Eur J Plant Pathol (2012) 134:415–430
DOI 10.1007/s10658-012-0001-x

Diversity among agrobacteria isolated from diseased plants of blueberry (*Vaccinium corymbosum*) in Argentina

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Accepted: 27 April 2012 / Published online: 24 May 2012

Abstract The aim of this study was to isolate, identify and analyze the diversity of the causative agents of crown galls and hairy roots from symptomatic plants of *Vaccinium corymbossum* by means of biological, biochemical and molecular tools. All the bacteria isolated from blueberries (n=78) were found to be *Agrobacterium* since they grew on three differential media, provoked cell and/or root proliferation on Kalanchoe, and contained a 730 bp partial sequence that codes for virulence genes within the *vir*C operon found on Ti and/or Ri plasmids. Isolates were highly variable considering the ERIC-PCR patterns as well as biochemical reactions and were all represented by 7 different restriction patterns of the 16SrDNA. While most of the isolates belonged to *Agrobacterium* bv. 1 (n=33) or *Agrobacterium* bv. 2 (n=31) only fourteen were *Agrobacterium rubi.* A representative isolate of each of these three groups was further identified by sequencing the approximately 400 bp 16SrDNA. We concluded that *Vaccinium* plants are particularly susceptible to *Agrobacterium* bv. 1, *Agrobacterium* bv. 2, and also to *Agrobacterium rubi.* To our knowledge this is the first survey of *Agrobacterium* affecting blueberries in Argentina.

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Keywords *Agrobacterium rubi* · *Agrobacterium tumefaciens* · *Agrobacterium rhizogenes* · Biovars · *Vaccinium corymbossum* · Blueberries · Crown gall disease · Diversity · PCR

Introduction

Agrobacteria are soil-borne Gram-negative bacteria within the alphaproteobacteria subclass that belong to the family Rhizobiaceae. The genus *Agrobacterium* comprise a diverse group of microorganisms, all of which, when harbouring the appropriate plasmids, provoke uncontrolled cell proliferations on dicotyledoneous plants (Farrand et al. 2003). Depending on the species and strains of *Agrobacterium* involved, the interaction might result in the development either of galls or hairy roots (Sawada et al. 1995; Moore et al. 2001) or they might not be pathogenic in plants (Llop et al. 2009). *Agrobacterium* species are particular in that they infect their hosts only through wounds that release compounds such as acetosyringone, that activate the complex and unique mechanism encoded by plasmids. This complex interaction results in the insertion of a Ti or Ri sequence in the plant's genome at the cell nucleus (Gelvin 2010).

Agrobacterium species were first classified by means of a pathogenicity based-taxonomy in *A. tumefaciens*, those strains that induce crown-gall, *A. rhizogenes*, those strains that induce hairy-roots, and *A. radiobacter* that are non-pathogenic strains (Moore et al. 2001). In addition to this, three other species

have been described: *A. vitis, A. rubi* and *A. larrymoorei.* These species induce galls in *Vitis* spp.; raspberries, blackberries, blueberries and cranberries; and *Ficus benjamina,* respectively (Bouzar and Jones 2001; Moore et al. 2001; Young et al. 2005; Alippi et al. 2010). Pulawska and co-workers (2006), based on the phylogenetic analysis of the 23S rRNA gene sequences, made a simpler classification of *Agrobacterium* in four taxa, *Agrobacterium* biovar 1, *Agrobacterium* biovar 2, *Agrobacterium vitis* (biovar 3), and *A. rubi*. *A. larrymoorei* was left as a separate species. However, Young and co-workers (2001), based on phylogenetic analysis of the 16SrDNA, suggested that *Agrobacterium* species should be renamed *Rhizobium radiobacter, Rhizobium rhizogenes, Rhizobium rubi,* and *Rhizobium vitis*. In support of this, researchers recently described the isolation of *Rhizobium skierniewicense* from tumours of chrysanthemum and *Prunus* spp. (Pulawska et al. 2012) and *Rhizobium pusense* from the rhizosphere of chickpea (Panday et al. 2011). Nevertheless, other authors such as Farrand and co-workers (2003) found that as a defined and distinct group within rhizobiaceae, the name should be kept as *Agrobacterium*. In any case, the phylogenetic-based classification of *Agrobacterium,* suggests that they can be separated in *Agrobacterium* biovar 1 that contains at least nine genomic species causing crown gall or hairy roots or are non-pathogenic (Costechareyre et al. 2010; Lasalle et al. 2011); and three closely related species, *A. rubi* that carries a Ti plasmid and induces galls mostly on *Rubus* spp. (Anderson and Moore 1979), *A. vitis* (formerly known as biovar 3) those strains that have a Ti plasmid that provokes galls exclusively on grape (Lim et al. 2009), and *A. larrymoorei,* carrying a Ti plasmid producing galls on *Ficus benjamina* (Bouzar and Jones 2001). However, the former *Agrobacterium* biovar 2 members that also induce galls or hairy roots, whether they have the Ti or Ri plasmid or are non-pathogenic, are considered *Rhizobium rhizogenes* (Portier et al. 2006). Recently, the Subcommittee on the Taxonomy of *Agrobacterium* and *Rhizobium,* confirmed that *Rhizobium rhizogenes* is a valid designation for biovar 2 (Costechareyre et al. 2010) and also for the *A. radiobacter* strains K84 and *A. tumefaciens* AK10 (Velázquez et al. 2010).

Since, the nomenclature and taxonomy of *Agrobacterium* remains obscure and under debate. The Subcommittee on *Agrobacterium* and *Rhizobium* taxonomy suggested that it is up to the authors to choose the nomenclature to be used (Portier et al. 2006). Here, we decided, mostly based on simplicity, to use the biovar divisions proposed by Pulawska and co-workers (2006) that included: *Agrobacterium* biovar 1 (Syn = *A. tumefaciens)* carrying Ti or Ri plasmids or non-pathogenic; *Agrobacterium* biovar 2 *(Syn = A. rhizogenes)* carrying Ti or Ri plasmids or non-pathogenic; *Agrobacterium rubi, Agrobacterium vitis* and *Agrobacterium larrymorei.*

An ample array of *Agrobacterium* strains may share the same soil environment where is most probably that horizontal DNA transfer occurs (Bouzar et al. 1993; Kechris et al. 2006). Considering this, great levels of diversity might be present in soils within isolates of the same species and/or within different species. This diversity might be reflected, among other things, in strain virulence - their capacity to infect and provoke disease on plants (Ryder et al. 1985; Nesme et al. 1987). However, in any plant microbe interaction, the crucial signals are provided by both partners (Kado 1991). Therefore, plants might also differ in their susceptibility to *Agrobacterium* (Gelvin 2010), i.e. some varieties of grapes, blueberries and raspberries are particularly susceptible to different species or biovars of *Agrobacterium* (Benjama et al. 2002).

Beginning in the 1990's, the production and culture of blueberries in Argentina has experienced a considerable growth. An expansion of the production area throughout the country as well as an increase in yield, has resulted in an 8,000 t annual export commodity. Blueberries are a group of native species of the northern hemisphere of America that belong to the genus *Vaccinium*, family Ericaceae; among them, a small group of species has commercial value i.e. *Vaccinium corymbosum*.

Nurseries located in different areas spread out within Argentina are providing *V. corymbosum* plants to farmers to initiate production. Symptomatic plants from nurseries as well as from commercial farms were sent to our laboratory between 2006 and 2008. Preliminary analysis showed that almost all of them presented, at the base of the shoot or on the root, crown gall-like structures. Therefore, the aim of this study was to evaluate if these galls were induced by bacteria. If this proved to be true, then we wanted to confirm the identity of the isolates, determine their pathogenic ability and analyze their diversity.

Materials and methods

Bacterial isolates

The isolates of *Agrobacterium* were obtained from diseased plants of *Vaccinium corymbosum* (n=78) from nurseries (n=44) and also from commercial farms (n=34) that presented galls close to the root crown and/or hairy roots (Table 1); therefore the survey was unevenly distributed across these sites. Three representatives of *Agrobacterium* that were isolated from black raspberries (*Rubus spp.*) and also *Agrobacterium tumefaciens* strains ATCC15955, LBA 958 and K198 and *Agrobacterium radiobacter* (K1026) were included as controls, so the total number of agrobacteria studied was 85. In addition, two strains of *Rhizobium*, two *Ensifer,* one *Bradyrhizobium* and one strain of *Pantoea* were included for comparison (see Table 1).

Isolations from symptomatic plants

Bacterial isolation from diseased plants was performed as follows; galls were surface sterilized by immersing them in 1 % sodium hypochlorite for 15–20 min and then, were washed three times with sterile distilled water for 15 min each. Then, galls were cut in 2× 2 cm squares by means of a sterile scalpel, mixed with 15 ml of sterile distilled water and homogenized by vortexing for 5 min. The aqueous suspension was incubated at room temperature for 45 min and then, slants of each homogenate were made on the following media: D1 (Moore et al. 2001), D1-M (Perry and Kado 1982) and YEM Congo red (Vincent 1970) supplemented with potassium tellurite, to increase the medium selectivity (Mougel et al. 2001). Then, the plates were incubated in the darkness at 27 °C for 7 days and observations of culture development were made every day. All the colonies that developed in the different media assayed, whose morphological characteristics were similar to those of *Agrobacterium,* were purified in yeast dextrose carbonate agar (YDC) (Moore et al. 2001). Isolates were cultured on YEM and then aliquots were stored in 20 % glycerol (V/V) at −70 °C.

Isolates characterization

All the isolates of *Agrobacterium* from *Vaccinium corymbosum* (n=78) were characterized morphologically, biochemically and physiologically by means of the following reactions: Gram staining, catalase production, oxidase activity from isolates growing on both Nutrient agar (Ox-NA) and glucose nutrient agar (Ox-GNA) acid production on YDC, utilization of Keto-lactose, and oxidative/fermentative metabolism of glucose (OF-O and OX-F) following standard procedures (Moore et al. 2001). In addition, the isolates were characterized by means of the API 20E strips (Biomerieux,®), which included β-galactosidase utilization (ONPG), arginine dihidrolase (ADH), lysine decarboxilase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), hydrogen sulfide production (H_2S), urease production (URE), tryptophane deaminase (TDA), indole production (IND), acetoine production (Voges-Proskauer, VP), gelatinase activity (GEL), utilization of d-Glucose (GLU), d-mannitol (MAN), inositol (INO), d-sorbitol (SOR), l-rhamnose (RHA), d-sucrose (SAC), d-melibiose (MEL), amygdalin (AMY), l-arabinose (ARA) and nitrate reduction (NO_3-NO_2). In all cases, strains ATCC15955, LBA 958 and K198 and K1026 were included as controls.

Pathogenicity tests

Two assays were conducted, one consisted in using a cut leaf of Kalanchoe (*Bryophyllum daigremontiana*) (Minnemeyer et al. 2006). Briefly, 3–4 cm long young leaves were surface sterilized by immersing them in 2.5 % chlorine bleach solution for 15 min and were subsequently rinsed thoroughly in sterile distilled water. Leaves were injured with a sterile scalpel and 5 µl of 10^8 CFU/ml bacterial suspensions in sterile distilled water made by adjusting the cell concentration of 48 h old YEM cultures were pipette on each wound. Six leaves per strain were inoculated and placed in plates with water agar (2 %) supplemented with 50 µg/ml cicloheximide. They were incubated at 26 °C±2 °C under a 12 h light photoperiod achieved by eight Gro Klux MP100 tubes. In addition, root inducers isolates of *Agrobacterium* were evaluated by means of carrot discs assay (Ryder et al. 1985). Based on the analysis described above and those described below, we selected five representatives of *Agrobacterium rubi* (F210, F253, F304, F305 and F315), two of *A. tumefaciens* bv. 1 (F268 and F293) and two of *A. rhizogenes* bv. 2 (F288 and F289) and evaluated their pathogenicity on several hosts such as Blueberry (*Vaccinium corymbosum* cv. Misty) (50–60 cm high), 5-week old pepper (*Capsicum annuum* cv. California wonder), 5-week

Table 1 Bacterial isolates obtained from diseased blueberries and control strains of *Agrobacterium* and other strains used in this paper

Strain[a]	Species[b]	Biovar[c]	Host[d]	Cultivar[e]	Place of isolation[f]	Source[g]	Year[h]	RFLP pattern[i]
F 210	*Agrobacterium rubi*	rubi	blueberry	U	Buenos Aires	N	1997	G
F 253	*A. rubi*	rubi	blueberry	U	Tucumán	N	2006	A
F 254	*A. rubi*	rubi	blueberry	U	Tucumán	N	2006	A
F 255	*A. rhizogenes*	2	blueberry	Blue Crisp	Tucumán	F	2006	E
F 256	*A. rhizogenes*	2	blueberry	Misty	Buenos Aires	N	2006	D
F 258	*A. rhizogenes*	2	blueberry	U	Tucumán	F	2006	B
F 259	*A. tumefaciens*	1	blueberry	U	Tucuman	F	2006	A
F 260	*A. rhizogenes*	2	blueberry	Misty	Buenos Aires	N	2006	G
F 261	*A. rhizogenes*	2	blueberry	U	Tucumán	F	2006	B
F 262	*A. rhizogenes*	2	blueberry	O'Neil	Tucumán	F	2006	B
F 263	*A. rhizogenes*	2	blueberry	U	Buenos Aires	N	2006	A
F 264	*A. rhizogenes*	2	blueberry	O'Neil	Tucumán	F	2006	G
F 265	*A. rhizogenes*	2	blueberry	Misty	Tucumán	F	2006	E
F 266	*A. rubi* atypical strain	rubi	blueberry	U	Buenos Aires	N	2006	E
F 268	*A. tumefaciens*	1	blueberry	U	Entre Ríos	F	2006	A
F 269	*A. rhizogenes*	2	blueberry	U	Entre Ríos	F	2006	B
F 270	*A. rubi*	rubi	blueberry	U	Entre Ríos	F	2006	B
F 271	*A. rubi*	rubi	blueberry	U	cvo. in vitro	N	2006	B
F 272	*A. rubi*	rubi	blueberry	O'Neil	E. Rios	N	2006	C
F 273	*A. rubi*	rubi	blueberry	O'Neil	E. Rios	N	2006	C
F 274	*A. rhizogenes*	2	blueberry	O'Neil	Tucumán	F	2006	B
F 276	*A. tumefaciens*	1	blueberry	Millenia	Tucumán	F	2006	A
F 277	*A. rhizogenes*	2	blueberry	O'Neil	Tucumán	F	2006	B
F 278	*A. rhizogenes*	2	blueberry	Blue Crisp	Tucumán	F	2006	B
F 279	*A. tumefaciens*	1	blueberry	Blue Crisp	Tucumán	F	2006	B
F 280	*A. rhizogenes*	2	blueberry	U	Entre Ríos	N	2006	D
F 281	*A. tumefaciens*	1	blueberry	U	Entre Ríos	N	2006	B
F 282	*A. tumefaciens*	1	blueberry	Blue Crisp	Tucumán	F	2006	B
F 283	*A. rhizogenes*	2	blueberry	U	Entre Ríos	N	2006	E
F 284	*A. tumefaciens*	1	blueberry	U	Entre Ríos	N	2006	B
F 285	*A. tumefaciens*	1	blueberry	U	Tucumán	F	2006	B
F 286	*A. rhizogenes*	2	blueberry	Misty	Buenos Aires	F	2006	E
F 287	*A. rhizogenes*	2	blueberry	Misty	Buenos Aires	F	2006	E
F 288	*A. rhizogenes*	2	blueberry	Millenia	Tucumán	F	2006	C
F 289	*A. rhizogenes*	2	blueberry	O' Neil	Tucuman	F	2006	C
F 291	*A. tumefaciens*	1	blueberry	Millenia	Tucumán	F	2006	B
F 292	*A. tumefaciens*	1	blueberry	Misty	Buenos Aires	N	2006	H
F 293	*A. tumefaciens*	1	blueberry	Blue Crisp	Buenos Aires	N	2006	A
F 294	*A. rhizogenes*	2	blueberry	O'Neil	Buenos Aires	N	2006	G
F 295	*A. tumefaciens*	1	blueberry	O'Neil	Buenos Aires	N	2006	B
F 296	*A. tumefaciens*	1	blueberry	O'Neil	Buenos Aires	N	2006	B
F 297	*A. tumefaciens*	1	blueberry	Misty	Buenos Aires	N	2006	G
F 298	*A. tumefaciens*	1	blueberry	Misty	Buenos Aires	N	2006	B
F 299	*A. tumefaciens*	1	blueberry	Blue Crisp	Buenos Aires	N	2006	D

Table 1 (continued)

Strain[a]	Species[b]	Biovar[c]	Host[d]	Cultivar[e]	Place of isolation[f]	Source[g]	Year[h]	RFLP pattern[i]
F300	*A. tumefaciens*	1	blueberry	O' Neil	Buenos Aires	N	2006	D
F 301	*A. rhizogenes*	2	blueberry	O'Neil	Buenos Aires	N	2006	D
F 302	*A. rhizogenes*	2	blueberry	Blue Crisp	Buenos Aires	N	2006	E
F 303	*A. rhizogenes*	2	blueberry	Blue Crisp	Buenos Aires	N	2006	E
F 304	*A. rubi*	rubi	blueberry	Misty	Buenos Aires	N	2006	A
F 305	*A. rubi*	rubi	blueberry	Misty	Buenos Aires	N	2006	E
F 306	*A. tumefaciens*	1	blueberry	Blue Crisp	Buenos Aires	N	2006	B
F 309	*A. tumefaciens*	1	blueberry	Misty	Tucumán	F	2006	B
F 310	*A. tumefaciens*	1	blueberry	Misty	Buenos Aires	N	2006	B
F 311	*A. rhizogenes*	2	blueberry	Blue Crisp	Tucumán	N	2006	G
F312	*A. rhizogeness*	2	blueberry	Blue Crisp	Buenos Aires	N	2006	B
F 313	*A. rhizogenes*	2	blueberry	Misty	Buenos Aires	N	2006	B
F 314	*A. tumefaciens*	1	blueberry	U	Tucumán	F	2006	B
F 315	*A. rubi*	rubi	blueberry	U	Tucumán	F	2006	A
F 316	*A. tumefaciens*	1	blueberry	U	Tucumán	F	2006	B
F 317	*A. rhizogenes*	2	blueberry	U	Tucumán	F	2006	B
F 319	*A. tumefaciens*	1	blueberry	Blue Crisp	Buenos Aires	N	2006	A
F 320	*A. tumefaciens*	1	blueberry	U	Tucumán	F	2006	A
F 321	*A. tumefaciens*	1	blueberry	U	Buenos Aires	F	2006	B
F 332	*A. tumefaciens*	1	blueberry	U	Corrientes	F	2007	B
F 333	*A. tumefaciens*	1	blueberry	O' Neill	Buenos Aires	F	2007	B
F 334	*A. tumefaciens*	1	blueberry	Misty	Buenos Aires	N	2007	B
F 335	*A. rubi*	rubi	blueberry	U	Tucumán	N	2007	B
F 336	*A. tumefaciens*	1	blueberry	U	Tucumán	N	2007	B
F 337	*A. rubi*	rubi	blueberry	U	Tucumán	N	2007	G
F 338	*A. rubi*	rubi	blueberry	U	Tucumán	N	2007	B
F 339	*A. rhizogenes*	2	blueberry	U	Tucumán	N	2007	H
F 340	*A. rhizogenes*	2	blueberry	U	Tucumán	N	2007	H
F 341	*A. tumefaciens*	1	blueberry	U	Tucumán	N	2007	A
F 346	*A. tumefaciens*	1	blueberry	Misty	Tucumán	F	2007	B
F 347	*A. tumefaciens*	1	blueberry	Yewell	Tucumán	F	2007	B
F 348	*A. tumefaciens*	1	blueberry	Emerald	Tucumán	N	2007	B
F 349	*A. rhizogenes*	2	blueberry	Gold Coast	Tucumán	N	2007	B
F 400	*A. rhizogenes*	2	blueberry	U	Bs. As.	F	2008	B
F 239	*A. tumefaciens*	1	raspberry	Heritage	Buenos Aires	F	2004	B
F 240	*A. tumefaciens*	1	raspberry	Bliss	Buenos Aires	F	2004	B
F 241	*A. tumefaciens*	1	raspberry	Rubi	Buenos Aires	F	2004	B
K 1026	*A. radiobacter*	2	U	U	U	U	U	B
LBA 958	*A. tumefaciens*	1	U	U	U	U	U	B
ATCC 15955	*A. tumefaciens*	1	U	U	U	U	U	B
K198	*A. tumefaciens*	1	peach	U	Australia	U	U	B
Hambi540	*Rhizobium galegae*	–	*Galega* sp.	U	Finland	U	U	A
CFN4	*R. etli bv. phaseoli*		bean	U	U	U	U	A
109	*Ensifer japonicum*	–	soybean	U	U	U	U	A

Table 1 (continued)

Strain[a]	Species[b]	Biovar[c]	Host[d]	Cultivar[e]	Place of isolation[f]	Source[g]	Year[h]	RFLP pattern[i]
103-1 HH	*Ensifer fredii*	–	soybean	U	U	U	U	A
USDA 1002T	*E. meliloti*	–	lucerne	U	U	U	U	H
F 347	*Pantoea ananatis*	–	maize	U	Argentina	U	2009	F

[a] dentification number

[b] Species of *Agrobacterium* isolated

[c] biovar designation according to multiplex PCR

[d] host of isolation

[e] cultivar of *Vaccinium corymbosum* or U unknown

[f] place of isolation

[g] *N* nursery- *F* farm; *h*: year of isolation

[i] Restriction patterns of the 16SrDNA generated by combining the fragments obtained by means of *Taq*I and *Hae*III

old tomato (*Solanum lycopersicum,* formerly *Lycopersicum esculentum* cv. Presto), sunflower (*Helianthus annuum* cv. Dekalb 4040) and 2- to 3- months old tobacco (30–40 cm high) (*Nicotiana tabacum cv.* Xanthi) as described by Peluso and co-workers (2003). Briefly, plants were inoculated by a stabbing a needle containing a bacterial cell suspension (10^8 CFU/ml) of each strain tested or with sterile distilled water (control treatment). Each strain was inoculated on two plants of each species. Inoculated plants were maintained in a greenhouse at 24±5 °C and the appearance of tumours or hairy roots was visually assessed in herbaceous species 4–6 weeks after inoculation and in blueberries after 3 to 6 months.

Analysis of diversity and identification of the isolates by molecular tools

DNA preparation

Genomic DNA was isolated by using an interchange ionic resin as described by Alippi et al. (2003). Briefly, all bacterial strains listed in Table 1 were grown in YEM for 24 h at 28 °C. Cells from about one to two single colonies were picked with a sterile toothpick and suspended in 300 µl NaCl 1 M. Each sample was vortex-mixed and centrifuged at 16,000 g for 4 min, and once, supernatant was removed and the pellet resuspended in 300 µl double-distilled water, vortex-mixed and centrifuged at 16,000 g for 3 min. The supernatant was removed and the pellet was resuspended in 150 µl of an aqueous suspension of 6 % resin (Chelex® 100, 200–400 mesh, Bio-Rad). The mixture of cells and resin was incubated at 56 °C for 20 min and vortex-mixed for 30 s. Finally the mixture was incubated at 99 °C for 8 min and vortex-mixed for 1 min. Bacterial debris and resin were removed by centrifugation, and 5 µl of the supernatant containing DNA was used as template for PCR amplifications in a final volume of 25 µl.

PCR detection of virC operon located in Ti and Ri plasmids

A 730 bp fragment representing the partial sequence of *vir*C operon found in Ti and Ri plasmids present on pathogenic strains of *Agrobacterium* species was amplified by means of VCF/VCR primers (Sawada et al. 1995). Five 5 µl of genomic DNA isolated as described before was used as template in a PCR reaction with a final volume of 25 µl. PCR products were resolved in 1.6 % agarose gels in TBE buffer 0.5 X, and observed under UV light after staining with ethidium bromide. All the isolates listed in Table 1 were compared in the same manner.

Multiplex PCR

A PCR multiplex reaction based on differences at the 23SrDNA allowed us to discriminate between *Agrobacterium* biovar 1, *Agrobacterium* biovar 2, *A. rubi* and *A. vitis,* respectively. (Pulawska et al. 2006). PCR mixtures and amplifications were performed as described by Pulawska and co-workers (2006), but using

as template 5 μl of genomic DNA isolated as specified before. PCR reactions were performed in a thermal cycler (Mastercycler Personal; Eppendorf, Hamburg, Germany) programmed as described (Pulawska et al. 2006). PCR products were resolved in 1.6 % agarose gels in TBE buffer 0.5 X, stain with ethidium bromide and observed under UV light. In addition to all the isolates listed in Table 1, strains K 306 from Australia and S4 from Hungary were included as positive controls of *Agrobacterium vitis*.

RFLP analysis of PCR-amplified 16S rDNA

We used genomic DNA as template to amplify the full sequence of the 16SrDNA by means of primers fD1 and rD1 (Weisburg et al. 1991). 4-μl aliquots of the amplified 16SrDNA were incubated overnight with restriction enzymes *Taq* I (Promega Biotech) and *Hae* III (Promega Biotech) at 65 °C and 37 °C, respectively according to the conditions suggested by the manufacturer. RFLP was resolved by electrophoresis in 2 % agarose gels, the DNA was stained with ethidium bromide and visualized with a UV transiluminator (UVP). All the isolates listed in Table 1 were analyzed.

Analysis of the diversity of isolates by rep-PCR using primers ERIC

Diversity among isolates was assessed based upon fingerprints generated by means of primers associated to Enterobacterial Repetitive Consensus sequences (ERIC) (Versalovic et al. 1994). All PCR reactions were performed in a thermal cycler (Mastercycler Personal; Eppendorf, Hamburg, Germany) as described by López and Alippi (2007). A total of 82 agrobacteria were compared (78 from blueberries and four from Culture Collections) Reactions were resolved in 1.5 % agarose gels that were visualized and analyzed as previously described. Gel images were digitalized and photographed using a digital image capture gel documentation system (Digi Doc-it, UVP, v. 1.1.25) and analyzed by means of Gelcompar II (v 5.1. Applied Math, Kortrijk, Belgium). Cluster analysis was performed using the DICE similarity coefficient and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering algorithm with a band tolerance of 5 %.

Sequence analysis

By sequencing the 16SrDNA (Sanger, et al. 1977) one representative of *A. rubi* (F266, GU580894), one representative of *Agrobacterium rhizogenes* bv. 2 carrying Ri plasmid (F289, GU580895) (Alippi et al. 2010) and one representative of *Agrobacterium tumefaciens* bv.1 carrying Ti plasmid (F268, GU580896) (this paper) were analyzed further. Individual sequences were assembled by means of the GCG Software (University of Wisconsin) and aligned by means of BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The dendrogram was based on the multiple alignment of the 16SrDNA full sequences by means of the Clustal W version 1.81. This information was processed to build a tree based on the UPGMA by means of Mega 5.0. The support of the groups within the tree was evaluated through bootstrap (Felsenstein 1985), with 1,000 replications. The values obtained are given on the basis of the branches.

Results

Bacterial isolations and colony characteristics in different media

All the plants analyzed presented either gall-like lesion on the crown roots or at the base of the shoot or hairy roots. From all these lesions we isolated Gram (−) bacteria, that developed colonies that presented, on the different media tested, morphological characteristics typical of *Agrobacterium*. On D1 they were of a green-yellowish colour, darker in the centres with creamy white edges. The initial light blue colonies turned to olive green as they aged but always with lighter edges. On D1M colonies were convex, bright, mucoid, of a faint blue colour with darker centres. In YEM-CRT, colonies appeared black, mucous and convex, with entire margins. On YDC, colonies were highly mucous, bright, convex and white cream to light beige in colour, some isolates acidified the medium as revealed by clear halos around the colonies. Based upon this, we decided to confirm by means of biological as well as molecular tools that the identity of the isolates was *Agrobacterium* and also analyzed their diversity. We obtained 78 *Agrobacterium* isolates from *Vaccinium corymbosum* plants showing symptoms,

44 from nurseries and 34 from commercial blueberry crops.

Characterization of bacteria by biochemical and physiological tests

We characterized the *Agrobacterium* isolates further by means of biochemical reactions. In Fig. 1 we present the data indicating the percentage of isolates of *Agrobacterium* bv. 1, *Agrobacterium* bv. 2 and *A. rubi* that gave a positive response to each of the biochemical reactions tested, including those from the API20E strips on a total of 78 isolates. Among 27 different biochemical reactions there were only four that were positive among all the isolates like glucose (GLU), arabinose (ARA), oxidative metabolism of glucose (OX-O), and oxidase activity of cultures growing in NA (OxNA). Only three other reactions, lysine decarboxilase (LDC), hydrogen sulphide production (H_2S), and indole (IND) were negative for all the isolates, except for the utilization of 3-ketolactose (KETOLAC) that was only positive for *Agrobacterium* bv. 1 isolates (Fig. 1). Analysis of the remaining reactions shows it is obvious that there is no characteristic pattern for *Agrobacterium tumefaciens* bv.1, *Agrobacterium rhizogenes* bv. 2 or *A. rubi* isolated from *Vaccinium corymbosum* (Fig. 1).

Pathogenicity tests

A virulence assay was performed by inoculating leaves of Kalanchoe with a bacterial suspension of each of the *Agrobacterium* isolates. All of them provoked cell proliferations, mostly after 10–12 days of incubation at room temperature. On the contrary, *Agrobacterium radiobacter* K1026 strain, *Pantoea ananatis* or rhizobia such as *Rhizobium galegae*, *Rhizobium etli* bv. *phaseoli*, *Ensifer melilotii*, *Ensifer japonicum*, and *Sinorhizobium freedii* included as controls, did not induce either galls or hairy roots formation. The symptoms developed by two plants suggested that the causative agents might be representatives of *Agrobacterium rhizogenes* or *Agrobacterium* bv. 2. Therefore, these two isolates F288 and F289, five *A. rubi* isolates (F210, F253, F304, F305, F315) and two *Agrobacterium* bv.1 isolates (F268 and F293) were evaluated by means of additional biological tests. *A. rubi* and *A. tumefaciens* bv. 1 isolates developed gall like structures on *V. corymbosum* (highbush blueberry), *Bryophyllum daigremontiana* (Kalanchoe, whole plants), *Nicotiana tabacum* (Tobacco), *Lycopersicum esculentum* (Tomato), *Capsicum annuum* (Pepper) and *Helianthus annuum* (Sunflower), while isolates F288 and F289 only induced proliferation of roots in all the inoculated hosts which was also observed in the carrot disk assay.

PCR detection of *vir*C operon

All the Agrobacterium isolates from blueberries and control strains (LBA958, ATCC15955, and K198) proved to have in their genome a 730 bp PCR sequence coding for virulence gene *vir*C (Sawada et al. 1995). On the contrary, *Agrobacterium radiobacter* K1026 strain as well as *Pantoea ananatis* or rhizobia (*Bradyrhizobium japonicum*, *Rhizobium galegae*, *Rhizobium etli* bv. *phaseoli*, *Ensifer melilotii* and *Ensifer freedii)* lacked this sequence.

Multiplex PCR

The multiplex reaction consisted of amplifying DNA fragments of four different sizes from the 23SrDNA template. Within the *Agrobacterium* strains isolated from blueberries, 14 generated an amplicon of 1,006 bp, 31 generated an amplicon of 1,066 bp and 33 generated an amplicon of 184 bp, that corresponded to *Agrobacterium rubi*, *Agrobacterium* bv. 2 and *Agrobacterium* bv. 1, respectively (Pulawska et al. 2006). The three isolates from raspberries corresponded to bv. 1, reference strains LBA958, ATCC15955 and K 198 were bv. 1, while strain K1026 (previously named *A. radiobacter*) belonged to bv. 2, and *A. vitis* strains S4 and K306 generated an amplicon of 478 bp. Genomic DNA of *Bradyrhizobium japonicum*, *Rhizobium galegae*, *Rhizobium etli* bv. *phaseoli*, *Ensifer melilotii*, *Ensifer freedii* and *Pantoea ananatis* did not act as templates of any of the described amplicons (Table 1).

RFLP analysis of PCR-amplified 16S rDNA

Considering that all the isolates belonged to *Agrobacterium* species we amplified the full sequence of the 16SrDNA and made an enzyme restriction analysis. Even though the 16SrDNA is a universal conserved sequence it varied considerably among *Agrobacterium* isolates; seven different restriction patterns were found among the agrobacteria isolated from blueberries ($n=78$) and those strains of agrobacteria ($n=7$) and

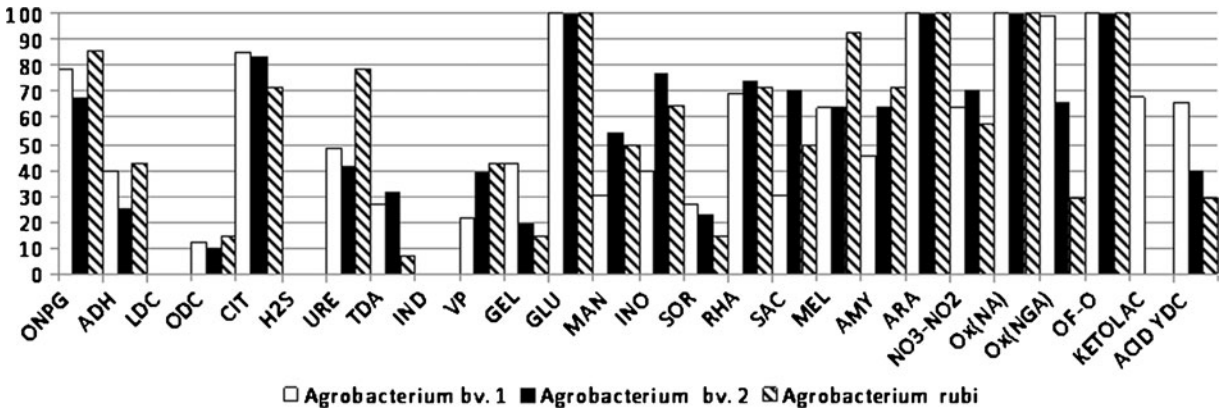


Fig. 1 Biochemical characterization of *Agrobacterium* isolates. Percentage of isolates of *A. tumefaciens* biovars 1 and 2 and *A. rubi* that gave a positive response in the analysis. Names of each reaction are listed in Materials and Methods section

rhizobia ($n=5$) that were used as controls (Table 1 and Fig. 1). Each restriction pattern occurred at different frequency within agrobacteria. *Agrobacterium tumefaciens* bv. 1 strains carrying the Ti plasmid presented the following patterns: A, B, D, G and H; *Agrobacterium rhizogenes* bv.2 carrying the Ti plasmid presented A, B, D, E, G and H. *Agrobacterium* bv. 2 carrying the Ri plasmid presented only pattern C most probably due to the fact that there were only two isolates and those strains characterized as *A. rubi* presented the following 16SrDNA patterns: A, B, C, E and G. The most common pattern found within the agrobacteria populations was B with a total of 45 isolates (52 %), which also included all the reference strains, while patterns A, C, D, E, G and H represent 14 %, 5 %, 6 %, 11 % and 8 % respectively (Table 1 and Fig. 2). When analyzing the rhizobial isolates that were used as controls, we found the presence of pattern A in 4 strains (*R. galegae, R. etli* bv. *phaseoli*, *B. japonicum* and *E. fredii)* and pattern H in one strain of *E. meliloti* (USDA 1002 T). Additionally, *Pantoea ananatis* had the F pattern (Table 1).

Analysis of the diversity of isolates by rep-PCR using primers ERIC

The diversity of the isolates from blueberries and Culture Collections ($n=82$) was analyzed by ERIC-PCR generating 72 different patterns that included four to 13 amplified bands that range in size between 50 and 1,500 bp (Fig. 3). Although the fingerprint patterns were highly diverse, they were clustered at a low similarity level (about 45 %), two main clusters, named I and II were separated at a similarity index of 65 % and 60 % respectively. The rest of the strains ($n=10$) clustered at a similarity level of 45 % (Fig. 3).

Sequence analysis

Identity of three selected isolates was confirmed by sequencing the 16SrDNA of strains F 266, *Agrobacterium rubi* (GenBank No. GU580894), F 289 *Agrobacterium rhizogenes* bv. 2 (GU580895) and F268, *Agrobacterium tumefaciens* bv.1 (GU580896). These sequences were compared with those of *Agrobacterium tumefaciens, Agrobacterium rhizogenes, Agrobacterium rubi, Agrobacterium vitis,* and *Agrobacterium larrymoorei*, and also with those of the new described species *Rhizobium pusense* and *Rhizobium skierniwicense.* In addition, the 16S rDNA sequence of *Bradyrhizobium japonicum* type strain was used as an outgroup. These sequences were aligned by means of the Clustal W multiple alignment and then a tree was built based on the Maximum Likelihood algorithm. As expected, the sequences were highly related (Fig. 4) and were monophyletic, though grouped in three clusters supported with 1,000 boostraps. Cluster A included four representatives of *A. rubi* (AY626394.1; X67228.1-type strain-; D14503.1 and EU281314.1), two representatives of *A. larrymorei* (AY626382.1 and Z30542.1 –type strain-), four representatives of *A. tumefaciens* (F268 GU580896.1; AY623686.1; GU645017.1 and D14500), *R. pusense* (FJ969841.2) and *R. skierniewicense* (HQ 823552.1 –type strain-); cluster B included three representatives of *A. vitis* (AY636399.1, D14502.1 and AY626401.1) clearly

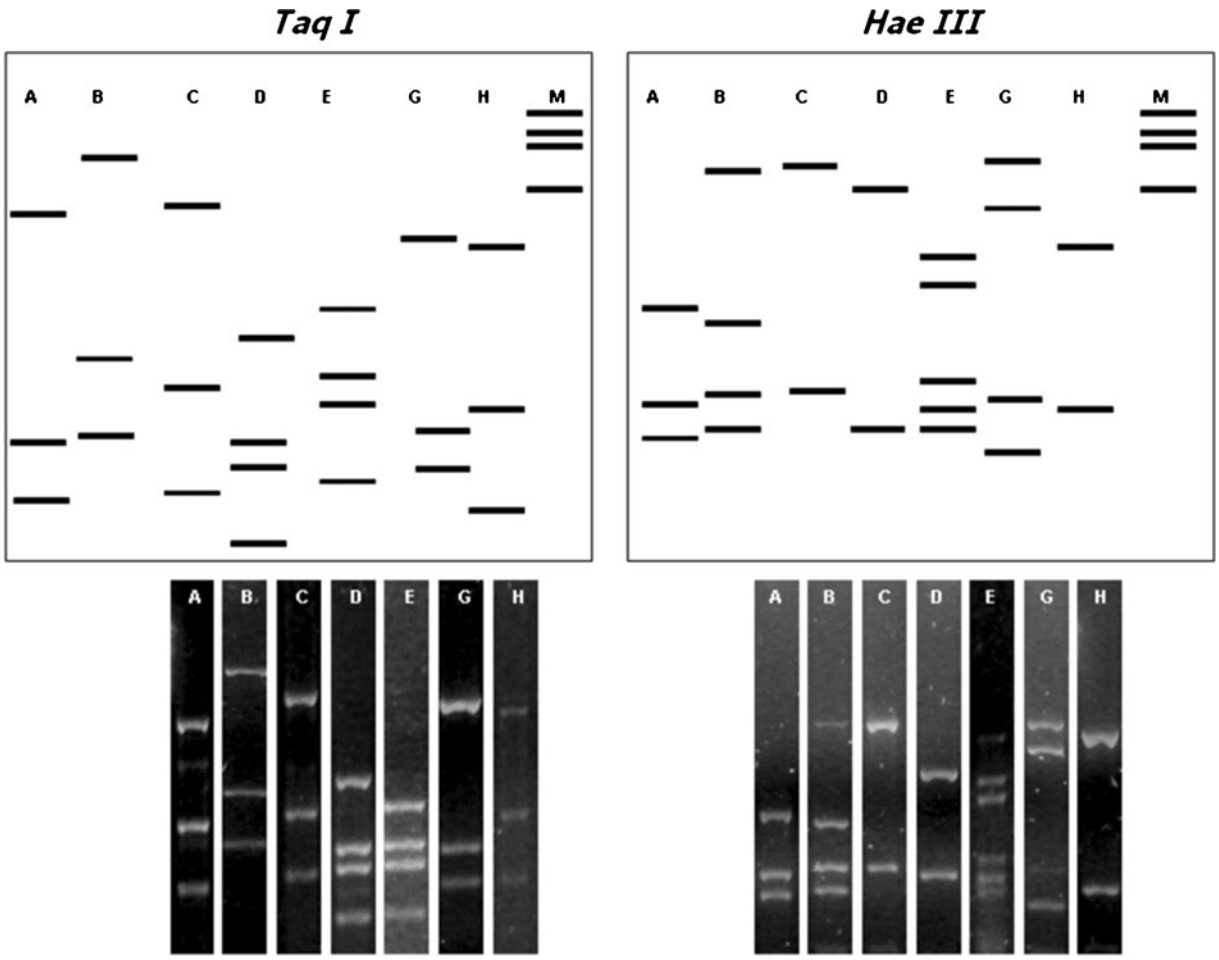


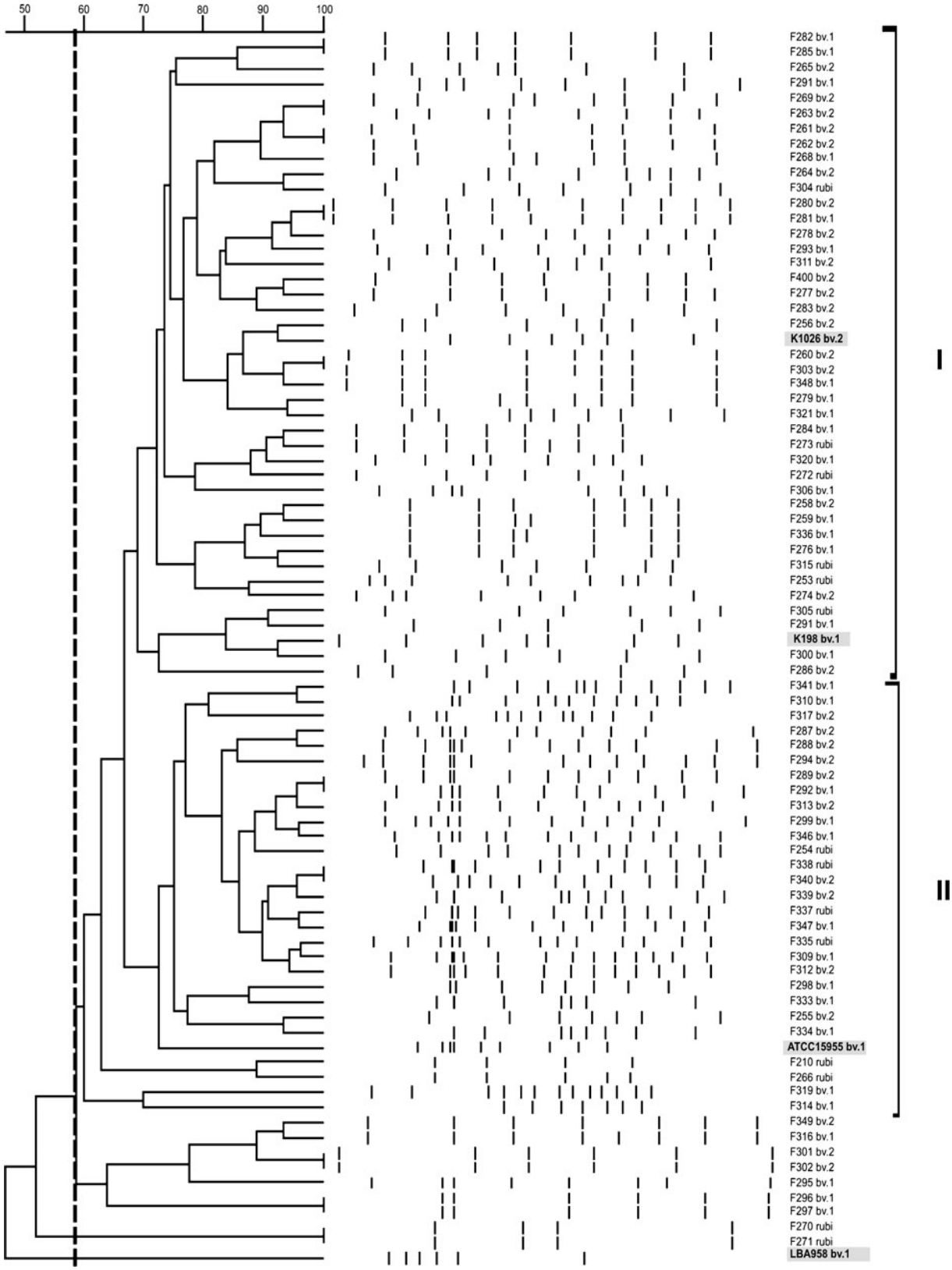
Fig. 2 Restriction fragment lenght polymorphism (RFLP) patterns of PCR-amplified 16SrDNA found among all the isolates from blueberries tested (n=78). The full sequence of the 16SrDNA was digested with two restriction enzymes *Taq*I and *Hae*III, respectively. Reference strains of *Agrobacterium* (n=7) showed a pattern B and the rhizobia used as controls showed patterns A (4 strains) and H (1 strain), respectively

separated from the rest of the isolates; and cluster C included *A. rubi* F266 *(*atypical strain) (GU580894.2), one representative of *A. rubi* AY626395.1) and five representatives of *A. rhizogenes* (F289, GU580895.1; AY626391.1; AY626389.1,D14501.1 and AY945955.1 –type strain-) (Fig. 4).

Discussion

The 78 bacterial isolates obtained from diseased blueberry plants with symptoms such as root proliferations or galls, grew on selective media and provoked cell proliferation in detached leaves of Kalanchoe. They presented a variable biochemical pattern and even key enzymes were variable, while a few of them were hardly present in a few isolates within *Agrobacterium* bv. 1 and *Agrobacterium* bv. 2 strains and *A. rubi* (Fig. 1). Diversity was confirmed by molecular tools such as ERIC-PCR where there was no correlation between biovars and fingerprint patterns and/or geographical origin of the isolates, since representatives of *Agrobacterium* bv. 1, *Agrobacterium* bv. 2 and *A.*

Fig. 3 Diversity at the genetic level among 78 strains of *Agrobacterium* isolated from blueberries based on the presence or absence of bands generated by means of ERIC markers. Pairwise comparisons were calculated using the DICE similarity coefficient. A dendrogram was generated from a similarity matrix using unweighted pair group method with arithmetical averages (UPGMA) with a band tolerance of 5 % by means of Gelcompar II v 5.1


50
60
70
80
90
100
F282 bv.1
F285 bv.1
F265 bv.2
F291 bv.1
F269 bv.2
F263 bv.2
F261 bv.2
F262 bv.2
F268 bv.1
F264 bv.2
F304 rubi
F280 bv.2
F281 bv.1
F278 bv.2
F293 bv.1
F311 bv.2
F400 bv.2
F277 bv.2
F283 bv.2
F256 bv.2
K1026 bv.2
F260 bv.2
F303 bv.2
F348 bv.1
F279 bv.1
F321 bv.1
F284 bv.1
F273 rubi
F320 bv.1
F272 rubi
F306 bv.1
F258 bv.2
F259 bv.1
F336 bv.1
F276 bv.1
F315 rubi
F253 rubi
F274 bv.2
F305 rubi
F291 bv.1
K198 bv.1
F300 bv.1
F286 bv.2
F341 bv.1
F310 bv.1
F317 bv.2
F287 bv.2
F288 bv.2
F294 bv.2
F289 bv.2
F292 bv.1
F313 bv.2
F299 bv.1
F346 bv.1
F254 rubi
F338 rubi
F340 bv.2
F339 bv.2
F337 rubi
F347 bv.1
F335 rubi
F309 bv.1
F312 bv.2
F298 bv.1
F333 bv.1
F255 bv.2
F334 bv.1
ATCC15955 bv.1
F210 rubi
F266 rubi
F319 bv.1
F314 bv.1
F349 bv.2
F316 bv.1
F301 bv.2
F302 bv.2
F295 bv.1
F296 bv.1
F297 bv.1
F270 rubi
F271 rubi
LBA958 bv.1
I
II

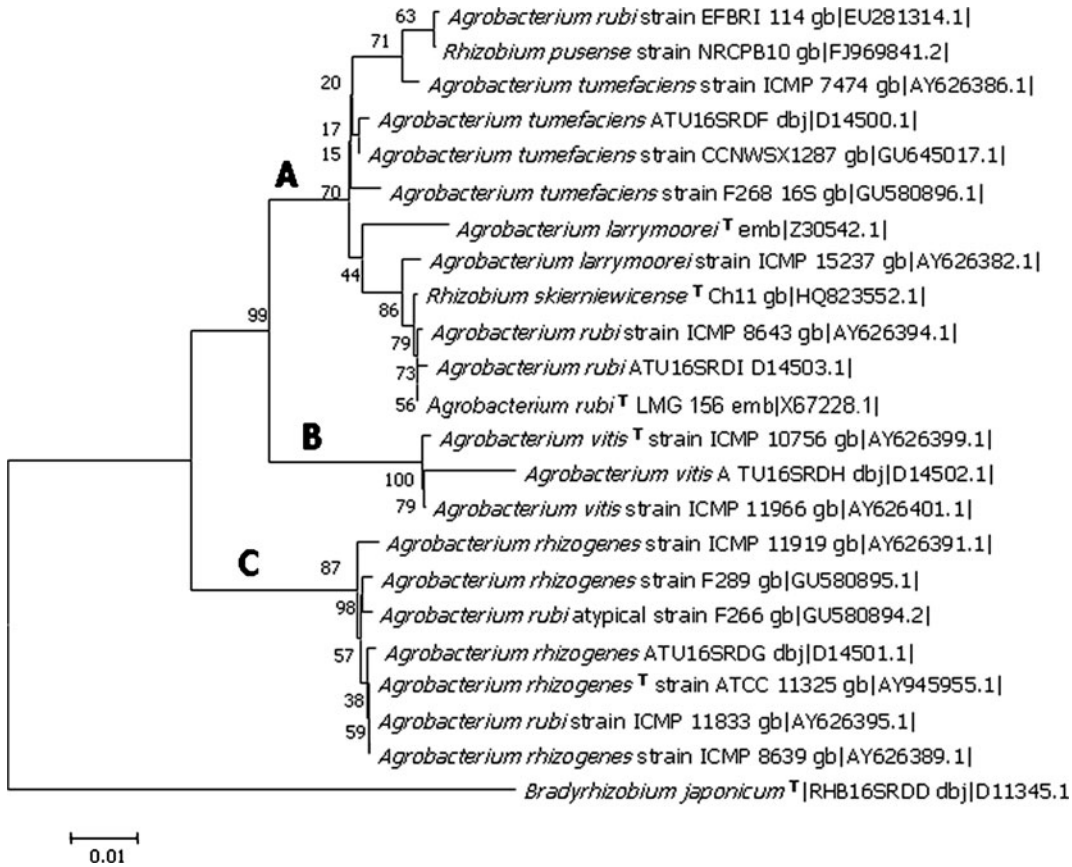


Fig. 4 Rooted phylogenetic tree generated based on the 16SrDNA showing the relationship of three *Agrobacterium* isolates each representing *A. tumefaciens* (GU580896), *A. rhizogenes* (GU580895) and *A. rubi* atypical strain (GU580894) provoking symptoms on blueberries, with annotated sequences of representatives of *Agrobacterium Rhizobium* and *Bradyrrizobium* species. The support of the groups within the tree was evaluated through bootstrap, with 1,000 replications. The values obtained are given on the basis of the branches

rubi, as well as strains from Culture Collections were distributed along the different clusters (Fig. 3). This is in agreement with other authors that also reported high levels of diversity within *Agrobacterium* representatives (Nesme et al. 1987; Peluso et al. 2003). Therefore, the isolates of *Agrobacterium* affecting blueberries in Argentina, are like other isolates, quite variable. Some biochemical reaction characteristics of *Agrobacterium* were negative, which might be related to the methodology used. Based on the phenotypic analysis *A. rubi* seems to be atypical, though more closely related to *Agrobacterium* bv.1 (Tighe et al. 2000). Therefore, these results confirmed the variable nature of *Agrobacterium* isolated from diseased blueberries from Argentina and argue against the reliability of an identification or characterization of *Agrobacterium* based only on such tools.

Among the nomenclatures of the genus *Agrobacterium,* the traditional pathogenicity-based classification that relies on the pathogenic ability of the isolates, which is coded by genes located on plasmids, might not be the most accurate, considering that plasmids are dispensable and transferable between organisms in their environment. The other nomenclature is the one that refers phenotypic differences to biovars (Holmes and Roberts 1981; Moore et al. 2001; Young et al. 2005), which was repeatedly confirmed by both sequence comparison (Pulawska et al. 2006; Slater et al. 2009; Pulawska et al. 2012) and fatty acid analysis (Tighe et al. 2000).

The multiplex PCR of the blueberries isolates (n=78) indicated that 33 were *Agrobacterium* bv. 1 (Syn. *A. tumefaciens)* (42 %), 31 *Agrobacterium* bv. 2 (40 %) and 14 isolates were *Agrobacterium rubi* (18 %).

Regarding reference strains of *Agrobacterium*, all of them including the three isolates from *Rubus* spp. belonged to bv. 1 while the K 1026 strain of *A. radiobacter* belonged to bv. 2, and as expected, the strains S4 and K306 were *A. vitis*.

Among all the isolated bacteria, we selected five representatives of *A. rubi,* two of *Agrobacterium* bv. 1 and two of *Agrobacterium* bv. 2 for further analysis. Five *A. rubi* and two *Agrobacterium* bv. 1 induced galls on *V. corymbossum* (blueberry), *Bryophyllum daigremontiana* (Kalanchoe), *Nicotiana tabacum* (Tobacco), *Lycopersicum esculentum* (Tomato), *Capsicum annuum* (Pepper) and *Helianthus annuum* (Sunflower) *Agrobacterium* bv.2 isolates (F288 and F 289) only induced root proliferations in those hosts and in carrot disks. Therefore the identity of the isolates was *A. rubi*, *A. tumefaciens* and *A. rhizogenes,* respectively. Both the biological assays as well as polymorphisms at the 5′ sequence of the 23SrDNA (Bautista-Zapanta et al. 2009), support that 33 isolates from blueberries were *Agrobacterium* bv. 1 (42 %), 31 isolates were *Agrobacterium* bv. 2 (40 %) and 14 were *A. rubi* (18 %).

The identity of an *Agrobacterium* bv. 1 (F268) that induced galls (this paper) and one representative of *Agrobacterium* bv. 2 that provoked root proliferation (F289) (Alippi et al. 2010) were confirmed by the 16SrDNA full sequence. In relation to the strain F266, previously classified as *A. rubi* based on a multiplex PCR and biochemical tests, the phylogenetic analysis indicated that it is closely related to strain F289 (*A. rhizogenes* bv. 2) and that they both formed part of a cluster that include other representatives of *Agrobacterium rhizogenes* (n=5) and one *A. rubi* strains (Fig. 4). However, isolate F 266, with the exception of its ability to metabolize citrate, which was a variable trait among putative *A. rubi* isolates, showed a biochemical pattern similar to those reported for *A. rubi* (i.e. positive for β–galactosidase, D-glucose, D-arabinose, n-acetyl-glucosamine, maltose, mannitol, malonate, urease but negative for L-arabinose, saccharose, lysine decarboxylase, H_2S production, indole and 3-ketolactose production). Additionally, the sequence of *A. tumefaciens* isolate F268 was phylogenetically closely related to other *A. tumefaciens* strains and also to other representatives of *A. rubi, A larrymoorei* and *R. skierniewicense.* Young et al. (2004) also found an *Agrobacterium rubi* strain closely related to *Agrobacterium rhizogenes*, suggesting that it might be *A. rhizogenes* rather than *A. rubi*. On the other hand, Farrand et al. (2003) while analyzing the 16SrDNA sequences stated that *A. rubi* is atypical but more closely related to biovar 1 isolates than to biovar 2 ones. The fact that the phylogenetic analysis based on the 16SrDNA suggest a close association of isolate F266 with *A. rhizogenes* bv. 2 while the multiplex PCR and biochemical tests suggest that the isolate is *A. rubi*, raises some questions about the identity of the isolate, that we called atypical, therefore further work should be done to clarify this.

Even though all these organisms are closely related, the phylogenetic analysis confirmed the complexity and variability of the genus *Agrobacterium*. In addition to this, the analysis of Table 1 concomitantly with the identity of the isolates of agrobacteria showed that there was no correlation between the place of origin of sampled plants and the *Agrobacterium* species infecting them. *Agrobacterium* bv. 1, *Agrobacterium* bv. 2 and *A. rubi* were isolated from diseased plants that were sent from nurseries (n=44) and also from field commercial crops (n=34) within four provinces that is Tucumán, Entre Ríos, Corrientes and Buenos Aires.

In Argentina, there is no information about the diversity of agrobacteria affecting *Vaccinium corymbosum* and not much is known about the number and diversity of the *Agrobacterium* population in the soils. Diseased blueberry plants are probably the main way *Agrobacterium* is disseminating into new areas. As many of the analyzed blueberry plants originate in nurseries (n=44) from several places in Argentina; therefore, unknowingly nurseries might be threatening new areas of production by introducing the pathogen through infected plants. The number of isolates of each *Agrobacterium* species suggested that the evaluated nurseries are producing *Vaccinium* plants in infected soils and/or substrates containing mainly *Agrobacterium* bv. 1 and bv. 2 and low levels of *A. rubi*.

Like other bacterial-plant interactions, the genome of both *Agrobacterium* and the plant might contribute to the occurrence of the disease. While *A. tumefaciens* and *A. rhizogenes* have been found to infect many different hosts, *A. rubi, A. vitis* and *A. larrymoorei* have been associated mostly with *Rubus* spp. and *Vaccinium* spp., *Vitis* spp. and *Ficus* spp., respectively, suggesting that each species has a genetic component that is contributing to infection. It has been proposed that plants differ in their susceptibility to *Agrobacterium*. Some plants and/or cultivars are particularly susceptible to *Agrobacterium* (Gelvin 2010); for

instance, compatibility of *A. tumefaciens* and Leuce poplar is controlled at the infraespecific level (Nesme et al. 1987). Regarding this, blueberries seem to be particularly susceptible to *Agrobacterium* since the frequency of pathogenic populations of *Agrobacterium* bv. 1 and bv. 2 isolated from *Vaccinium* were similar (42 % and 40 %, respectively), with *A. rubi* represented in a minor proportion (18 %). It is not clear, at this moment, if blueberries are particularly resistant to *Agrobacterium* isolates carrying the Ri plasmid or if the isolation of only two strains with this characteristic indirectly reflects the frequency of this organism in the soils of those farms. Soil samples from fields cultivated with blueberries (n=15) contained only pathogenic *Agrobacterium* bv. 1 (94 %) and *A. rubi* (6 %) (data not shown). Future studies should include the isolation of *Agrobacterium* from soil sampled based on a systematic survey of the area with plant nurseries, which might help to more knowledge about the ecology of these organisms in the soils and also to estimate the risk of spreading the bacteria to other areas.

Agrobacterium is a complex genus and as such its taxonomy is still an issue under discussion (Young et al. 2001; Farrand et al. 2003; Young et al. 2004; Young et al. 2005; Gelvin 2010). In addition to this, it has been demonstrated that the plant mediates instability of *A. tumefaciens* populations (Dion et al. 1996). It has been demonstrated that non-pathogenic mutants appear in response to cultivation of *Agrobacterium* in the presence of substances naturally present in plant wounds (Goodner et al. 2001). In this study all the isolates obtained were pathogenic, though it is worth mentioning that we worked with only one colony of each gall, and this is contrary to the proposal of Anderson and Moore (1979) and Nesme and co-workers (1987) that the *Agrobacterium* population obtained from tumours consists mostly of nonpathogenic strains. Recently, Llop and co-workers (2009) isolated mutants derived from *Agrobacterium* strains inoculated in pepper and tomato. They found that the appearance of non-pathogenic mutants of *Agrobacterium* in tumours occurred at low frequency and differed within host species; furthermore, they demonstrated that the genetic changes occurred mostly in large areas of the Ti plasmid. This might explain, at least in part, why all the isolates studied in this work were pathogenic on different hosts.

We conclude that *Vaccinium* plants appear to be highly susceptible to *Agrobacterium* isolates, whether they are *Agrobacterium* bv. 1, *Agrobacterium* bv. 2 or *A. rubi.* The frequency of each *Agrobacterium* species infecting nurseries and/or field plots of *Vaccinium* might indirectly reflect their presence in the soil. Further work should be done to clarify this.

Acknowledgments A.M.A and P.B.A. are members of the Scientific Research Career of Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) (Argentina) and A.C.L. is a Member of CONICET (CCT La Plata). This research was supported by a grant from CIC. The authors are grateful Drs. D. H. Grasso (Instituto de suelos, INTA Castelar, Argentina) and S. Süle (Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary) for providing the reference strains *A. tumefaciens* LBA 956 and *A. vitis* K306 and S4 respectively.

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