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Characteristics of Portuguese strains of *Agrobacterium tumefaciens* isolated from grapevine and stone fruit trees

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RESUMO

Prospecções efectuadas em vinhas e pomares no País revelaram a presença do agente causal do tumor bacteriano, *Agrobacterium tumefaciens*. Com base no estudo das características morfológicas, culturais, bioquímicas e de patogenicidade, quarenta isolamentos de *Agrobacterium* spp. foram identificados e separados nos respectivos biótipos. A maioria das estirpes isoladas apresentaram características inerentes aos biótipos 1 e 2. No caso da videira, foi também detectada a presença da bactéria no translocado xilémico, o que evidencia a natureza sistémica desta doença.

RÉSUMÉ

Au Portugal, des prospections effectuées aux vignobles et aux vergers ont permis la détection de l'agent de la tumeur bactérienne ou galle du collet, *Agrobacterium tumefaciens*. On a étudié les caractères morphologiques, culturaux, biochimiques et de pathogénicité de quarente souches d'*Agrobacterium* spp., ce qui a permis de les identifier et de les regrouper, le plus souvent, aux biotypes 1 et 2. Chez la vigne, on a réussi à isoler la bactérie à partir des vaisseaux, ce qui met en évidence la nature vasculaire de la maladie.

SYNOPSIS

In Portugal, some vineyards and orchards were surveyed and the causal agent of crown gall disease, *Agrobacterium tumefaciens*, was detected. Forty isolates were identified and biotyped, based on morphological, cultural, biochemical and pathogenical characteristics. The majority of *Agrobacterium* spp. isolates, obtained both from grapevine and stone fruit trees, were allocated to biotypes 1 and 2. The pathogen was also isolated from vascular fluids of grapevine, which suggests the systemic infection in this host.

1. INTRODUCTION

Crown gall, caused by *Agrobacterium tumefaciens* (Smith & Townsend) Conn, is one of the most important and widely distributed bacterial diseases of cultivated plants (ELLIOTT, 1951). All virulent *A. tumefaciens* strains contain a large tumor-inducing *(Ti)* plasmid which is required for tumorigenicity. If the *Ti* plasmid is eliminated, the remaining strain becomes completely avirulent and phenotypically the same as the nontumorigenic soil organism *Agrobacterium radiobacter*. Conversely, if the *Ti* plasmid is transferred into *A. radiobacter*, the recipient strain becomes a virulent *A. tumefaciens* (WATSON *et al.*, 1975; KERSTERS & DE LEY, 1984).

The host range of *A. tumefaciens* is remarkably wide. KERSTERS & DE LEY (1984) reported that at least 643 host plants from more than 90 families were susceptible to crown gall disease. Most of these hosts are dicotyledonous, some gymnospermous and only a few species belonging to *Liliaceae* and *Araceae* families, of monocotyledonous plants, are affected (HAYWARD & WATERSTON, 1965). Despite the broad host range of *A. tumefaciens*, crown gall disease causes major economical losses throughout the world on pome and stone fruit trees, grapevines and roses (KERR, 1980; DU PLESSIS *et al.*, 1984; BURR *et al.*, 1988; PSALLIDAS, 1988). However, some strains of *A. tumefaciens* exhibit a very high host specificity and a limited host range (THOMASHOW *et al.*, 1980).

It has been established that strains of *A. tumefaciens* can be separated into three distinct biotypes or biovars, on the basis of their physiological characteristics (KERR & PANAGOPOULOS, 1977; KERSTERS & DE LEY, 1984). Strains affecting fruit trees belong to biotypes 1 and 2, while those found on grapevines belong mainly to biotype 3 (WEBSTER *et al.*, 1986). An interesting recent development in the taxonomy of *Agrobacterium* spp. is the proposed inclusion of biotype 3 strains in a new species, *Agrobacterium vitis* (OPHEL & KERR, 1990). The new specific name is justified because the strains now classified as biotype 3, tumorigenic and nontumorigenic, are closely related but show low levels of DNA homology when compared with *A. tumefaciens* and other *Agrobacterium* spp. strains (OPHEL & KERR, 1990). However, in the present work, the old nomenclature (*A. tumefaciens* and *A. radiobacter*, biotypes 1, 2 and 3) is still used.

It should be noted that grapevine is the only known host on which all three biotypes can be found naturally (PANAGOPOULOS *et al.*, 1978; SÜLE, 1978). Although host specificity of biotype 3 strains to grapevine has been widely reported (KERR & PANAGOPOULOS, 1977; PANAGOPOULOS *et al.*, 1978; SÜLE, 1978; LOPER & KADO, 1979), some European and Californian strains appear to be not host specific or limited in their host range (PANAGOPOULOS *et al.*, 1978; PERRY & KADO, 1982).

A review, focusing on the biochemical features of the interaction between *A. tumefaciens* and host plants, was recently published in Portugal (VARENNES, 1991). To date, however, no research work has been undertaken in our country concerning crown gall disease, although plants with gall-like formations on vineyards, orchards and nurseries are frequently observed. So, the aim of the present study is the characterization of *A. tumefaciens* strains isolated from grapevine and stone-fruit plants, showing symptoms of crown gall.

2. MATERIALS AND METHODS

2.1. ISOLATION OF AGROBACTERIUM FROM GRAPEVINE AND STONE-FRUIT GALLS

Gall tissues were washed in tap water, immersed in NaOCl (0.5%) during 10 min, rinsed by three times in sterile distilled water (SDW) and macerated in a pestle and mortar. The crushed gall was left for 4-6 hours and, after appropriate dilution, streaked on petri dishes containing the selective media described by SCHROTH *et al.* (1965), NEW & KERR (1971) and ROY & SASSER (1983) and hereafter referred as SCH, NK and RS media, respectively. A small modification was made on RS medium, on which chlorothalonil was replaced by cycloheximide (BURR *et al.*, 1987). Plates were incubated for five days at 28°C and checked for growth of potential *Agrobacterium* colonies.

2.2. ISOLATION OF AGROBACTERIUM FROM GRAPEVINE VASCULAR FLUIDS

Stem segments (15 cm long) were surface-sterilized by flame after immersion in 95% ethanol. A pressure chamber, in which SDW is forced acropetally at 0.1 MPa through the stem tissue, was used (GOODMAN *et al.*, 1987). Displaced vascular fluids and bacteria were collected at the apical end with a sterile capilary pipette and placed in a sterile tube (TARBAH & GOODMAN, 1986). Triplicate 0.1 ml aliquots from the first 1.5-2.0 ml of fluid were spread on petri dishes containing SCH, NK and RS media. Plates were incubated for five days at 28°C and also checked for growth of potential *Agrobacterium* colonies.

2.3. CHARACTERIZATION OF STRAINS

To clone for purity, individual and typical colonies from selective media were suspended in SDW and streaked on potato dextrose agar (PDA-Difco). Single colonies from PDA were again selected and then transferred to PDA slants and stored at about 4°C, until ulterior utilization. Prior to further characterization, all strains were tested for Gram reaction, fluorescein production on King's medium B (KMB) (KING *et al.*, 1954) and type of metabolism (HUGH & LEIFSON, 1953).

Strains were characterized to a specific biotype by the determinative procedures of KERR & PANAGOPOULOS (1977) which include: production of 3-ketolactose (BERNAERTS & DE LEY, 1963), sodium chloride tolerance, growth at 35°C, action on litmus milk, acid production from erythritol and melezitose and alkali production from malonate and tartrate. All tests were replicated and repeated at least twice. Four strains classified as *A. tumefaciens* were used for comparative purposes (Table 1). These strains were obtained from Dr. C. Bazzi, Istituto di Patologia Vegetale, Università degli Studi, Bologna, Italy.

TABLE 1

Description of Agrobacterium tumefaciens strains used for comparative purposes on biochemical and pathogenicity tests

Strains	Isolated from	Host plant	Biotype
IPV-BO 2150a	gall	grapevine	1
IPV-BO $FC_2$14	vascular fluid	grapevine	3
IPV-BO 1506	gall	rose	2
IPV-BO 2028	gall	*Chrysanthemum* sp.	1

2.4. *PATHOGENICITY TESTS*

Strains were streaked on nutrient agar (NA-Oxoid) slants supplemented with 1% D-glucose to prepare inoculum for all pathogenicity tests, except those concerning grapevine detached leaves. In the last case, the method described by THIES *et al.* (1991) was used.

Inoculations were made on all or some of the following hosts: *Chrysanthemum* sp., sunflower (*Helianthus annus* "Florasol"), tomato (*Lycopersicon esculentum* "Marmande") and grapevine (*Vitis vinifera* "Jaen") potted plants, carrot slices *(Daucus carota)* and grapevine leaves. The grapevine cultivars chosen to carry out this last assay mostly coincided with those from which isolates were obtained. At least three plants, slices or leaves, of each host were inoculated with each strain and tests were repeated twice.

Sunflower, tomato and young shoots of grapevine plants were inoculated according to the folowing method: stems or shoots were repeatedly punctured with a fine sterile needle and, subsequently, a heavy smear of cells of each isolate was spread in the wounds (KNAUF *et al.*, 1982). Control plants were similarly treated, but SDW was used instead of inoculum. All plants were covered with transparent polyethylene bags for 48 hours and, subsequently, maintained under greenhouse conditions until symptoms appearance.

Leaves and stems of chrysanthemum were inoculated with hypodermic needle injections of each bacterial suspension in SDW (approximately 10^8 c.f.u./ml). Leaf injections were made by inserting the needle into the midvein or lateral vein, until a small water-soaked area around the point of injection was visible (MILLER, 1975). Plants treated with SDW were used as negative controls. Only four isolates from grapevine and IPV-BO 2028 positive control were tested in chrysanthemum plants. All plants were covered with transparent polyethylene bags and maintained at room temperature, during incubation period.

Carrot slices were inoculated following the method of ARK & SCHROTH (1958). Fresh young roots were selected and, after surface-sterilization, cutted on slices 5 mm thick. Slices were then placed inside petri plates containing a moistened filter paper. Inoculation was made by spreading the inoculum on the slice surface and plates were wrapped with Parafilm during incubation. IPV-BO 2028 and IPV-BO 1056 were used as positive controls.

Detached leaf assay was only used to test for pathogenicity the grapevine isolates and was based on procedures described by THIES *et al.* (1991). However, in the present study, leaves were collected from grapevine potted plants, originated from rooted cuttings, instead of those of THIES *et al.* (1991), produced by meristem tissue culture. In a preliminary experiment, water agar (WA) and wood plant medium (LLOYD & MCCOWN, 1980), modified by THIES *et al.* (1991), were compared for leaf maintenance. As no difference was observed between both media, when plates were incubated at 24°C under fluorescent light (16 hours of photoperiod) for 4 weeks, WA was chosen to be used in this experiment. Negative controls were injected with nutrient glucose broth, the medium used on inoculum production. Positive controls, IPV-BO 2150a and IPV-BO $FC_2$14, were also included.

3. RESULTS

3.1. ISOLATES AND CHARACTERIZATION

Twenty six strains of *Agrobacterium* spp. were isolated from grapevine galls, six from vascular fluids or sap and eight from galls of cultivar/rootstock combination of peach and plum (Tables 2 and 3).

TABLE 2 – *Strains of Agrobacterium spp. isolated from galls (Ag) or vascular fluids (Av) of grapevine cultivars and/or rootstocks*

Strains	Isolated from	Cultivar/Rootstock	Region*
Ag1, Ag15, Ag16	gall from graft union	Arinto / 1103 P	West
Ag2, Ag14	gall from base of rootstock	— / 1103 P	West
Ag4, Ag12, Ag13a**, Ag13b	gall from graft union	Touriga Francesa / 99R	North
Ag5, Ag9a, Ag9b	gall	Trincadeira / —	South
Ag8	gall	Fernão Pires / —	South
Ag10	gall	unknown	South
Ag11	gall from graft union	Bical / —	North
Ag17a, Ag17b	gall strand	— / 1103 P	West
Ag18, Ag21	gall strand	— / 99 R	South
Ag22	gall from graft union	Negra Mole / 99 R	South
Ag19a, Ag19b, Ag20, Ag23, Ag26a, Ag26b	gall from graft union	Periquita / 99 R	South
Av3, Av8	vascular fluids	unknown	South
Av11	vascular fluids	Touriga Francesa /99 R	North
Av17, Av18, Av19	vascular fluids	— / 1103 P	West

*All isolates were obtained in Portugal.

**Isolates with names differing only in a/b termination were obtained from the same gall.

Typical colonies were produced on SCH, NK and RS selective media, after 5 days of incubation. On SCH, colonies were round, domed, smooth, entire and yellowish coloured; on NK, colonies were round, domed, entire and pearly-white becoming tan-coloured; on RS, colonies were convex and slightly mucoid, with red centers and a narrow white margin. An exuberant growth was observed on PDA and colonies were round, convex, mucous, glistening, opaque, with entire edge, white to beige coloured. All isolated strains were Gram-negative and aerobic. No fluorescent pigment was produced on KMB and glucose was oxidatively metabolised.

TABLE 3 – *Strains of Agrobacterium spp. isolated from galls of rootstocks and cultivars of peach and plum*

Strains	Isolated from	Cultivar/Rootstock	Region*
Ap2, Ap3a**, Ap3b	gall from roots	Rubidoux / —	North
Ap4a, Ap4b	gall from roots	— / GF 305	Center
Ap6	gall from crown	Stanley / Mariana	Center
Ap7	gall from crown	Super Angelino / Mariana	Center
Ap8	gall from crown	— / GF 305	South

*All isolates were obtained in Portugal.

**Isolates with names only differing in a/b termination were obtained from the same gall.

Based on growth on selective media and biochemical characteristics, strains were separated into biotypes. Of the twenty six strains obtained from grapevine galls, twelve were identified as biotype 1, eight as biotype 2, two as biotype 3 and four were not identified. However, the majority of strains allocated to biotype 1 was L-tartrate positive. This result is in agreement with the response of biotype 1 IPV-BO 2028 strain, isolated from chrysanthemum, but disagrees with the response of IPV-BO 2150a, also a biotype 1 strain, but isolated from grapevine (Table 4). In other respects, however, they correspond to the criteria of biotype 1.

Among the strains isolated from vascular fluids, biotype 2 was prevalent but, due to the small number of strains collected and studied, this result cannot be generalized. Some less common results from biochemical tests were obtained for Ag2, Ag8, Ag10, Ag11, Av8 and Av19 grapevine isolates. Although Ag10 and Av8 isolates have shown some characteristics of biotype 3, they failed to grow in 2% NaCl and in alkali production from malonate and for this reason were not classified. On the other hand, strains isolated from galls and vascular fluids of the same plant (Ag10-Av8, Ag15-Av17, Ag17a-Av18) showed identical biochemical characteristics and strains belonging to distinct biotypes were obtained from the same gall.

Of the eight strains isolated from galls of cultivar/rootstock combinations of peach and plum, five were identified as biotype 2 and three as biotype 1 (Table 5). Results of biochemical tests of these strains were completely in agreement with those obtained

for the isolates used for comparative purposes. None of the peach and plum strains identified as biotype 1 utilized L-tartrate. These results generally contradict those obtained for grapevine isolates. Similarly, for grapevine and peach and plum isolates, distinct biotypes were found to be present in the same gall.

TABLE 4 – *Biochemical characteristics of Agrobacterium spp. strains isolated from galls and vascular fluids of grapevine and those used for comparative purposes*

Strains	3-keto-lactose	NaCl 2%	Growth 35°C	Litmus milk	Erythritol	Melezitose	Malonate	L-tartrate	Biotype*
Ag1	–	–	–	Acid	+	–	+	+	2
Ag2	–	–	–	Acid	+	–	–	–	...**
Ag4	+	+	+	Alkal.	–	+	–	–	1
Ag5	–	+	+	Alkal.	–	–	+	+	3
Ag8	–	–	–	Acid	–	–	–	–	...
Ag9a***	+	+	+	Alkal.	–	+	–	+	1
Ag9b	–	–	–	Acid	+	–	+	+	2
Ag10	–	–	+	Alkal.	–	–	–	+	...
Ag11	–	–	–	Alkal.	–	–	–	–	...
Ag12	+	+	+	Alkal.	–	+	–	+	1
Ag13a	–	+	+	Alkal.	–	–	+	+	3
Ag13b	+	+	+	Alkal.	–	+	–	+	1
Ag14	–	–	–	Acid	+	–	+	+	2
Ag15	–	–	–	Acid	+	–	+	+	2
Ag16	+	+	+	Alkal.	–	+	–	+	1
Ag17a	+	+	+	Alkal.	–	+	–	+	1
Ag17b	–	–	–	Acid	+	–	+	+	2
Ag18	–	–	–	Acid	+	–	+	+	2
Ag19a	–	–	–	Acid	+	–	+	+	2
Ag19b	+	+	+	Alkal.	–	+	–	–	1
Ag20	+	+	+	Alkal.	–	+	–	+	1
Ag21	+	+	+	Alkal.	–	+	–	–	1
Ag22	+	+	+	Alkal.	–	+	–	+	1
Ag23	+	+	+	Alkal.	–	+	–	+	1
Ag26a	–	–	–	Acid	+	–	+	+	2
Ag26b	+	+	+	Alkal.	–	+	–	+	1
Av3	–	–	–	Acid	+	–	+	+	2
Av8	–	–	+	Alkal.	–	–	–	+	...
Av11	–	–	–	Acid	+	–	+	+	2
Av17	–	–	–	Acid	+	–	+	+	2
Av18	+	+	+	Alkal.	–	+	–	+	1
Av19	–	–	–	Acid	+	–	–	–	...
IPV-BO 2028	+	+	+	Alkal.	–	+	–	+	1
IPV-BO 2150a	+	+	+	Alkal.	–	+	–	–	1
IPV-BO $PC_2$14	–	–	–	Alkal.	–	–	+	+	3
IPV-BO 1506	–	–	–	Acid	+	–	+	+	2

*Proposed identification.
**Unidentified biotype.
***Isolates with names only differing in a/b termination were obtained from the same gall.

TABLE 5

Biochemical characteristics of Agrobacterium spp. strains isolated from galls of rootstocks and cultivars of peach and plum

Strains	3-keto-lactose	NaCl 2%	Growth 35°C	Litmus milk	Erythritol	Melesitose	Malonate	L-tartrate	Biotype*
Ap2	–	–	–	Acid	+	–	+	+	2
Ap3a**	+	+	+	Alkal.	–	+	–	–	1
Ap3b	–	–	–	Acid	+	–	+	+	2
Ap4a	+	+	+	Alkal.	–	+	–	–	1
Ap4b	–	–	–	Acid	+	–	+	+	2
Ap6	–	–	–	Acid	+	–	+	+	2
Ap7	+	+	+	Alkal.	–	+	–	–	1
Ap8	–	–	–	Acid	+	–	+	+	2
IPV-BO 2150a	+	+	+	Alkal.	–	+	–	–	1
IPV-BO 1506	–	–	–	Acid	+	–	+	+	2

*Proposed identification.
**Isolates with names only differing in a/b termination were obtained from the same gall.

3.2. *PATHOGENICITY TESTS*

Results concerning the pathogenicity tests of grapevine isolates are summarized in Table 6. Among the isolated strains, 65.6% were pathogenic, at least, to one host plant and for this reason classified as *A. tumefaciens*. On carrot slices, only Ag12, IPV-BO 1056 and IPV-BO 2028 strains proved to be pathogenic by gall development 30 days after inoculation (Figure 1A). A similar result was obtained when green shoots of potted grapevines were inoculated. Failure to obtain more positive results of pathogenicity, in young shoots of grapevine, does not prove the avirulence of inoculated strains. In fact, some of these strains, when inoculated in grapevine leaves (Figure 1B) and/or sunflower, showed to be tumorigenic. So, it will be reasonable to conceive that the cultivar choice and/or environmental conditions (uncontrolled) during the incubation period have not been the most adequate. As previously referred, only four isolates were inoculated on chrysanthemum plants and typical galls were produced by Ag4, Ag9a and IPV-BO 2028 on stems and leaves.

The Ag12 isolate of *A. tumefaciens*, although not a typical member of biotype 1, showed to be the most virulent strain tested and to be not host-specific. It was pathogenic for sunflower, tomato, carrot slices and young shoots and detached leaves of grapevine.

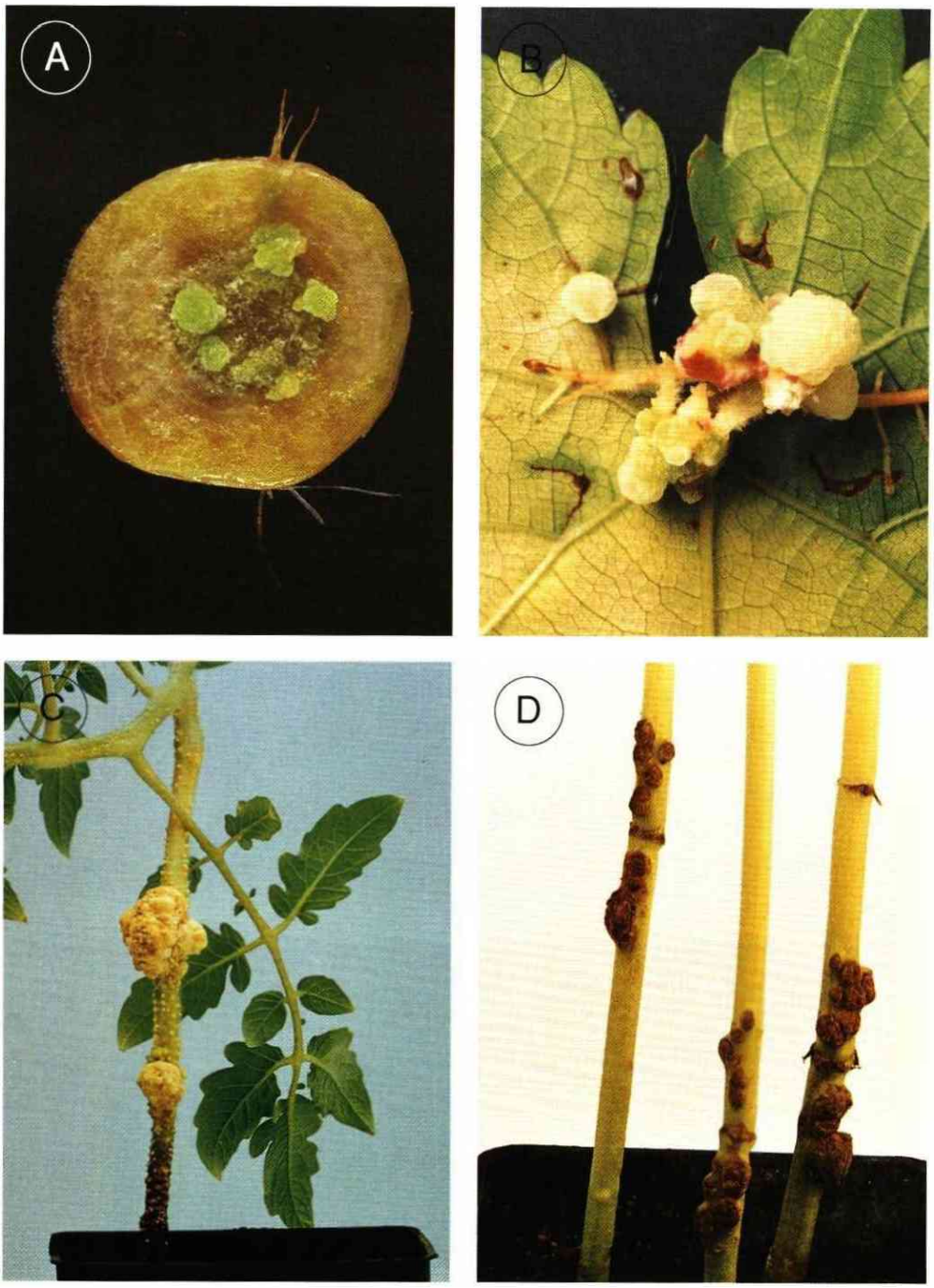

FIGURE 1 - Galls induced by *A. tumefaciens* isolates, 3 weeks after inoculation, on: (A) carrot slice (Ag12, biotype 1); (B) grapevine detached leaf (Ag12); (C) tomato stem (Ap2, biotype 2); (D) sunflower stems (Ap8, biotype 2).

TABLE 6

Pathogenicity tests of Agrobacterium spp. strains isolated from grapevine galls (Ag) and vascular fluids (Av)

Strains	Sunflower	Tomato	Grapevine leaves
IPV-BO $FC_2$14, Ag12	+	+	+
Ag4, Ag9a*, Ag9b, Ag11, Ag14, Ag16, Ag17a	+	−	+
IPV-BO 2150a, Ag10, Ag13a, Ag19b, Ag20, Ag21, Ag22, Av11, Av17, Av18	+	−	−**
Ag1, Ag2, Ag8, Ag15	−	−	+
Ag5, Ag13b, Ag17b, Ag18, Ag19a, Ag23, Ag26a, Ag26b, Av3, Av8, Av19	−	−	−

*Isolates with names only differing in a/b termination were obtained from the same gall.

**Strains Av11 and Av17 were not tested.

TABLE 7

Pathogenicity tests of Agrobacterium spp. strains isolated from peach and plum (Ap) cultivars or rootstocks

Strains	Sunflower	Tomato	Carrot slices
IPV-BO 1506, Ap2, Ap7	+	+	+
Ap 8	+	+	NT*
IPV-BO 2028	NT	+	+
IPV-BO 2150a, Ap4a**	+	−	−
Ap3a, Ap3b	NT	−	−
Ap4b, Ap6	−	−	−

*Not tested.

**Isolates with names only differing in a/b termination were obtained from the same gall.

Results of pathogenicity tests of strains isolated from peach and plum cultivars/rootstocks are listed in Table 7. Of the eight isolates obtained from galls, 50% were pathogenic at least to one host plant, and a few isolates were pathogenic to all tested host plants. Well developed galls were obtained for Ap2, Ap7 and Ap8 isolates on tomato and sunflower plants (Figures 1C and 1D).

4. DISCUSSION

The SCH, NK and RS media were very effective for *Agrobacterium* isolation. In most cases, pure colonies were obtained with almost total supression of other microrganisms, as it was confirmed by streaking single colonies on non-selective medium PDA. However, biotypes 1 and 2 also grew on RS medium, although at a slower rate than biotype 3, and some biotype 1 strains grew on NK medium. These findings were also referred by BURR & KATZ (1984) and BURR *et al.* (1987). Thus, despite of the suitability of selective media for *Agrobacterium* isolation and preliminary identification, additional key tests for biotype characterization and for pathogenicity are required, to confirm the accurate identity of isolates. In the present work, strains with the same biochemical characteristics of *A. tumefaciens*, but avirulent to the inoculated host plants, may presumably be *A. radiobacter*. As previously referred, the species *A. radiobacter* and *A. tumefaciens* differ only in lacking and containing, respectively, the *Ti* plasmid, which is transferable between strains. So, further studies should be carried out in order to confirm the absence of *Ti* plasmid or the inexistence of any other host plants for the strains that showed to be nontumorigenic.

The pathogenicity studies are usually carried out on potted plants, under greenhouse conditions, with the exception of carrot or other few root slices which are maintained at room temperature (MOORE *et al.*, 1988). Our results suggest that pathogenicity of grapevine isolates may be easily tested on grapevine detached leaves. This simple method, previously described by THIES *et al.* (1991) for strains of *Agrobacterium* isolated from muscadine, is advisable mainly when strains are host-specific and when greenhouses with controlled conditions are not available.

It has long been recognized the dominance of *A. tumefaciens* biotype 3 on *Vitis vinifera* and other *Vitis* spp. in different grape growing regions (LOUBSER, 1978; PANAGOPOULOS *et al.*, 1978; SÜLE, 1978; PERRY & KADO, 1982; BURR & KATZ, 1983; THIES *et al.*, 1991). However, biotype 1 and, less frequently, biotype 2 have also been isolated from grapevine in other countries (PANAGOPOULOS & PSALLIDAS, 1973; SÜLE, 1978; FAIVRE-AMIOT, 1984; SZEGEDI, 1985; MA, 1987). For example, BURR & KATZ (1983) found that biotypes 1 and 2 may readily initiate galls on potted grapevines which are indistinguishable from those produced by biotype 3. Furthermore, FAIVRE-AMIOT (1984) reports the occurrence of one strain belonging to biotype 2, on grapevine, that is highly virulent to grapevine and other hosts and having, consequently, a broad-host range. Interestingly, BURR & KATZ (1984), working on the same infected vineyard, found a net predominance of biotype 3 when sampling was made during the growing season, and of biotype 1 strains when isolates were obtained from dormant cuttings.

Our results suggest that strains belonging to biotypes 1 and 2 may be the main responsible for crown gall disease of grapevines, in Portugal. In fact, only Ag5 and Ag13a isolates fitted the biochemical characteristics of biotype 3, as described by KERR & PANAGOPOULOS (1977) and PANAGOPOULOS *et al.* (1978), and Ag5 isolate showed to be nontumorigenic. Strains identified as biotypes 1 and 2 were the most frequently isolated from grapevine galls. However, and as previously referred, the majority of strains allocated to biotype 1 utilized L-tartrate, which is a typical characteristic of biotypes 2 and 3 strains. Similar findings were obtained by SÜLE (1978), but this author pointed out that those strains should not be considered as typical members of biotype 1. BURR & KATZ (1983) and KERSTERS & DE LEY (1984) admitted a variable response of members of biotype 1 to L-tartrate test and, more recently, this test has not been used for biotype separation (BURR *et al.*, 1987; THIES *et al.*, 1991).

One doubt remains, however, regarding the utilization of L-tartrate by the majority of biotype 1 grapevine strains. MOORE *et al.* (1988) referred that it should be expected to isolate some strains with intermediate characteristics between the different biotypes, because genetic mutation and recombination may occur in nature. It will also be possible that some strain adaptation has occurred

due to the predominance of tartaric acid in all parts of *V. vinifera* (KLIEWER, 1966). On the contrary, very homogeneous responses to biochemical tests were obtained for strains isolated from peach and plum and these strains should be considered as typical members of biotypes 1 and 2.

Despite the low number of samples collected from vascular fluids, our results suggest the prevalence of *A. tumefaciens* biotypes 1 and 2 and their systemic movement in grapevine. Systemic movement of *A. tumefaciens* has been also reported in chrysanthemum (MILLER, 1975) and clearly demonstrated for biotype 3 in grapevine (LEHOCZKY, 1971; BURR & KATZ, 1984). The pathogen can survive systemically in symptomatic and asymptomatic *Vitis* spp. plants and be transmitted by propagation material (TARBAH & GOODMAN, 1986; BURR *et al.*, 1988; THIES *et al.*, 1991).

Grape growers extensivelly use propagating material from vineyards assumed to be free of the crown gall pathogen; however, much of these materials are contaminated as it has been detected in several grape regions of United States and Europe (LEHOCZKY, 1968; BURR & KATZ, 1983; BAZZI *et al.*, 1987). The use of contaminated propagation material appearing to be the major source of inoculum for crown gall development, indicates the need for the improvement of propagation systems which would allow to produce *Agrobacterium*-free plants for new vineyards establishement. Attempts to exclude the pathogen from grapevine have been made by repetitive propagation and indexing of green shoot cuttings (GOODMAN *et al.*, 1987), by the use of shoot tip culture (BURR *et al.*, 1988) and by hot water treatment of dormant grape cuttings (BURR *et al.*, 1989). However, the first two methods are very time-consuming and the last has proved to be effective only against biotype 3 strains, which are generally more heat sensitive than strains of biotypes 1 and 2.

In summary, the presence of *A. tumefaciens* in grapevine and stone-fruit trees and evidences of systemic infection of grapevine were confirmed. Our results also showed that, thus far, *A. tumefaciens* biotype 3 has a sporadic occurrence. However, further work is required to ensure that the obtained results represent accurately the relative predominance of biotypes 1 and 2 in comparison with biotype 3, rather than an occasional occurrence in the surveyed portuguese vineyards.

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