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# Physiological, biochemical and molecular analyses of an Italian collection of *Agrobacterium tumefaciens* strains

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**Abstract**

Physiological, biochemical and molecular characteristics of *Agrobacterium tumefaciens* strains isolated in Italy from different host plants were analysed. Diseased plants were collected from several nurseries located in nine different regions. Out of 1293 strains isolated from 12 fruit tree and six ornamental plant species, a group of 120 strains was chosen as representative of the whole collection. The majority of the strains were biovar 2 (82.5%), agrocin 84 sensitive, and were isolated from stone fruit trees. Most of the strains identified as biovar 1 were isolated from ornamental plants and were insensitive to *A. radiobacter* antagonistic strain K84. Some strains that were isolated from *Euonymus* spp, *Prunus* GF 677 and *Pyrus communis* (pear) OHF tumours could not be allocated to any of the three *Agrobacterium* biovars. PCR-restriction fragment length polymorphism of the *rrs* gene plus the intergenic spacer was used for strain fingerprinting and characterisation. Results showed a wide genetic variability within the biovar 1 strains and homogeneity within the biovar 2 group. Biovar 2 strains from Sardinia were highly variable and differed from the biovar 2 strains isolated from the other regions of Italy.

## Introduction

Crown gall is a plant disease that is widespread all over the world, mainly in temperate areas, where it causes heavy economic losses to many crops. The disease is particularly serious in nurseries because the infected plants become unsalable (Lippincott et al., 1983; Moore, 1988). The causal agent of the disease is *Agrobacterium tumefaciens*. At present, nomenclature and classification of bacterial species belonging to the genus *Agrobacterium* is controversial. Epithets like '*tumefaciens*', '*radiobacter*', '*rhizogenes*', '*vitis*', '*rubi*' and '*larrymoorei*' have been proposed by many authors (Keane et al., 1970; White, 1972; Kerr and Panagoupolos, 1977; Holmes and Roberts, 1981; Kersters and De Ley, 1984; Ophel and Kerr, 1990; Sawada et al., 1993; Willems and Collins, 1993; Bouzar, 1994; Bouzar and Jones, 2001) to indicate different species, but also varieties, biotypes, biovars or even pathovars. Recently Young et al. (2001) proposed that the genus *Agrobacterium* should be included in the genus *Rhizobium* since both belong to an unique phylogenetic cluster on the basis of the analysis of their 16S rRNA sequences. As all this matter is still under discussion the name *Agrobacterium tumefaciens* will be used in this work.

*Agrobacterium tumefaciens* is a soil-borne bacterium that induces tumours on many dicotyledons and on some monocotyledons and gymnosperms (DeCleene and De Ley, 1976). The mechanism of tumourigenesis involves the transfer of a fragment (T-DNA) of the tumour inducing plasmid (pTi) from the bacteria to a wounded plant cell where it is integrated into the genome and expressed (Gelvin, 2000). The transferred DNA portion of the Ti plasmid carries the genes *tms* and *tmr*, which encode proteins involved in the synthesis of phytohormones (auxins and cytokinins), which are responsible for uncontrolled cell

proliferation during neoplastic growth (Lipp-Nissinen, 1993). The T-DNA also encode the synthesis of opines, novel tumour-specific compounds. These compounds are released by the tumour tissues and are preferentially utilised by *A. tumefaciens* strains as the sole carbon and/or nitrogen source (Clarke et al., 1992).

Agrobacteria are part of the common soil microflora and occur either as saprophytes or as plant pathogens (Schroth et al., 1971). Up to now very little is known about survival of tumourigenic agrobacteria in the soil, the environmental factors responsible for epidemic outbreaks, and the structure of soil agrobacteria populations. The lack of information is in part due to the difficulty in detecting the tumourigenic strains in soil where disease outbreaks occur and where the level of non-tumourigenic agrobacteria populations is always high (Schroth et al., 1971; Bouzar and Moore, 1987).

A collection of *Agrobacterium* isolates, obtained from diseased plants collected in many regions of Italy, was developed with the aim of studying the physiological, pathological and molecular characteristics of the isolates and thus providing an understanding of the *Agrobacterium* populations that occur in Italy.

Several PCR-based methods are commonly employed to measure genetic diversity in phytopathogenic bacteria including arbitrarily primed PCR (AP-PCR), randomly amplified polymorphic DNA (RAPDs), repetitive extragenic palindromic PCR (rep-PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), or amplified fragment length polymorphism (AFLP) (Louws et al., 1999). In this study, molecular analysis was carried out using the PCR-RFLP analysis of the ribosomal region consisting of the *rrs* gene plus the intergenic spacer region (IGS) located between *rrs* and *rrl* gene (Navarro et al., 1992; Normand et al., 1992; Languerre et al., 1996; Vineusa et al., 1998). Analysis of the IGS region was particularly useful for *Agrobacterium* strains since these bacteria have a relatively long IGS region (Ponsonnet and Nesme, 1994) that allows a clear identification and a very accurate typing of the isolates to be obtained. The use of the IGS region in PCR-RFLP analysis may yield significant results about the diversity of closely related *Agrobacterium* strains (Momol et al., 1998).

Molecular analyses were used to study the composition of the natural Italian populations of agrobacteria, and verify if any correlation between a given bacterial genotype and a specific host plant or geographic area of origin could be found. The analysis may provide a useful tool for epidemiological studies on crown gall disease in Italy. This work represents the Italian contribution to the characterisation of a large collection of agrobacterium strains isolated from several Mediterranean countries within a research project supported by the EC.

## Materials and methods

### *Plant material*

A total of 183 diseased plant samples, including fruit tree rootstocks and ornamentals, were collected during 1997 and 1998 from 30 nurseries located in 20 different areas of Northern, Central and Southern Italy. The list of plants and the region of origin is reported in Table 1.

### *Isolation and storage of* Agrobacterium *isolates*

Fresh tumours were washed under tap water, surface sterilised for 15 min by NaClO solution (1%) and rinsed with sterile water. Tissue fragments were ground in a mortar and pestle in 1–2 ml of sterile distilled water (SDW). The homogenates were left standing for 30 min and then streaked on mannitol-glutamate-agar medium amended with 0.1% yeast extract (MGY) (Moore et al., 1988), and on selective media for agrobacteria biovar 1 (Schroth et al., 1965) and biovar 2 (Brisbane and Kerr, 1983). Six to twelve colonies from each tumour were chosen on the basis of their morphology and purified until pure cultures were obtained. Strains were indicated as 'At' followed by an identification number and stored both in glycerol 30% (v/v) at $-80\,^{\circ}$C and in sterile double-distilled water at $4\,^{\circ}$C. All the agrobacteria isolates were deposited in the collection of phytopathogenic bacteria of the Department of Botany, Horticulture and Plant Pathology of the University of Naples 'Federico II' (Italy).

### *Pathogenicity tests*

Bacterial isolates were grown for 3 days on MGY medium and individually inoculated at three different points into the stem of two 4-week-old tomato (*Lycopersicon esculentum*) cv. Marmande plants. Strains that did not induce tumours on tomato were

*Table 1.* Number of samples, geographic origin of host plants and some characteristics of *Agrobacterium* strains of the Italian collection

| Host plant | Number of samples | Virulent strains | | Prevalent opine catabolism | Region of origin |
|---|---|---|---|---|---|
| | | bv1 | bv2 | | |
| *Castanea sativa* | 2 | | 14 | Nopaline | Campania |
| *Chrysanthemum frutescens* | 3 | 2 | | Mannopine | Campania, Liguria |
| *Chrysanthemum* spp. | 7 | 28 | 2 | None | Lazio |
| *Diospyrus kaki* | 5 | | | | Campania, Sardinia |
| *Euonymus* spp. | 2 | | 2 | Nopaline | Toscana |
| *Juglans regia* | 1 | | | | Veneto |
| *Malus communis* (M9) | 5 | | | | Campania, Emilia-Romagna, Friuli |
| *Prunus armeniaca* | 8 | | 29 | Nopaline | Campania |
| *P. cerasifera* (Myrobalan) | 22 | 1 | 38 | Nopaline | Campania, Emilia-Romagna, Sardinia |
| *P. munsonniana* × *P. cerasifera* (Mariana GF 8-1) | 2 | 2 | 5 | Nopaline | Campania |
| *P. persica* | 73 | 4 | 307 | Nopaline | Campania, Emilia-Romagna, Lazio, Sardinia |
| *P. persica* × *P. amygdalus* (GF677) | 31 | 7 | 153 | Nopaline | Campania, Sardinia |
| *Pyrus beatulifolia* | 1 | | 1 | Nopaline | Campania |
| *P. communis* (OHF) | 3 | | 6 | | Sardinia |
| *Rosa indica major* | 14 | | 68 | | Campania, Lazio, Liguria |
| *Rubus ideaus* | 3 | | 39 | None | Campania |
| *Solidago* spp. | 1 | | 8 | Octopine/mannopine | Campania |

inoculated into datura (*Datura stramonium*) plants. Tumour formation was visually assessed 4 and 6 weeks after inoculation.

### *Characterisation of bacteria*

#### *Biovar characterisation*

All agrobacteria-like isolates were preliminarily submitted to aesculine utilisation and urease production tests and assayed for 3-ketolactose production (Moore et al., 1988). Previous studies indicated that the latter test is highly reliable for the preliminary assignment of isolates to biovar 1 or biovar 2 group (Kersters et al., 1973; Popoff et al., 1981; Ridé et al., 2000).

One-hundred and twenty isolates were chosen as representatives of the whole collection on the basis of host plant, geographic origin, isolation medium, pathogenicity and 3-ketolactose production. They were submitted to the following biochemical and physiological tests for biovar determination: acid production from erythritol and melezitose, growth at 35 °C, growth in 5% NaCl broth, alkali production from mucic, malonic and tartaric acids, growth and pigmentation in ferric ammonium citrate broth and citrate utilisation (Kerr and Panagopoulos, 1977). Strains were considered to belong to a given biovar when at least 7 out of the 10 tests matched with the corresponding profile. When the correspondence was lower, the strains were assigned to an intermediate biovar. *Agrobacterium* strains bv 1 C58 and B6 (ATCC 33970; ATCC 23308) and bv 2 B49c/83 (L.W. Moore, Oregon State University) were included in all tests as the reference strains.

#### *Opine catabolism*

Strains were screened for opine catabolism on a basal salt medium supplemented with 5 mM octopine, nopaline or mannopine and solidified by addition of Gelrite Gellan Gum (Sigma). Strains that were able to grow on solid medium were tested for opine catabolism in a liquid medium containing opine as the sole carbon and nitrogen source. A density of 0.20 O.D. at 600 nm wavelength in 24 h, was the minimum threshold value indicating opine utilisation (Canfield and Moore, 1991). *Agrobacterium* spp. strains B49c/83 (mannopine/nopaline), C58 (agrocinopine/nopaline) and B6 (octopine/mannopine) were included in the tests as most appropriate controls. All opine utilisation tests were repeated at least twice.

#### *Sensitivity to K84*

The 120 strains were tested for their sensitivity *in vitro* to agrocin 84 produced by *A. radiobacter*

strain K84 (New and Kerr, 1972). The test was performed according to the protocol described by Stonier (1960).

*PCR-RFLP analysis of 16S + IGS ribosomal region*

Primers FGPS6 and FGPL132′ (Normand et al., 1992) were used to amplify a fragment of 2500–2700 bp of the ribosomal region that included the gene 16S, the intergenic spacer between 16S rDNA and 23S rDNA (IGS) and a 132 bp piece of 23S rDNA. Preparation of PCR reaction mixtures and conditions of amplification were as described by Ponsonnet and Nesme (1994). Ten microlitres of the PCR products were digested with 10U of each restriction enzyme. Digestion of PCR amplicons was performed using *Cfo*I (GibcoBRL), *Hae*III (Biolabs), *Nde*II (GibcoBRL) *Taq*$^{\alpha}$I (Biolabs) restriction enzymes. Restriction fragments obtained after 1 h of digestion were separated by horizontal electrophoresis in TBE buffer on a 2.5% (w/v) Nusieve agarose gel containing 1 µg/ml ethidium bromide. Gels were run at 2.3 V/cm for 3 h and photographed under UV light using Polaroid film type 57. Fragments smaller than 123 bp were not considered for comparison of restriction patterns. The restriction fragments obtained from the isolates of the Italian collection were compared with type strains C58 and B6 for biovar 1, B49c/83 for biovar 2, *A. vitis* (CFBP 2736) and *A. rubi* (ATCC 13335). The patterns were analysed by cluster analysis using the software SYN-TAX-PC 5 (Podani, 1993). An average linkage agglomeration criterion was applied to a resemblance matrix of Gowen coefficients of distances between strains.

## Results

*Isolation and characterisation of agrobacteria*

A total of 1293 putative *Agrobacterium* isolates was collected. One thousand and ninety-four isolates were obtained from 12 woody plant species and 199 from six ornamental species. All the isolates were considered to be agrobacteria because they exhibited urease activity and were able to degrade aesculine. According to 3-ketolactose production, 243 isolates (18.8%) behaved as biovar 1 and 1050 (81.2%) as biovar 2. Pathogenicity tests showed that 44 biovar 1 and 672 biovar 2 strains were tumourigenic on tomato or datura. Strains isolated from M9 rootstock plants and from walnut and persimmon seedlings were unable to induce tumours on tomato or datura and were considered non-pathogenic (Table 1).

On the basis of results from the other nine tests to differentiate among biovars performed on 120 representative isolates, 17 strains were biovar 1, 99 were biovar 2 and four strains were put in an intermediate biovar. This third biovar group included two isolates (At 148rk2 and At 149rk4) from *Euonymus* plants, one (At 137n8) from a *Prunus* GF677 and one (At 141n8) from Pear OHF rootstock. These four strains reacted as biovar 2 for 3-ketolactose test and for the ability to produce alkali from mucic acid, while they reacted as biovar 1 on ferric ammonium citrate broth. In the remaining biochemical assays, the four strains responded differently. In fact, the two strains from *Euonymus* were similar to biovar 1, 2 and 3 in 5, 2 and 3 of tests respectively. The other two strains showed a similarity of 3, 4 and 3 tests with biovar 1, 2 and 3, respectively. No strain was identified as biovar 3.

*Opine catabolism*

Eighty-five strains (78 biovar 2 and seven biovar 1 strains) isolated from stone fruit rootstocks, all 13 of the biovar 2 strains obtained from *Rosa indica* major, pear OHF, *Pyrus baetulifolia* and chestnut, and the four intermediate biovar strains catabolised nopaline. The biovar 1 At 18s7 strain from peach utilised octopine while the two biovar 1 strains isolated from *Chrysanthemum frutescens* (At 172n7 and At 174b1) catabolised mannopine. Three strains were octopine/mannopine utilisers (two biovar 2 strains from *Solidago* spp. and one biovar 1 strain from peach). Twelve strains (six biovar 1 and six biovar 2) were not able to utilise any of the three opines tested; among these, the six biovar 2 strains were all isolated from raspberry.

*Sensitivity to K84*

The agrocin 84 sensitivity test showed that 102 strains were sensitive, while the remaining 18 strains were insensitive to this compound. Of the 18 insensitive strains, 10 were biovar 1 and were not able to utilise nopaline, whilst eight were isolated from *Chrysanthemum* spp. All biovar 2 agrocin insensitive strains were obtained from raspberry and *Solidago*. All the agrocin 84 sensitive strains utilised nopaline,

confirming that nopaline catabolism and agrocin 84 sensitivity were closely related characteristics.

*PCR-RFLP analysis*

Amplification performed with the forward primer FGPS6 and reverse primer FGPL132′ gave a DNA fragment of about 2700 bp from all strains tested. Digestion of the fragments from the 120 strains tested using *Cfo*I, *Hae*III, *Nde*II and *Taq*$^{\alpha}$I restriction enzymes generated 31 different restriction patterns with five to 14 sites for each enzyme.

Thirteen different patterns were obtained by PCR-RFLP analysis of the 17 biovar 1 strains. Three of them showed the same profile as the reference strain C58 while three others were similar to B6. Each of the remaining 11 biovar 1 strains (four from stone fruit and seven from ornamental plants) showed unique restriction profiles (Figure 1). Ninety-nine biovar 2 strains were divided into 16 different restriction profiles. Seventy-nine biovar 2 strains produced a RFLP profile that was identical to the reference strain B49c/83. The remaining 20 biovar 2 strains were dispersed into 15 different profiles (Figure 2). Two intermediate biovar strains had the same restriction profile as the reference strain C58, while the two other intermediate strains showed two patterns different from all the other tested strains.

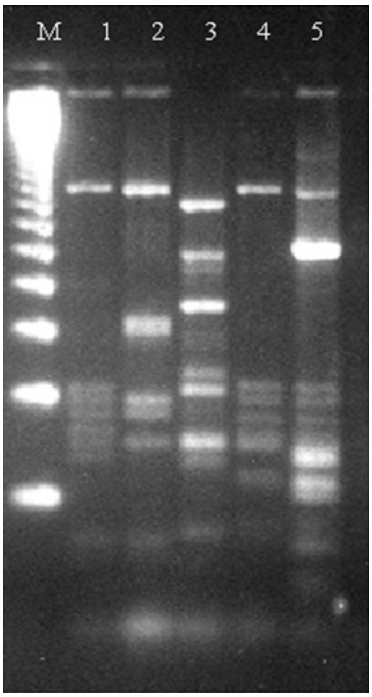


*Figure 1.* Restriction profiles of biovar 1 strains obtained with *Nde*II restriction enzyme. M = 123 bp; 1 = At 82s12; 2 = At 85s9; 3 = At 88s10; 4 = At 172n7; 5 = At 174b1.

Cluster analysis of PCR-RFLP patterns of 16S+IGS ribosomal region of the representative strains led to the dendrogram illustrated in Figure 3. Strains were separated in many different clusters revealing a genetic heterogeneity within the collection of 120 agrobacteria. The largest group, located in the upper part of Figure 3, included 82 biovar 2 strains, 79 of which were grouped together with B49c/83 reference strain. The great majority (68) of them were isolated from stone fruit rootstocks. Three biovar 2 strains belonging to this main cluster were isolated from chestnut (At 14p4, At 14n8 and At 169b6), one strain from *Pyrus beatulifolia* (At 178n5) and seven additional strains from *Rosa indica major* (At 31n1, At 33n6, At 34n2, At 38s4, At 40n4, At 153s1 and At 153n4).

Strains that clustered differently from this large group formed several subgroups that included 17 biovar 1, 17 biovar 2 and the four intermediate biovar strains. Biovar 1 strains showed a wide variability and were distributed in the bottom part of the dendrogram with different levels of similarity. Ten biovar 1 strains were from peach rootstocks while most of the remaining strains were from ornamentals. The 17 biovar 2 strains fell into 12 different genotypes, these strains were isolated from such uncommon hosts as *Solidago*, Pear OHF and raspberry and, interestingly, strains from stone fruit rootstocks were all isolated from diseased plants collected in Sardinia. Eight different genotypes were obtained from the 10 Sardinian strains and the same number of genotypes were identified in the much larger group of 89 biovar 2 strains gathered from all the other Italian regions. The two intermediate biovar strains from *Euonymus* (At 148rk2 and At 149rk4) clustered with C58, while the At 137n8 intermediate strain isolated from GF677 rootstock in Sardinia was included in one subgroup formed with other biovar 2 strains isolated from stone fruit rootstocks from the same region. The At 141n8 strain, isolated from pear OHF in Sardinia, represented an unique genotype.

## Discussion

A collection was made of 1293 *A. tumefaciens* strains, isolated from 183 galled plants belonging to

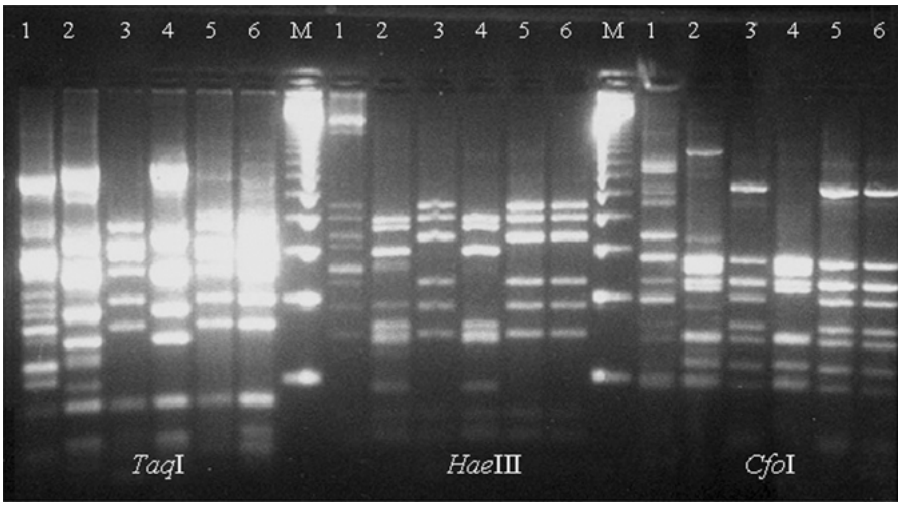


*Figure 2*. Restriction profiles of biovar 2 strains obtained with *Cfo*I, *Hae*III and *Taq*I restriction enzymes. M = 123 bp; 1 = At 126n5; 2 = At 129n5; 3 = At 40n4; 4 = At 137s9; 5 = At 45n3; 6 = At 162s8.

several species. A preliminary screening using the 3-ketolactose test identified 243 strains as putative biovar 1 and 1050 as biovar 2. Inoculations of tomato and datura plantlets showed that 716 strains of the 1293 were tumourogenic. No virulent strains were obtained from apple rootstock M9 plants or from walnut or persimmon. The finding of only avirulent agrobacteria in apple tumours agrees with previous studies showing that phenolic compounds, like acetosyringone, cause mutation of tumourogenic strains to avirulence (Canfield and Moore, 1989; Bélanger et al., 1995). Notwithstanding, pathogenic agrobacteria from walnut have been obtained previously in our (unpublished data) and in other laboratories (Moore and Canfield, 1996; Nesme and Mougel, 1997). Walnut tumours that were processed in this study were probably too old and strongly colonised by saprophytic microflora to yield tumourogenic strains of *Agrobacterium*. Forty-six strains were isolated from persimmon and all were non-pathogenic. To our knowledge this is the first report of natural occurrence of crown gall on this host plant. However, since all the agrobacteria from persimmon were unable to induce tumours on tomato or datura, it is possible that these strains could harbour a narrow host range pTi type (Anderson and Moore, 1979; Thomashow et al., 1980) or perhaps, persimmon also induces non-tumourigenicity (Bélanger et al., 1995).

The physiological characterisation of the 120 representative *A. tumefaciens* strains (Kerr and Panagopoulos, 1977) showed that the majority could be included in the biovar 2 phenotype. This result agrees with the composition of pathogenic *Agrobacterium* populations of other countries (Kersters et al., 1973; Panagopoulos and Psallidas, 1973; Bouzar et al., 1983; Lopez et al., 1983; Du Plessis et al., 1984; Bouzar et al., 1991; Sobiczewski, 1996), where biovar 2 strains are always predominant. The rather high frequency of biovar 2 strains obtained in our research may also be related to the large number of stone fruit plants processed for isolation. In fact, it is well known that this group of plants is preferentially infected by biovar 2 strains (Lopez et al., 1988; Zoina et al., 1992). Most of the biovar 2 isolates were sensitive to agrocin 84 and able to utilise nopaline as sole nitrogen and carbon source, confirming a strong correlation among these three characteristics (Du Plessis et al., 1984; Lopez et al., 1988; Zoina et al., 1992). These results are in agreement with those regarding the efficacy of K84 in controlling crown gall of stone fruit trees in Southern Italy (Raio and Zoina, 1992; Zoina et al., 1992). A wider use of the biological control method is predicted in all the other Italian regions.

The majority of the biovar 1 strains were obtained from the two *Chrysanthemum* species analysed in this study, while only six agrobacteria from stone fruit rootstocks belonged to this biovar. Biovar 2 strains were also predominant in the two other ornamental plants tested (*Solidago* and *Rosa indica major*).

A few strains could be not allocated to any of the three recognised biovars on the basis of the biochemical and physiological tests. The occurrence of strains with intermediate characters has been frequently reported

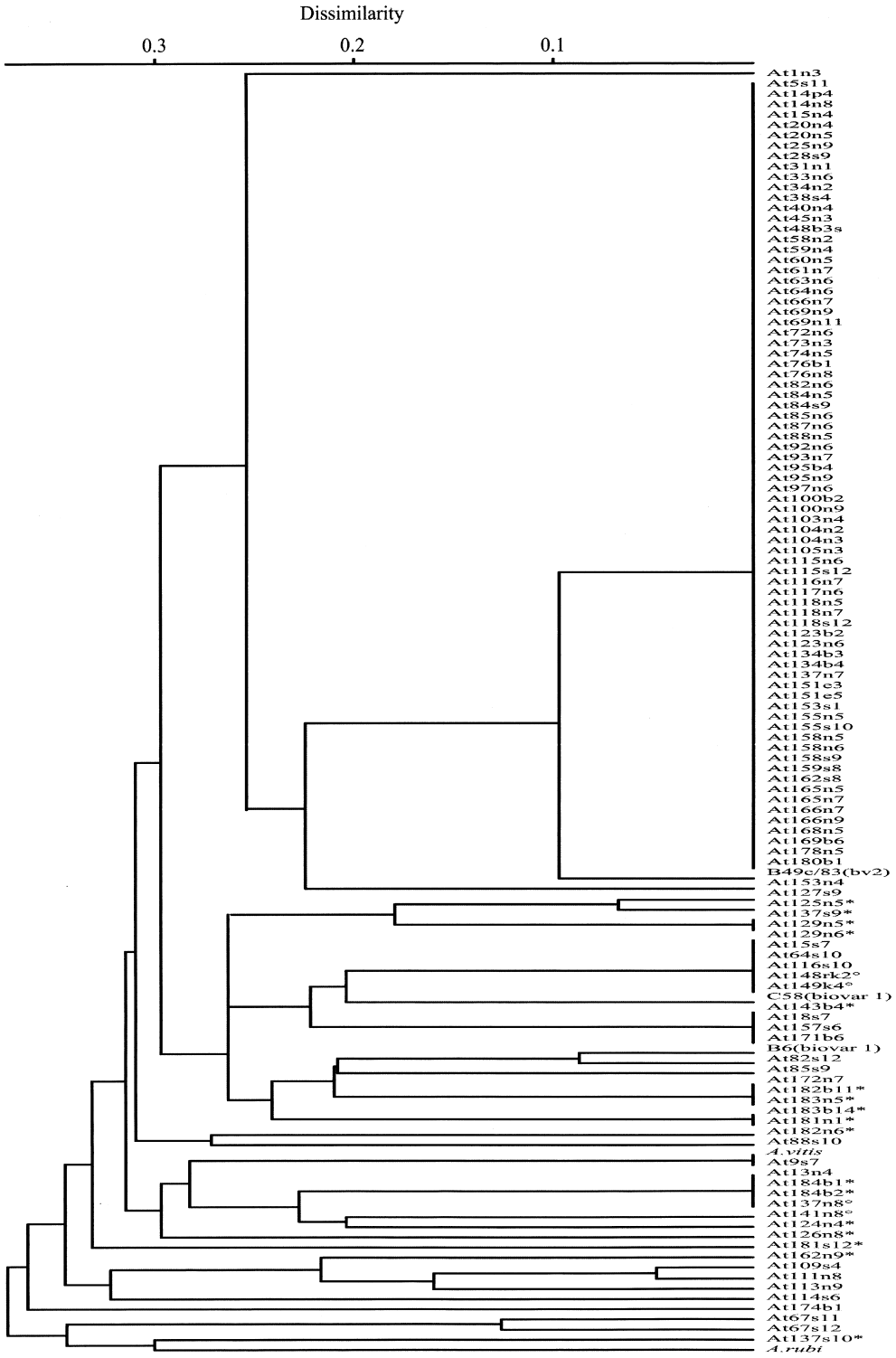


*Figure 3*. Dendrogram showing genetic relationships between *Agrobacterium* strains based on PCR-16S + IGS ribosomal region. *: Biovar 2 from Sardinia or from uncommon hosts. °: Biovar intermediate.

(Du Plessis et al., 1984; Bouzar and Moore, 1987; Raio and Zoina, 1992; Zoina et al., 2001), and, probably, a more accurate identification of these agrobacteria would need additional tests (Du Plessis et al., 1984; Sobiczewski, 1996).

The ribosomal region composed of the *rrs* gene plus the IGS was analysed by PCR-RFLP. Of the restriction enzymes tested, *Cfo*I and *Hae*III were the most effective because they produced the highest number of different patterns and allowed clear discrimination of many *Agrobacterium* strains. Cluster analysis of PCR-RFLP patterns from the 16S + IGS *rrs* gene revealed several groups of strains. The greatest majority of biovar 2 strains were included in a wide and homogeneous cluster while biovar 1 strains, intermediate strains and a few biovar 2 strains were distributed in several sub-clusters. Low genetic variability within the biovar 2 strains had been already observed by Ponsonnet and Nesme (1994) and the present study using a large number of biovar 2 strains, fully confirms those findings. However, the genetic homogeneity of biovar 2 strains analysed in this study could in part be due to the fact that most of them originated from stone fruit rootstocks. It is also possible that the number of IGS polymorphisms could be increased by testing additional restriction enzymes as suggested by Ponsonnet and Nesme (1994). However, even if the selection of other tetrameric restriction enzymes could enhance the information gained at the strain level, the use of a large number of enzymes is not useful for routine identification (Moyer et al., 1996; Oger et al., 1998). Seventeen biovar 2 strains were distributed in many small clusters with several levels of similarity but they were clearly separated from the large homogeneous biovar 2 cluster. Our data suggest that the biovar 2 strains from Sardinia are genetically distinct from other Italian biovar 2 strains. Moreover, a higher genetic variability was observed within the Sardinian strains compared with other biovar 2 agrobacteria.

PCR-RFLP analysis of the IGS region allowed the differentiation of 17 biovar 1 strains into 13 genotypes. Although the majority of the biovar 1 strains came from the same region, a considerable genetic variability was observed. This finding confirms that a wide genetic variability is typical of biovar 1 agrobacteria (Popoff et al., 1981; Ponsonnet and Nesme, 1994). Analysis of agrobacteria by PCR-RFLP allowed precise taxonomic allocation of three out of four 'intermediate' biovar strains. This genetic characteristic allowed grouping of the two strains from *Euonymus* (At 148rk2 and At 149rk4) with C58, which is a biovar 1 strain, while strain At 137n8 was clustered together with the biovar 2 strains from Sardinia. The fourth intermediate strain, At 141n8 isolated in Sardinia from pear, represented a unique genotype.

This is the first study regarding both phenotypic and molecular characterisation of agrobacteria isolated from several hosts and from all areas of Italy with a relevant plant nursery industry. Previous investigations (Bazzi and Mazzucchi, 1977; Bazzi et al., 1980; Raio and Zoina, 1992; Zoina et al., 1992) were concerned with physiological and biochemical characters of agrobacteria only from apple and stone fruit trees in some of regions of Northern and Southern Italy.

The PCR-RFLP analysis of 16S + IGS ribosomal region used in the present study proved to be a powerful tool for distinguishing *Agrobacterium* strains, more reliable and much faster than biotyping. Moreover, this technique precisely allocated strains that had an intermediate biovar based on the tests of Kerr and Panagopoulos (1977). PCR-RFLP allowed the distinction of closely related strains belonging to the same biovar, and interestingly showed that all biovar 2 agrobacteria isolated from Sardinia were genetically different from those isolated from other regions of Italy. It is possible that *Agrobacterium* populations in Sardinia evolved separately and maintained their own genetic characteristics through time.

PCR-RFLP of 16S+IGS was a reliable technique for fingerprinting of *Agrobacterium* population. However, our data show that this technique is not suitable for studies on epidemiology of crown gall since no specific markers were found in the ribosomal regions analysed. The analysis of conserved regions of Ti plasmids (Pionnat et al., 1999) may represent a more appropriate tool for epidemiological and ecological studies on agrobacteria.

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