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. *Plant Dis.* 73:15–18.
12. Kerr, A. and Panagopoulos, C.G. (1977) Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. *Phytopathol. Z.* 90:172–179.
13. Kersters, K. and De Ley, J. (1984) Genus III. *Agrobacterium* Conn 1942, 359^{AL}. *in*: Krieg, N.R. [Ed.] Bergey's Manual of Systematic Bacteriology, Vol. 1. Williams & Wilkins, Baltimore, MD.
14. Lehoczky, J. (1971) Further evidence concerning the systemic spreading of *Agrobacterium tumefaciens* in the vascular system of grapevines. *Vitis* 10:215–221.
15. Lehoczky, J. (1978) Root system of the grapevine as a reservoir of *Agrobacterium tumefaciens* cells. *Proc. 4th Int. Conf. Plant Pathogenic Bacteria* (Angers, France), vol. 1, pp. 239–243.
16. Moore, L.W., Kado, C.I. and Bouzar, H. (1988) Gram-negative bacteria. Agrobacterium. *in*: Schaad, N.W. [Ed.] Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. APS Press, St. Paul, MN.
17. Ophel, K. and Kerr, A. (1990) *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. *Int. J. Syst. Bacteriol.* 40:236–241.
18. Panagopoulos, C.G. and Psallidas, P.G. (1973) Characteristics of Greek isolates of *Agrobacterium tumefaciens* (E.F. Smith & Townsend) Conn. *J. Appl. Bacteriol.* 36:233–240.

19. Panagopoulos, C.G., Psallidas, P.G. and Alivizatos, A.S. (1978) Studies on biotype 3 of *Agrobacterium radiobacter* var. *tumefaciens*. *Proc. 4th Int. Conf. on Plant Pathogenic Bacteria* (Angers, France), vol. 1, pp. 221–228.
20. Sawada, H., Ieki, H. and Takikawa, Y. (1990) Identification of grapevine crown gall bacteria isolated in Japan. *Ann. Phytopathol. Soc. Jpn.* 56:199–206.
21. Stonier, T. (1960) *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. *J. Bacteriol.* 79:889–898.
22. Sule, S. (1978) Biotypes of *Agrobacterium tumefaciens* in Hungary. *J. Appl. Bacteriol.* 44:207–213.
23. Tarbah, F.A. and Goodman, R.N. (1986) Rapid detection of *Agrobacterium tumefaciens* in grapevine propagating material and the basis for an efficient indexing system. *Plant Dis.* 70:566–568.
24. Volcani, Z. (1961) Crown gall disease in Israel. *Plant Dis. Rep.* 45:823.
25. Volcani, Z. (1985) [Bacterial plant diseases in Israel.] Weizmann Science Press of Israel, Jerusalem (in Hebrew).
26. Wabiko, H., Kagaya, M., Kodama, I., Masuda, K., Kodama, Y., Yamamoto, H., Shibano, Y. and Sano, H. (1989) Isolation and characterization of diverse nopaline type Ti plasmids of *Agrobacterium tumefaciens* from Japan. *Arch. Microbiol.* 152:119–124.
27. Yang, N.S., Platt, S.G. and Christou, P. (1987) Detection of opines by colorimetric assay. *Anal. Biochem.* 160:342–345.
28. Zutra, D. and Orion, D. (1982) Crown gall bacteria *(Agrobacterium radiobacter* var. *tumefaciens)* on cotton roots in Israel. *Plant Dis.* 66:1200–1201.