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# Characterization of *Agrobacterium tumefaciens* Strains Isolated from Brazilian Plant Species

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**Abstract:** The morphology and development of Brazilian isolates-induced tumors were studied *in vitro* and *in vivo* using marker plants, which are highly susceptible to *Agrobacterium* sp. infection. Tumor tissues were submitted to biochemical analysis to identify opine compounds and bacterial isolates were tested to their ability to utilize different opine types. 16S rDNA region and Intergene Space Sequence (IGS) of these isolates were amplified, producing a single fragment of 1,500 and 2,600 bp, respectively. From Ti plasmid, nopaline synthase (*nos*) region was amplified for all isolates, with a fragment of 256 bp. The results from test-plants showed that tumor phenotype characteristics, such as form (wart and root), color and post-inoculation appearance period were distinct for each isolate. Bacteriological tests allowed to classify all the eight isolates as hosting a nopaline type Ti plasmid. Sequence analysis of PCR products from 16S rDNA region showed that these *Agrobacterium* isolates have high sequence similarity to previously described *A. tumefaciens* isolates, belonging most of them to biovar 1.

**Key words:** Tumor phenotype, *Agrobacterium tumefaciens*, opine, nopaline, Ti plasmid

## Introduction

The plant pathogen *Agrobacterium tumefaciens* causes crown gall tumors in a variety of dicotyledonous plants by transferring discrete DNA fragments (T-DNAs) from its tumor-inducing (Ti) plasmid into plant cells (Zambryski, 1992; Long and Staskawicz, 1993). It is suggested that these bacteria are able to attach to plant cell during the early tumor formation stage, due to specific recognition between the bacteria and the wounded plant cell. In such tumors, compounds such as octopine and nopaline are present and they cannot be detected in healthy plant tissues. Opines are molecules resulting from the condensation of an amino acid with a sugar molecule. Once synthesized by the plant transformed cell, opines are secreted outside the cell and specifically catabolized by the infecting bacteria present in the intracellular space of the tumor as a nutrient source (Petit and Tempe, 1978; Dessaux *et al*., 1992). Several opines have been identified (nopaline, octopine, agropine, etc.) according to the type of amino acid and carbohydrate molecule conjugated, but only the tumor-inducing bacteria are able to catabolize its own opine (Hooykaas *et al*., 1984).

T-DNA transformed cells keep on dividing disordered due to hormone production and the more they divide, the more they produce opines that are utilized by the bacteria as nitrogen and carbon source (Hooykaas *et al*., 1984). This mechanism allows tumor-inducing bacteria to multiply. Opines

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also induce conjugative transfer of Ti plasmid from tumor inducing bacteria to those without Ti plasmid. These strains become virulent and they infect non-attacked plant cell. T-DNA-encoded enzymes involved in the biosynthetic production of cytokinins (Akiyoshi *et al*., 1984) and auxins (Thomashow *et al*., 1986) play an important role in the overproduction of these phytohormones.

The aim of this research was to study the importance of opines in plant infection process. For this purpose this study involved phytopathological, biochemical and molecular studies of the ethnological agent of tumors, *A. tumefaciens* naturally found in plants.

## Materials and Methods

*Culture Conditions*

The Brazilian isolates of *A. tumefaciens* studied in this work were obtained from the Experimental Center Collection of the Biological Institute of Campinas-SP and they are listed in Table 1. *A. tumefaciens* C58 was used as a nopaline positive control (Sciaky *et al*., 1978) and R10 was used as nopaline negative control. *A. tumefaciens* strains were grown at 27°C for 48 h in either Luria-Bertani (LB) medium or AT minimal salts medium (Petit and Tempé, 1978).

*Opine Catabolism and Analysis*

Opine catabolism experiments were carried out in AT liquid medium containing 1) 200 mM of nopaline; 2) 200 mM nopaline plus 100 g $L^{-1}$ $(NH_4)_2SO_4$ and 3) 200 mM nopaline, 2 g $L^{-1}$ $(NH_4)_2SO_4$ and 0.5 g $L^{-1}$ yeast extract. The flasks were supplemented with 2 g $L^{-1}$ mannitol. Tubes containing 3 mL of medium were inoculated with 100 μL of the appropriate *Agrobacterium* strain grown overnight at 28°C. Aliquots (0.1 mL) were collected at intervals up to 168 h, centrifuged and analyzed by High Voltage Paper Electrophoresis (HVPE).

*Greenhouse Assay*

*Kalanchoe tubiflora, K. daigremontiana* and *Datura stramonium* plants were used in this experiment. All the assays were performed under adequate light, at 27°Cand 70% air moisture. Three experiments were carried out, each one consisting of 3 plants of each plant species. A control plant was included (the plant was injured, but no bacteria were inoculated).

Isolates of *Agrobacterium* were grown on LB agar medium at 28°Cfor 48 h. Plants were washed with distilled water, then sprayed with 70% ethanol, wounded with a blade and inoculated mechanically in the internodal region. The total period of experimental observation of tumor appearance was established for 60 days.

*In Vitro Assay*

*Daucus carota* roots (carrot) were used in *in vitro* assays. Carrots were washed with 5% sodium hypochlorite (v/v), then sliced and placed in Petri dishes containing 4% agar. Transversal sections of carrots were inoculated with *Agrobacterium* isolates and incubated in a controlled photoperiod room at 27°Cfor 16 h. Each assay was performed in triplicate.

Table 1: List of all *A. tumefaciens* strains used in this study

| Strains | Host source | Geographical origin | Biovar |
|---|---|---|---|
| 305 | *Lactuca sativa* | Mogi das Cruzes-SP | 1 |
| 500 | *Inga fagifolia* | Miguel Pereira-Ceropédica-RJ | 1 |
| 501 | *Inga fagifolia* | Miguel Pereira-Ceropédica-RJ | 1 |
| 523 | *Chrysantemum morifolium* | Itaquera-SP | 3 |
| 709 | *Chrysantemum morifolium* | Holambra-SP | ni* |
| 711 | *Chrysantemum morifolium* | Holambra-SP | 1 |
| 823 | *Rubus idaens* | Ibiúna-SP | 1 |
| 949 | *Rubus idaens* | Caxias do Sul-RS | 1 |
| C58 | Cherry | USA | 1 |
| R10 | Cherry | USA | ni* |

*Not identified

Table 2: Oligonucleotides utilized as primers in the PCR analysis (Ponsonnet and Nesme, 1994)

| Gene | Primers | Oligonucleotide sequence (5-3) |
|---|---|---|
| Chromosomal | FGPS 6 (Forward 16S and IGS) | GGAGAGTTAGATCTTGGCTCAG |
| | FGPS 1509' (Reverse 16S) | AAGGAGGGGATCCAGCCGCA |
| | FGPL 132' (Reverse IGS) | CCGGGTTTCCCCATTCGG |
| Ti Plasmid | FGP *nos* 975 | CATAACGTGCATCATGCATG |
| | FGP *nos* 1236' | CACCATCTCGTCCTTATTGA |
| | FGP *vir* A 2275 | TCAAAAGGCAAGCAAGCAGATCTGG |
| | FGP *vir* $B_2$ 164' | TCAGTGCCGCCACCTGCAGATTG |

## Molecular Characterization

For each strain of *A. tumefaciens*, a small scale genomic DNA extraction method was used (Sambrook *et al*., 1989). To obtain plasmidial DNAs, a small scale DNA extraction method, from the technique of Birnboim and Doly (1979) was used. DNA extraction of tumor (transformed tissues) and healthy tissues of *A. tumefaciens* was prepared according (Mettler, 1989). Oligonucleotides used in this experiment are indicated in Table 2. PCR amplifications were performed according to Normand *et al*. (1992). Each PCR reaction was performed using 100 ng of agrobacterial total DNA for 16S, 23 S, *vir* and *nos* regions. PCR reactions were carried out in a total volume of 50 µL containing: 0.1 µg of genomic DNA (in 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$), 20 µM of each dNTPs, 0.1 µM of each primer and 2.5 U of Taq DNA polymerase (Gibco BRL, Cergy-Pontoise, France). Reaction temperatures and cycle times were: 95°Cfor 3 min 35 cycles at 95°Cfor 1 min, 55°C for 1 min and 72°C for 2 min., with a final step at 72°Cfor 3 min. Five microliter aliquots of the PCR products were visualized on ethidium bromide stained 1% agarose gel. The genomic and plasmidial DNAs of *A. tumefaciens* isolates were sequenced in the automatic sequencer ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems, HITACHI). The samples were prepared for sequencing (about 500 ng of DNA) according to the specifications of kit ABI Prism DNA Sequencing (PE Applied Biosystems). The sequences obtained were analyzed using the program Sequence Navigator (PE Applied Biosystems). Posterior analyses were carried out through Genbank database search using BLAST (NCBI - http://www.ncbi.nlm.nih.gov) to determine sequence similarities. For sequences comparisons the programs Blast 2.0, Align and Clustal W were used.

*Opines Extraction*

The methodology used in this study is based in the direct detection of the presence of opines in the plant tissues (Elkan, 1981). This technique involves the following steps: tissues samples (10 to 300 mg) from healthy plants (control) and from plants with tumor were placed in microtubes. Distilled water was added to the tissues (3 mL $g^{-1}$ of sample), macerated and kept at 100°Cfor 10 min. The macerated tissues were centrifuged at 10,000 × g for 5 min at 28°C. The supernatant was transferred to another microtube and the solution was lyophilized. The extracts were resuspended in distilled water (0.5 mL $mg^{-1}$ of initial sample) and kept at -20°Cfor posterior use.

*Opines Separation and Detection*

HPVE was performed according to Firmin and Fenwick (1978). Extracts of tumor and healthy tissues (6 µL) were applied to chromatographic high quality paper (Whatman 3 mm). Opine (nopaline and octopine) were used as standard. The paper was dried at room temperature and then dived carefully in the buffer (1.1 M acetic acid and 0.7 M formic acid, pH 1.9) and separation was performed in the acrylic electrophoresis vat (60 cm length x 40 cm width). The electrophoresis was performed at 2 kV, 25 mA and 50 Watts for 40 min (50 to 100 V $cm^{-1}$). The paper was primarily examined under UV light (312 nm) in order to visualize all the fluorescent compounds present in the tumor tissues extracts and then by diving the paper in a solution containing 10 mL of 0.1% phenantrenaquinone (in 90% ethanol) and 25 mL of 6 N NaOH, which reacts specifically with opines of monosubstituted guanidines group (Zweig *et al*., 1972). Chromatograms were photographed with Polaroid equipment.

## Results and Discussion

*Assays*

To confirm the pathogenic characteristics of the Brazilian isolates of *A. tumefaciens* and the phenotypic characteristics of the tumors, bioassays at greenhouse and *in vitro* were performed, in which plant species of *Kalanchoe tubiflora*, *K. daigremontiana*, *Datura stramonium* and *Daucus carota* were tested through inoculation in the plant internodal regions with fresh inocula of *A. tumefaciens*. The response to these wounds enabled to characterize the host plant species that could produce tumors in a shorter period of time and to study tumor morphology and the pathogenicity of the bacterial isolates. The morphology and characteristics of the tumors in plant species are presented in Table 3. *Kalanchoe tubiflora* plants were the most effective in plasmid Ti-induced tumor formation. Tumors could be detected as early as 8 days after inoculation with isolate 501, which was the first to induce phenotype changes in the plant. The other *Agrobacterium* isolates induced tumors within 8 to 20 days after inoculation and each one promoted a specific tumor phenotype, ranging from small, medium and big in size, light to dark green in color and some of them showed shoot and root development over the tumors (Fig. 1). An example is the isolate 523 that induced roots in the lower part of the tumors and isolate 823 that induced root in the lower part of the tumor and shoot in the upper part.

In *K. daigremontiana*, tumors could be detected after 12 days of inoculation. In this plant no isolate promoted shoot formation; however isolate 711 induced root formations in the lower part of

Table 3: Morphology and development of Brazilian *Agrobacterium* sp isolates-induced tumors *in vitro* and *in vivo* within marker plants

| Strains | Plant species | Period of tumor appearance (d) | Tumor size (mm) | Wart | Roots | Tumor color |
|---|---|---|---|---|---|---|
| 305 | *K. tubiflora* | 15 | 4 | + | + | Light green |
| | *K. daigremontiana* | 15 | 2 | - | - | Light green |
| | *D. stramonium* | 20 | 8 | - | - | Light green |
| | *D. carota* | 15 | 1 | - | - | Light green |
| 500 | *K. tubiflora* | 9 | 8 | + | - | Light green |
| | *K. daigremontiana* | 18 | 7 | + | - | Light green |
| | *D. stramonium* | 15 | 1 | - | - | Light green |
| | *D. carota* | 15 | 1 | - | - | Light green |
| 501 | *K. tubiflora* | 11 | 8 | + | - | Light green |
| | *K. daigremontiana* | 14 | 4 | - | - | Light green |
| | *D. stramonium* | 17 | 1 | - | - | Light green |
| | *D. carota* | 15 | 7 | - | - | Light green |
| 523 | *K. tubiflora* | 10 | 6 | + | - | Dark green |
| | *K. daigremontiana* | 15 | 3 | + | - | Dark green |
| | *D. stramonium* | 18 | 1 | + | - | Dark green |
| | *D. carota* | 15 | 3 | - | - | Dark green |
| 709 | *K. tubiflora* | 12 | 3 | - | - | Light green |
| | *K. daigremontiana* | 20 | 6 | - | + | Dark green |
| | *D. stramonium* | 15 | 5 | - | - | Light green |
| | *D. carota* | 16 | 7 | - | - | Dark green |
| 711 | *K. tubiflora* | 8 | 5 | - | + | Dark green |
| | *K. daigremontiana* | 18 | 8 | - | - | Dark green |
| | *D. stramonium* | 15 | 6 | - | - | Light green |
| | *D. carota* | 20 | 1 | - | - | Light green |
| 823 | *K. tubiflora* | 20 | 8 | + | + | Light green |
| | *K. daigremontiana* | 17 | 7 | - | + | Dark green |
| | *D. stramonium* | 20 | 7 | - | - | Light green |
| | *D. carota* | 18 | 7 | - | - | Light green |
| 949 | *K. tubiflora* | 11 | 6 | + | - | Light green |
| | *K. daigremontiana* | 12 | 1 | - | - | Light green |
| | *D. stramonium* | 15 | 7 | - | - | Light green |
| | D. carota | 18 | 1 | - | - | Light green |

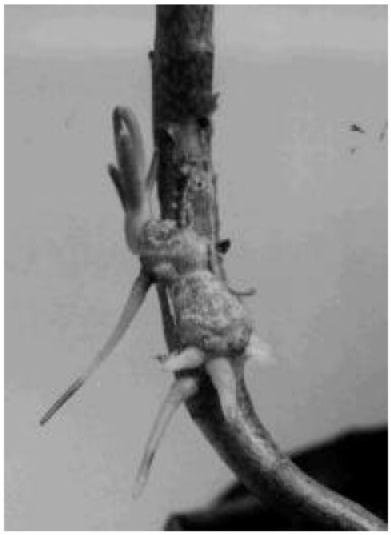

Fig. 1: Tumor induced by strain 501 on *K. tubiflora* 11 days after inoculation

Fig. 2: Tumor induced by strain 711 on K. *daigremontiana* 18 days after inoculation

the tumor (Fig. 2). In *D. stramonium* the isolate 949 induced earlier tumor formation at 11 days after inoculation. Tumor size ranged from very small (isolate 305) to big in size and all of them were light green in color (Fig. 3).

The phenotypic characteristics of each isolate inoculated in all plant species tested were distinct: 80% of *K. daigremontiana* showed phenotypic changes after 40 days of inoculation and tumors necrosis in the superior region was observed. *Datura stramonium* showed sensitivity after 20 days of inoculation, 90% of the plant was necrosed and dead and tumors were less developed (about 2 mm) in comparison with other plants.

*In vitro* tests showed no roots and sprouts appearance in the tumors surfaces. Isolates 305, 500, 501 and 523 induced tumors at approximately 15 days after inoculation and the other isolates induced

Fig. 3: Tumor induced by strain 305 on *D. stramonium* 20 days after inoculation

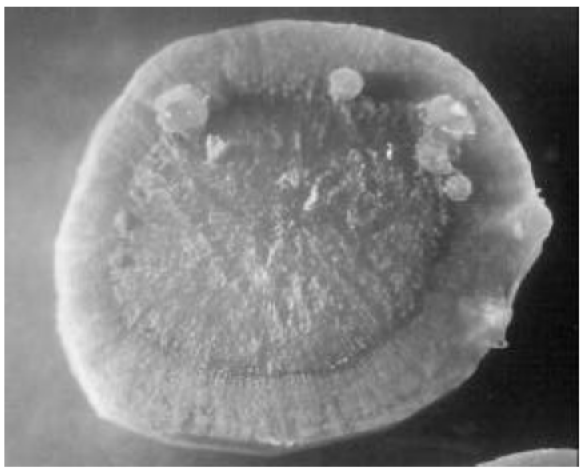

Fig. 4: Tumors induced by strain 711 on *D. carota* 20 days after inoculation

tumors until the 25th day (Table 3). For isolates 709 and 711, morphological characteristics of the tumors induced by these isolates were small in size and dark green in color, while the others were medium in size and light green in color. All isolates were inoculated onto the abaxial face of the carrot slices, due to the auxin flow, which favors tumor formation upon inoculation with *Agrobacterium* isolates (Fig. 4). Through these bioassays in greenhouse and *in vitro* it could be confirmed the pathogenic character of all of the Brazilian isolates of *A. tumefaciens* in all the plant species tested.

*Opines Detection*

In order to classify the Brazilian isolates, studies were conducted to detect opines produced by them in the transformed plant cells. The nature of opines found inside a tumor or in a transformed root, depend on the plasmid type found in the bacterium. This characteristic constitutes the basis of a plasmid classification system in function of its opine type. It was verified through the electrophoretic

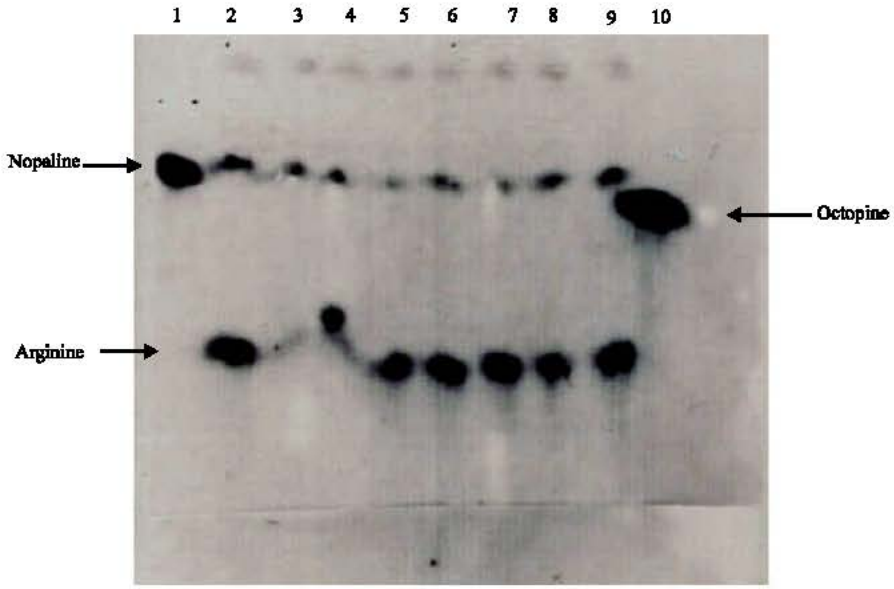


Fig. 5: Paper electrophoresis profile of extracts obtained after opines extraction. Lanes: 1) Nopaline control; 2) Isolate 305; 3) Isolate 500; 4) Isolate 501; 5) Isolate 523; 6) Isolate 709; 7) Isolate 711; 8) Isolate 823; 9) Isolate 949; 10) Octopine control

mobility of these molecules the similarity of them with the nopaline standard. Nopaline was the opine found in all the tumoral tissues extracts promoted by the Ti plasmids found in all agrobacteria tested (Fig. 5). Therefore, the classification of these plasmids can be identified as plasmids Ti of nopaline-type. No opines were detected in the extracts of the tissues of the healthy plants (internodal region).

Each *Agrobacterium* that has a pathogenic plasmid can be characterized by its opine-type. Opines present in the transformed plant cells are degraded by the isolate that induced the disease. The characterization of a specific relationship between the bacterium and opine nature detected in the transformed plant cells provides the base for the understanding of the interaction plant-microorganism, which promotes opines synthesis. A pathogenic *Agrobacterium* can specifically use them as growth substrates, recognizing specific nutrients for its development. However, opines synthesis in the plants cells cause pathogen multiplication and promotes pathogenicity spreading. This mechanism of opines functions is known as the opines concept, initially formulated to the interaction of *A. tumefaciens*-plant and also extended for the interaction of *A. rhizogenes* and for the symbiosis *Rhizobium*-leguminous (Murphy *et al.*, 1987). All the isolates of *A. tumefaciens*, as well as the C58 (positive control) strain were able to growth in the three selective media. The growth in AT+nop medium was favored by sulphate ammonium and yeast extract addition. It could be verified that only strains that carry the plasmid Ti of the nopaline-type uses this substratum to growth, while the isolate R10 (octopine positive) did not grow in none of the tested media (Table 4).

*PCR Analysis of Chromosomal and Plasmidial Regions*

The 16S, IGS and *nos* regions were amplified. In the sequence analyses of ribosomal DNA, the 16S gene appears highly conserved between the same genomic species and they can be used to differentiate species through the IGS region, located between 16S and 23S rDNA region, where the genes are sufficiently variable in certain bacterial species.

PCR amplifications of 16S rDNA regions of the eight *A. tumefaciens* isolates and strain C58 with primers FGPS 6 and FGPS 1509 resulted in only one fragment of about 1,500 bp. Amplifications

Table 4: Uptake of nopaline by all strains tested after growth in the presence or absence of nopaline

| | Growth in liquid culture | | |
|---|---|---|---|
| Strain | AT+nop | AT+nop+ammonium sulfate | AT+nop+ammonium sulfate+yeast extract |
| 305 | + | + | + |
| 500 | + | + | + |
| 501 | + | + | + |
| 523 | + | + | + |
| 709 | + | + | + |
| 711 | + | + | + |
| 823 | + | + | + |
| 949 | + | + | + |
| C58 | + | + | + |
| R10 | - | - | - |

using forward primer of the gene 16S FGPS 6 and reverse primer 23S FGPS 132 resulted in only one fragment of about 2,600 bp for the eight *A. tumefaciens* isolates and strain C58. No amplifications with primers FGP*vir* 2275 and FGP*vir* $B_2$ 164 was detected. The region *vir* of the wild Brazilian isolates does not show similarity with the ones of the standard primers.

Amplifications of *nos* region were performed using plasmidial DNAs of the eight isolates and through DNAs of the tumoral extracts of the transformed plant cells. It was obtained a single fragment of about 256 bp for all of the genomic DNAs and for the tumoral DNAs. The results obtained from the amplification products showed that the T-DNA region that has genes responsible for opines synthesis present in plasmids of *A. tumefaciens* can be verified in the genomic DNA and in the tumoral DNA. These data evidence the presence of the T-DNA region of the Ti plasmid of agrobacteria and also its incorporation in the plant genome.

*Partial Sequence of 16s rDNA Region*

It can be verified through comparisons in databases (NCBI), with other sequences of *A. tumefaciens*, that the isolates of our collection have a similarity in the rDNA 16S region among the isolates 500, 501, 711, 823, 949 and the strain C58. Besides the high similarity among them, it was verified that all of them belong to biovar 1 (Table 1). The analyses of the 16S rDNA regions of *A. tumefaciens* isolates were not enough to classify these isolates in different strains; however, they confirm its classification regarding biovar.

Several studies for the identification of *Agrobacterium* isolates are based in sequences of ribosomal DNA, since these regions can be amplified using specific primers. Undoubtedly, in the DNA sequence analyses, the 16S gene appears highly conserved among the isolates belonging to the same genomic species and can be used to differentiate species. The polymorphism of 16S rDNA region is not enough to classify and to identify rigorously isolates belonging to the same species (Normand *et al*., 1992). Thus, the best amplification occurs in the IGS region, since it has more variable regions. *Agrobacterium* isolates show IGS region ranging from 900 to 1,100 bp as the same as *Rhizobium* sp. When compared to other species such as *Escherichia coli, Frankia* sp and *Nitrobacter* sp, they show a fragment about 440, 411 and 800 bp, respectively (Normand *et al*., 1992). The importance in the size represents an advantage for studies using different DNA regions. Partial analyses of nucleotides sequences of 16S rDNA regions of *A. tumefaciens* isolates allowed the classification of the biovar of the isolates 501, 711, 823 and 949, that were not yet identified and the confirmation of isolate 500 and of C58 as belonging to biovar 1.

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