agronomy

MDPI

Article

In-Depth Characterization of Crown Gall Disease of Tobacco in Serbia

Renata Iličić [1], Aleksandra Jelušić [2], Goran Barać [1], Dušan Nikolić [3], Nemanja Stošić [4], Marco Scortichini [5] and Tatjana Popović Milovanović [6,*]

1 Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia; renatailicic@gmail.com (R.I.); goran.barac@polj.edu.rs (G.B.)
2 Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1, 11030 Belgrade, Serbia; jelusic.aleksandra@gmail.com
3 Institute for Science Application in Agriculture, Bulevar Despota Stefana 68b, 11108 Belgrade, Serbia; dnikolic@ipn.bg.ac.rs
4 Academy of Applied Studies, Dobropoljska 5, 15000 Šabac, Serbia; nemanjastosic.vpssa@gmail.com
5 Research Centre for Olive, Fruit and Citrus Crops, Council for Agronomical Research and Economics (CREA), Via di Fioranello, 52, I-00134 Roma, Italy; marco.scortichini@crea.gov.it
6 Institute for Plant Protection and Environment, Teodora Drajzera 9, 11040 Belgrade, Serbia
* Correspondence: tanjaizbis@gmail.com

Abstract: In August 2020, the unusual appearance of crown gall symptoms was observed on the tobacco plants (hybrid PVH2310) grown in fields in the Golubinci (Srem district, Serbia) locality. The causal agent isolated from galls located on tobacco roots formed circular, convex, and glistening light blue colonies, and then dark to olive-green-colored bacterial colonies on a semi-selective D1 medium. Molecular analysis based on multiplex PCR and multi-locus sequence analysis (MLSA) using concatenated sequences of the *atpD*, *dnaK*, *glnA*, and *rpoB* genes as well as 16S rRNA identified Serbian tobacco isolates such as *Agrobacterium tumefaciens* (biovar 1). Two duplex PCR methods confirmed the presence of the *virD2* and *virC* genes in tobacco isolates. Pathogenicity tests performed on carrot discs and squash fruits resulted in tumor/gall formation after 12 to 16 days post inoculation, respectively. Pathogenicity was also confirmed on tobacco plants, where isolates caused tumor development 21−25 days after inoculation. API 50 CH generated results regarding the biochemical features of the Serbian tobacco isolates. As *A. tumefaciens* (biovar 1) as a cause of tobacco crown gall has previously been documented solely in Japan, there is presently no data on its wider occurrence. Therefore, this first detailed investigation of *A. tumefaciens* isolated from naturally infected tobacco in Serbia will contribute to a better understanding of it at the global level.

Keywords: tobacco; *Agrobacterium*; natural infection; MLSA; pathogenicity

Citation: Iličić, R.; Jelušić, A.; Barać, G.; Nikolić, D.; Stošić, N.; Scortichini, M.; Milovanović, T.P. In-Depth Characterization of Crown Gall Disease of Tobacco in Serbia. *Agronomy* **2024**, *14*, 851. https://doi.org/10.3390/agronomy14040851

Academic Editors: Baoshan Chen and Yizhen Deng

Received: 15 March 2024
Revised: 12 April 2024
Accepted: 15 April 2024
Published: 19 April 2024

1. Introduction

Tobacco (*Nicotiana tabacum* L.) is considered to have originated from North and South America and has subsequently been introduced for cultivation in other parts of the globe. It is well represented in South America, Mesoamerica and the West Indies, as well as in the Pacific Islands, Asia, and Europe [1]. In 2023, 5.145 ha of land was under tobacco production in Serbia, with an average yield of 1.3 t/ha. Considered an industrial plant in this country, tobacco of the Virginia, Burley, and Oriental type is mostly grown [2].

Plant pathogenic bacteria from the genus *Agrobacterium* cause crown gall disease, which manifests as tumors and hairy root disease in a variety of crops, including those of high economic importance [3]. In addition to tumor formation, it may lead to symptoms such as dwarfing and the development of small and chlorotic leaves, which ultimately cause plants to wither and die [4].

Agrobacterium tumefaciens, a soil-borne, Gram-negative plant pathogenic bacterium, causes disease symptoms in a variety of plant species by integrating a transfer DNA

(T-DNA) from a tumor-inducing plasmid into the host genome [5,6]. The genes required for T-DNA processing and transfer are located in a virulence region with several *vir* operons (*virA-H*) on pTi [7]. The *A. tumefaciens* host range is extensive, and this bacterium has been reported to cause crown gall disease in more than 600 plants belonging to 331 genera [8]. *Agrobacterium* strains have been divided into three biovars based on their metabolic and growth characteristics: *A. tumefaciens* (biovar 1-bv1), *A. rhizogenes* (biovar 2-bv2), and *A. vitis* (biovar 3-bv3) [5]. Later, these were considered different genera/species in the *Rhizobiaceae* family, and were named as follows: *Agrobacterium* species complex (bv1), *Rhizobium rhizogenes* (bv2), and *Allorhizobium vitis* (bv3) [9]. At present, the *A. tumefaciens* species complex has been subdivided into multiple genomospecies with different numerical identifiers [10–16].

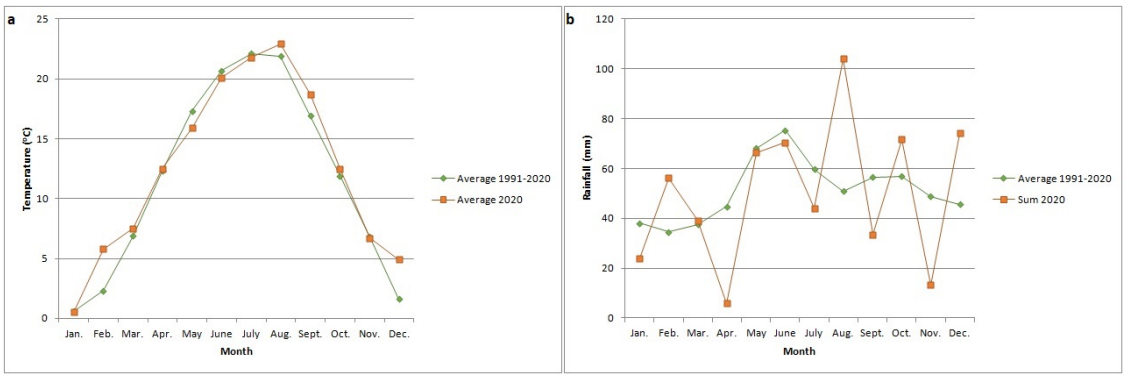
In tobacco, crown gall disease was first found on the roots of crops grown in a field in Kawai-mura, Iwate Prefecture, Japan, in 1995, and *A. tumefaciens* (bv1) was determined as the causative agent [17]. Since then, the disease has continued to occur in tobacco grown in the same field. On the other hand, the occurrence of this bacterium has not been reported on tobacco in any other country.

In Serbia, symptoms of crown gall disease on tobacco roots were observed in 2020 for the first time. Therefore, the aim of the present study was to determine the genotypic and phenotypic features of Serbian tobacco isolates obtained from infected plants in the field, thereby expanding the current knowledge of the causal agents closely related to *Agrobacterium* as a tobacco pathogen.

2. Materials and Methods

2.1. Sample Collection and Bacterial Isolation

In 2020, crown gall symptoms were observed on the roots of tobacco plants (hybrid PVH2310, ProfiGen do Brasil Ltd.a., Santa Cruz do Sul—RS, Brazil) grown in the Golubinci (Srem district, Serbia) production fields. Meteorological data were taken from the nearest meteorological station, Sremska Mitrovica, distanced 35 km from the sampling location. The average values of the three-decade air temperatures during the tobacco vegetation period (May–October) were similar to the annual average temperature, with the highest deviation being 1.4 °C in May (Scheme 1a). The total precipitation was lower in July and September, and higher in August and October compared to the perennial average for the observed tobacco vegetation period. Double the perennial average of precipitation was recorded in August (Scheme 1b).



Scheme 1. Meteorological data for sampling site: (**a**) Average monthly temperatures recorded for the period 1991–2020 and average monthly temperature in 2020; (**b**) Average monthly sum of precipitation recorded for the period 1991–2020 and the average sum of monthly precipitation in 2020.

Seven samples, each consisting of one tobacco plant with crown gall symptoms, were collected from the field for analysis. Prior to the bacterial isolation, tumors were rinsed under tap water, dried on filter paper, and treated with a commercial 10% bleach solution for 3−5 min; they were subsequently washed with sterilized distilled water (SDW). After

repeating this entire procedure, small fragments taken from the interior of the tumor tissues were macerated in SDW, plated onto semi-selective D1 medium [18] 60 min later and incubated at 26 °C for 10 days. All selected isolates were stored in Luria Bertani (LB) broth containing 20% (*v*/*v*) glycerol at a temperature of −20 °C.

In all tests and reactions, the reference strain C58–*A. tumefaciens* (bv1), currently *A. fabrum*, G8 [12,16], was used.

2.2. Molecular Characterization

2.2.1. DNA Isolation

Using a genomic DNA isolation kit (DNeasy Plant Mini Kit, Qiagen GmbH, Hilden, Germany), the genomic DNA of the tested Serbian isolates was extracted from cultures grown on Nutrient Agar (NA) for 48 h, following the manufacturer's instructions. The DNA was resuspended in 100 µL of TE buffer and long-term stored at −20 °C.

2.2.2. Multiplex PCR Detection of Agrobacterium Species/Biovars

As shown in Table 1, the DNA of each bacterial isolate was tested by conducting multiplex PCR, with UF + B1R + B2R + AvR + ArR serving as primers targeting 23S rRNA, in line with the approach described by [19]. PCR was performed using 25 µL of DreamTaq Green master mix (Thermo Scientific, Vilnius, Lithuania), 1 µL of bacterial DNA as a template, and 100 pM of appropriate primer. The amplified PCR products were visualized by gel electrophoresis on 1.5% agarose gel stained with Midori Green Advance under UV light. The fragment sizes were estimated in relation to the GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific™).

Table 1. Primers used in this study.

Primer	Primer Sequence	Region	Fragment Length (bp)
UF/ B1R	5′-GTAAGAAGCGAACGCAGGGAACT-3′ 5′-GACAATGACTGTTCTACGCGTAA-3′	Chromosomal (23S rRNA) gene, *A. tumefaciens*/biovar 1	184
UF/ B2R	5′-TCCGATACCTCCAGGGCCCCTCACA-3′	Chromosomal (23S rRNA) gene, *A. rhizogenes*/biovar 2	1066
UF/ AvR	5′-AACTAACTCAATCGCGCTATTAAC-3′	Chromosomal (23S rRNA) gene, *A. vitis*	478
UF/ ArR	5′-AAAACAGCCACTACGACTGTCTT-3′	Chromosomal (23S rRNA) gene, *A. rubi*	1006
A/ C	5′-ATGCCCGATCGAGCTCAAGT-3′ 5′-TCGTCTGGCTGACTTTCGTCATAA-3′	Ti and Ri plasmid *virD2* gene	224
CYT/ CYT	5′-GATCG(G/C)GTCCAATG(C/T)TGT-3′ 5′-GATATCCATCGATC(T/C)CTT-3′	Ti plasmid *ipt* gene	427
VCF3/ VCR3	5′-GGCGGGCGYGCYGAAAGRAARACYT-3′ 5′-AAGAACGYGGNATGTTGCATCTYAC-3′	Ti and Ri plasmid *virC* gene	414
PGF/ PGR	5′-GGGGCAGGATGCGTTTTTGAG-3′ 5′-GACGGCACTGGGGCTAAGGAT-3′	Chromosomal *pehA* gene, *A. vitis*	466
atpD (800F/ 1350R)	5′-GGCCAGGACGTTCTGTTCTT-3′ 5′-CTTGAAGCCCTTGATCGTGT-3′	F0-F1 ATP synthase subunit beta	465
dnaK (720F/ 1400R)	5′-GAAGACTTCGACATGCGTCT-3′ 5′-GCCGAGCAGCTTGTTGTC-3′	Heat shock protein, 70 kDa	480
glnA (144F/ 900R)	5′-GTCATGTTCGACGGCTCCT-3′ 5′-CCTTGGCATGCTTGATGAT-3′	Glutamine synthetase	474
rpoB (2040F/ 2718R)	5′-GAAAACGACGACGCCAAC-3′ 5′-GCGCAGAAGCTTTTCTTCC-3′	Beta subunit RNA polymerase	534
27F 1495R	5′-GAGAGTTTGATCCTGGCTCAG-3′ 5′-CTACGGCTACCTTGTTACGA-3′	16S rRNA	1550

2.2.3. Detection of Virulence Genes

Two duplex PCR analyses were carried out using the A/C (*virD2*) and CYT/CYT (*ipt*) primers [20] for the first reaction, and PGF/PGR (*pehA*) [21] and VCF3/VCR3 (*virC*) [22] primers for the second reaction (Table 1), adopting the amplification conditions outlined by Kuzmanović et al. [23]. The PCR products were separated by electrophoresis using 2% agarose gel, stained with Midori Green Advance. The fragment sizes were assessed by comparing them to the GeneRuler 100 bp DNA Ladder (Thermo Scientific™).

2.2.4. Multi-Locus Sequence Analysis (MLSA)

As shown in Table 1, multi-locus sequence analysis (MLSA) was performed based on the sequences of four housekeeping genes—*atpD*, *dnaK*, *glnA*, and *rpoB* [24]—as well as with partial sequences of 16S rRNA [25].

The PCR mix (25 µL) consisted of 12.5 µL of Color OptiTaq PCR Master Mix (2×), 9.5 µL of ultrapure DNases/RNase-free water, 1 µL of each primer (10 µM), and 1 µL of DNA. The following protocol was adopted for PCR amplifications: initial denaturation at 95 °C for 10 min (16S rRNA) or 3 min (*atpD*, *dnaK*, *glnA*, and *rpoB*), followed by 30 (16S rRNA) or 35 (*atpD*, *dnaK*, *glnA*, and *rpoB*) cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 90 s (16S rRNA) or at 60 °C (*atpD*), 65 °C (*dnaK* and *glnA*) and 68 °C (*rpoB*) for 1 min, extension at 72 °C for 150 s (16S rRNA, *dnaK*, *glnA*, and *rpoB*) or 90s (*atpD*), and a final extension step at 72 °C for 10 min.

The obtained PCR products were sent for sequencing to Eurofins Genomics, located in Hamburg (Germany). Upon receipt, all sequences were manually checked for quality using BioEdit software v.7.2.

The Basic Local Alignment Search Tool (BLASTn) available in the National Center for Biotechnology Information (NCBI) database was used for the preliminary identification of Serbian tobacco isolates based on the sequenced genes. Further, we performed phylogenetic analysis to finally identify and compare Serbian tobacco isolates with 15 different *Agrobacterium* spp. from the NCBI (Table 2). Concatenated sequences (3452 nt) of each of the five sequenced genes were used to construct a neighbor-joining (NJ) phylogenetic tree. The ClustalW multiple alignment tool from BioEdit v.7.2 was used to align sequences. The phylogenetic tree was generated in MegaX software version 10.0.5 using the Kimura two-parameter model [26]. The *Mesorhizobium huakuii* strain NZP2235 served as an out-group (Table 2) for rooting the tree. The sequences of the tobacco isolates were deposited in the NCBI database and the accession numbers were obtained.

Table 2. List of strains from the NCBI database used for phylogenetic analysis.

Species	Strain	Isolation Source	Country	Acc. No.
A. tumefaciens	HAMBI 105	soil	USA	CP139997
	Gle002	walnut	USA: California	CP048564
	Yol001	walnut	USA: California	CP048477
	Yol002	walnut	USA: California	CP048473
	12D1	-	-	CP033031
	183	almond	Tunisia	CP029044
	O54/95	cherry	-	CP124967
	BIM B-1315G	root endosphere of soybean	Belarus: Minsk	CP061003
A. larrymoorei	AF3.44	*Ficus benjamina*	USA: Florida	CP072167
	CFBP5477	-	Italy	CP124733
A. leguminum	CFBP4996	-	-	CP120211
A. fabrum	C58		USA	AE007869
	1D132	-		CP033022

Table 2. *Cont.*

Species	Strain	Isolation Source	Country	Acc. No.
A. vaccinii	B7.6	blueberry	Poland	CP054150
A. pusense	76	hyphae *Fusarium oxysporum* f. sp. *cucumerinum*	China: Beijing	CP053856
M. huakuii [a]	NZP2235	*Lotus japonicus*	New Zealand	CP139858

[a] Outgroup strain.

2.3. Phenotypic Characterization

2.3.1. Pathogenicity

The pathogenicity of the isolates was evaluated on carrot (*Daucas carota* L.) disc and squash (*Cucurbita pepo* var. *italica* L.) fruits. The tested isolates grown on NA at 28 °C for 48 h were suspended in SDW to a final concentration of 10^7 CFU mL^{-1}. SDW served as a negative control treatment. The carrot and squash fruits were washed, dried, and then disinfected with 70% ethanol before being artificially inoculated in line with the methods described by Ryder et al. [27] and Tolba and Soliman [28], respectively. The inoculated samples were stored in plastic boxes on filter paper at 25 °C for three weeks. Tumor formation was considered a positive reaction.

Selected Serbian tobacco isolates were also tested for pathogenicity to *N. tabacum* hybrid PVH2310. A bacterial suspension (at a final concentration of 10^8 CFU mL^{-1}) was prepared from cultures grown on NA at 28 °C for 48 h. The stems of 1-month-old tobacco plants grown in an air-conditioned greenhouse at a constant temperature of 28 °C were inoculated by the needle prick method. Three plants were inoculated with each strain, and the experiment was repeated twice. Upon symptom development, pathogenicity was evaluated based on tumor formation at the inoculation site [17]. Reisolations were performed on D1 medium as soon as symptoms were observed, and the congruence between the reisolates and the original isolates was assessed by multiplex PCR, according to the protocol described by Puławska et al. [6].

2.3.2. Biochemical Tests

The biochemical characterization of the Serbian tobacco isolates was performed according to conventional bacteriological methods described by Schaad et al. [29]. Additional tests were performed using the API 50 CH (bioMérieux, France), under the conditions indicated by the manufacturer, and the results were read after 72 to 96 h of incubation at 26 °C. The tested bacterial isolates were suspended in API 50 CHB/E medium (bioMérieux, Craponne, France), following the manufacturer's instructions.

3. Results

3.1. Symptoms and Bacterial Isolation

In August 2020, the appearance of crown gall symptoms was observed on the tobacco plants grown at the Golubinci production site. The diseased plants exhibited a lack of vigor, stunted growth, and a reduction in foliage. On the root system, the crown gall symptoms included round, white and soft tumors of different sizes (Figure 1a). In all cases, typical *Agrobacterium* circular, convex, glistening colonies with a characteristic light blue, and then dark to olive green color, were formed on D1 medium after 10 days of incubation (Figure 1b). Seven pure isolates (coded as DA5, DA17, DA21, DA34, DA40, DA52, and DA65) were selected for further testing.

Figure 1. *A. tumefaciens*. (**a**) Symptoms of naturally infected tobacco plants; (**b**) colonies on D1 medium 10 days after incubation.

3.2. Molecular Characterization

3.2.1. Multiplex PCR Detection of Agrobacterium Species/Biovars

In a multiplex PCR (using primers UF + B1R + B2R + AvR + ArR) assay targeting 23S rRNA gene sequences, a 184 bp fragment specific for *A. tumefaciens* (bv1) was amplified in all seven studied tobacco isolates. The C58 control strain yielded amplification products at the expected position of 184 bp (Figure 2a).

3.2.2. Detection of Virulence Genes

Among the four primer pairs (A/C and CYT/CYT; PGF/PGR and VCF3/VCR3) used in the pathogenicity evaluation, A/C′ and VCF3/VCR3 successfully amplified the fragment of the expected length (224 bp and 414 bp, respectively) in the tobacco isolates (Figure 2b,c). At the same time, a positive PCR reaction in the reference strain (C58) was observed for the primer pairs A/C′, CYT/CYT′, and VCF3/VCR3. Using the PGF/PGR primer pair, the gene for polygalacturonase (*pehA*) was not detected in any of the tobacco isolates, along with reference strain C58, since it is characteristic of *A. vitis*.

3.2.3. Multi-locus Sequence Analysis (MLSA)

According to the BLASTn analysis, seven Serbian tobacco isolates were preliminarily identified as *A. tumefaciens*, exhibiting the highest homology (99.84–100%) with *A. tumefaciens* strains originating from different hosts/countries (CFBP 5621, HAMBI 105, Gle002, Yol001, Yol002) for the *atpD*, *dnaK*, *glnA* and *rpoB* genes. According to the BLASTn analysis of 16S rRNA sequences, the tobacco isolates showed the highest homology (100%) with the *A. tumefaciens* strains obtained from the NCBI database (CFBP6623, CFBP6624, Yol001, Yol002, Gle002, HAMBI 105, 12D1, AR2, 186, 0176, 1D1460, 183, 175-HR29, etc.).

According to the constructed neighbor-joining phylogenetic tree shown in Figure 3, the seven Serbian tobacco isolates (DA5, DA17, DA21, DA34, DA40, DA52, and DA65) formed a homogeneous group and clustered with the HAMBI 105, Gle002, Yol001, and

Yol002 reference *A. tumefaciens* strains. The remaining four comparative *A. tumefaciens* strains (BIM B-1315G, 12D1, 183, and O54/95) formed a separate subcluster within the same cluster. Other comparative strains from the NCBI database (Table 2) were placed separately within different clusters, each corresponding to different *Agrobacterium* species (i.e., *A. pusense*, *A. leguminum*, *A. fabrum*, *A. vaccinii*, and *A. larrymoorei*). An outgroup strain, *R. rhizogenes* LBA9402, was clearly separated on a monophyletic tree branch. As all tested tobacco isolates were shown to be genetically homogenous, only one (DA5) was randomly selected and deposited into the NCBI database under the accession numbers PP471554 (*atpD*), PP471555 (*dnaK*), PP471556 (*glnA*), PP471557 (*rpoB*), and PP464228 (16S rRNA).

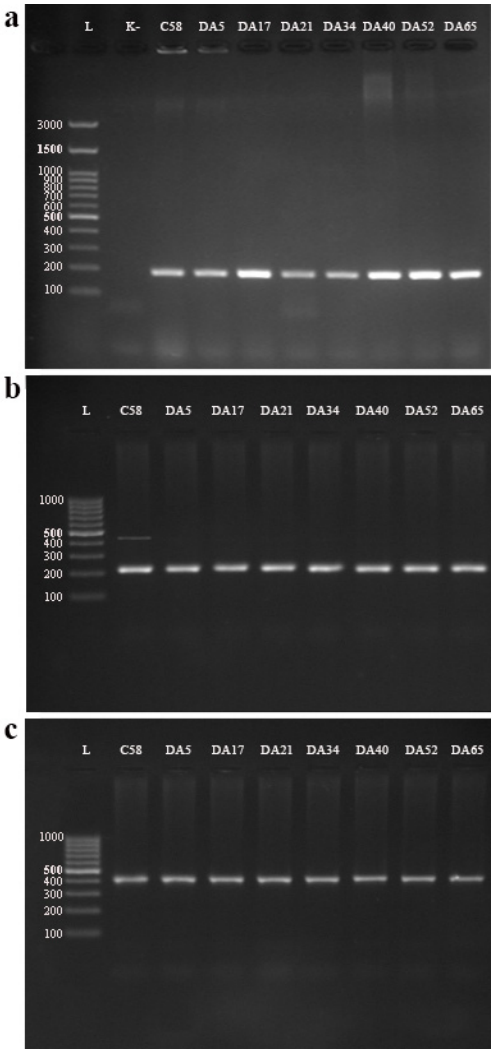


Figure 2. (**a**) Multiplex-PCR detection of *Agrobacterium* species/biovars using primers UF + B1R + B2R + AvR + ArR; (**b**) PCR detection of virulence genes *virD2* and *ipt*, using primers A/C and CYT/CYT, respectively; (**c**) PCR detection of virulence genes *virC* and *pehA*, using primers VCF3/VCR3 and PGF/PGR, respectively. Letter "L" represents DNA ladder—(**a**) GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific™) and (**b**,**c**) GeneRuler 100 bp DNA Ladder (Thermo Scientific™).

Figure 3. Neighbor-joining phylogenetic tree based on the concatenated sequences of 16S rRNA and the housekeeping genes *atpD*, *dnaK*, *glnA*, and *rpoB* for the seven Serbian tobacco isolates obtained in this study (marked with red triangles) and the 15 strains of different *Agrobacterium* spp. sourced from the NCBI database. The tree was rooted with *M. huakuii* NZP2235 from the NCBI.

3.3. Phenotypic Characterization of Tobacco Isolates

3.3.1. Pathogenicity

In the tumorigenicity tests, all tobacco isolates, along with reference strain C58, on the carrot discs and squash fruits were positive 12 to 16 days after inoculation. Positive responses were indicated by the formation of differently sized and shaped tumors/galls on the inoculated carrot discs (Figure 4a), as well as on the squash fruit (Figure 4b). Negative controls were symptomless.

In the pathogenicity assay involving tobacco plants, symptoms associated with crown gall disease emerged 21 to 25 days after inoculation, as indicated by the formation of galls with different sizes at the inoculation site (Figure 4c). No symptoms were observed on the plants that were inoculated with SDW. To fully adhere to Koch's postulates, the pathogen was reisolated from young tumor tissue on D1 medium and was identified by the amplification of a 184 bp fragment via multiplex PCR.

Figure 4. *A. tumefaciens*. Pathogenicity of isolate DA21 on: (**a**) carrot discs; (**b**) squash fruit; (**c**) tobacco plant.

3.3.2. Biochemical Tests

The biochemical test results showed that the isolates were Gram negative, catalase positive, and oxidase negative, and did not produce fluorescent pigment on King's B medium. In the API test (API 50 CH), the *Agrobacterium* tobacco isolates were positive for the fermentation of glycerol, d-arabinose, l-arabinose, d-ribose, d-xylose, d-adonitol, methyl-β-d-xylopyranoside, d-galactose, d-glucose, d-fructose, d-mannose, l-rhamnose, dulcitol, inositol, d-mannitol, d-sorbitol, aesculin ferric citrate, salicin, d-cellobiose, d-maltose, d-lactose, d-melibiose, d-sucrose, d-trehalose, d-raffinose, d-turanose, d-lyxose, d-tagatose, d-fucose, l-fucose, d-arabitol, and l-arabitol, while being negative for erythritol, l-xylose, l-sorbose, methyl α-d-mannopyranoside, methyl α-d-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, inulin, d-melezitose, amidon (starch), glycogen, xylitol, gentiobiose, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate. The API test results for strain C58 differed only in glycerol (−), methyl-β-d-xylopyranoside (−), d-galactose (−), l-sorbose (+), d-fucose (−), l-fucose (−) and d-arabitol (−) fermentation (Table 3).

Table 3. Biochemical characteristics of the Serbian tobacco isolates.

	Isolate							
Test	**DA5**	**DA17**	**DA21**	**DA34**	**DA40**	**DA52**	**DA65**	**C58**
Gram reaction	−	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+
Oxidase	−	−	−	−	−	−	−	+
Production of fluorescent pigment	−	−	−	−	−	−	−	−
Fermentation of:								
glycerol	+	+	+	+	+	+	+	−
d-arabinose	+	+	+	+	+	+	+	+
l-arabinose	+	+	+	+	+	+	+	+
d-ribose	+	+	+	+	+	+	+	+

Table 3. *Cont.*

	Isolate							
Test	**DA5**	**DA17**	**DA21**	**DA34**	**DA40**	**DA52**	**DA65**	**C58**
d-xylose	+	+	+	+	+	+	+	+
d-adonitol	+	+	+	+	+	+	+	+
methyl-β-d-xylopyranoside	+	+	+	+	+	+	+	−
d-galactose	+	+	+	+	+	+	+	−
d-glucose	+	+	+	+	+	+	+	+
d-fructose	+	+	+	+	+	+	+	+
d-mannose	+	+	+	+	+	+	+	+
l-rhamnose	+	+	+	+	+	+	+	+
dulcitol	+	+	+	+	+	+	+	+
inositol	+	+	+	+	+	+	+	+
d-mannitol	+	+	+	+	+	+	+	+
d-sorbitol	+	+	+	+	+	+	+	+
aesculin ferric citrate	+	+	+	+	+	+	+	+
salicin	+	+	+	+	+	+	+	+
d-cellobiose	+	+	+	+	+	+	+	+
d-maltose	+	+	+	+	+	+	+	+
d-lactose	+	+	+	+	+	+	+	+
d-melibiose	+	+	+	+	+	+	+	+
d-sucrose	+	+	+	+	+	+	+	+
d-trehalose	+	+	+	+	+	+	+	+
d-raffinose	+	+	+	+	+	+	+	+
d-turanose	+	+	+	+	+	+	+	+
d-lyxose	+	+	+	+	+	+	+	+
d-tagatose	+	+	+	+	+	+	+	+
d-fucose	+	+	+	+	+	+	+	−
l-fucose	+	+	+	+	+	+	+	−
d-arabitol	+	+	+	+	+	+	+	−
l-arabitol	+	+	+	+	+	+	+	+
erythritol	−	−	−	−	−	−	−	−
l-xylose	−	−	−	−	−	−	−	−
l-sorbose	−	−	−	−	−	−	−	+
methyl α-d-mannopyranoside	−	−	−	−	−	−	−	−
methyl α-d-glucopyranoside	−	−	−	−	−	−	−	−
N-acetylglucosamine	−	−	−	−	−	−	−	−
amygdalin	−	−	−	−	−	−	−	−
arbutin	−	−	−	−	−	−	−	−
inulin	−	−	−	−	−	−	−	−
d-melezitose	−	−	−	−	−	−	−	−
amidon (starch)	−	−	−	−	−	−	−	−
glycogen	−	−	−	−	−	−	−	−
xylitol	−	−	−	−	−	−	−	−
gentiobiose	−	−	−	−	−	−	−	−
potassium gluconate	−	−	−	−	−	−	−	−
potassium 2-ketogluconate	−	−	−	−	−	−	−	−
potassium 5-ketogluconate	−	−	−	−	−	−	−	−

Legend: +: positive; −: negative.

4. Discussion

The Gram-negative soil bacterium *A. tumefaciens* is widely distributed globally and is known to induce crown gall disease on various plants, including fruit trees as well as ornamental and herbaceous plants across different regions [3,13,30,31]. The present study marks the first attempt to examine *A. tumefaciens* as the causative agent of tobacco crown gall in Serbia. Thus far, this bacterial pathogen in tobacco fields has only been observed in Japan, with the first report dating back to 1995 [17]. Thus, by offering a more comprehensive understanding of tobacco isolates through genotyping, the present study

fills an important gap in the extant knowledge regarding this pathogen's interaction with tobacco. Although the relationship between tobacco plants and the bacterium *A. tumefaciens* is widely recognized and utilized as a model system for studying pathogenicity and virulence under controlled conditions, natural infections of tobacco by this bacterium have not garnered sufficient scholarly attention.

Based on the phylogenetic analysis performed as a part of the present study, the Serbian tobacco isolates exhibited the closest association with *A. tumefaciens* strains originating from soil and walnut trees in the USA. This finding may indicate a common evolutionary history, a pathway for dissemination, or adaptation across different geographical regions. Genetic diversity was not observed among the *A. tumefaciens* tobacco isolates from Serbia based on the genes used for sequencing. In the research conducted by Furuya et al. [17], tobacco isolates were solely identified through PCR, utilizing the universal primers VCF/VCR for detecting pathogenic *Agrobacterium* spp. It is noteworthy that there are no publicly accessible genomic data on isolates from tobacco when studied as an *A. tumefaciens* host; therefore, this study provides pioneering genomic information regarding *A. tumefaciens* strains originating from tobacco.

According to Aujoulat et al. [24], the MLSA scheme, using seven housekeeping genes encoding proteins involved in transcription (*rpoB*), the stress response (*dnaK*, *groEL*), amino acid biosynthesis (*glnA*, *trpE*), and energy metabolism (*atpD*, *zwf*), is suitable for analyzing strains belonging to the three biovars in the *Agrobacterium* genus—bv1, bv2, and *A. vitis* (bv3). In this study, we provided sequences from 16S rRNA with gene sets used in MLSA (*rpoB*, *dnaK*, *glnA*, *atpD*). Considering that the phylogenetic analysis clearly clustered *A. tumefaciens* in a clade separated from other *Agrobacterium* spp., the MLSA scheme adopted in our work displayed good discriminatory power for *Agrobacterium* phylogeny. On the other hand, taxon-specific sets of genes for the MLSA of taxa with gall-causing bacteria were identified by consulting the pertinent literature [11,32–35].

Furthermore, using a set of specific primers [19], the tobacco isolates were identified at the species/biovar level (*A. tumefaciens*, bv1), indicating that these tobacco isolates were members of this species. Two duplex PCR methods conducted with four different primer pairs confirmed the presence of the plasmid *virD2* and *virC* pathogenicity gene, indicating the presence of Ti or Ri plasmids in the tested isolates [16,22,23]. Kuzmanović et al. [36] stated that VCF3/VCR3 primers (targeting *virC1-virC2* gene) are applicable in a wide variety of tumorigenic agrobacteria and are therefore considered a reliable tool for detecting crown gall pathogens. The *VirD2* gene plays an important role in T-DNA processing and transfer, which is important for virulence [37].

Serbian tobacco isolates displayed phenotypic characteristics consistent with those of *A. tumefaciens* [17]. All isolates induced tumors on carrot discs, squash fruits, and young tobacco plants following artificial inoculation. Likewise, in the study conducted by Furuya et al. [17], all tested *Nicotiana* species were susceptible to the *A. tumefaciens* isolated from naturally infected tobacco plants. Based on their biochemical characteristics, the Serbian tobacco isolates most closely resembled *A. tumefaciens*, but the biovar phenotype features differed in some cases [29]. Strains with intermediate/atypical biochemical characters have been frequently reported, suggesting that more accurate additional tests are needed for precise phenotypic identification [38,39]. Regarding the results yielded by pathogenicity and biochemical tests, the molecular phylogenies of housekeeping genes and the determined presence of pathogenicity genes, the Serbian tobacco isolates were identified as *A. tumefaciens*.

The ease of *Agrobacterium* spread through tobacco transplants presents a latent threat not only to tobacco crops, but also to overall agricultural production. Due to the difficulty of controlling crown gall disease, this study highlights the importance of early and accurate detection for mitigating crop damage.

5. Conclusions

In conclusion, the results obtained in the present study provide novel insight into the taxonomic position of *Agrobacterium* isolates associated with tobacco, enhancing the current understanding of the *Agrobacterium* population in Serbia. To reduce the risk of significant crop losses resulting from crown gall infection, rigorous surveys are advised, especially in regions experiencing frequent outbreaks of economic significance (in this particular case, crucial tobacco plantations grown in monoculture).

Author Contributions: Conceptualization, R.I., M.S. and T.P.M.; methodology, R.I., A.J., G.B. and T.P.M.; software, A.J. and N.S.; validation, R.I., A.J., G.B. and T.P.M.; formal analysis, R.I., A.J., G.B. and T.P.M.; investigation, R.I., A.J., G.B. and T.P.M.; resources, R.I., D.N. and T.P.M.; data curation, R.I., A.J., G.B. and T.P.M.; writing—original draft preparation, R.I., A.J., G.B., D.N., N.S., M.S. and T.P.M.; writing—review and editing, R.I., A.J., G.B., M.S. and T.P.M.; visualization, R.I., A.J., G.B., D.N., N.S., M.S. and T.P.M.; supervision, R.I., A.J., M.S. and T.P.M.; funding acquisition, R.I., D.N. and T.P.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia [contract numbers 451-03-66/2024-03/200117, 451-03-66/2024-03/200053, 451-03-66/2024-03/200010].

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. CABI. *Nicotiana tabacum* (tobacco). 2014. Available online: https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.36326 (accessed on 10 December 2023).
2. SYRS. *Statistical Yearbook of the Republic of Serbia*; Statistical Office of the Republic of Serbia: Belgrade, Serbia, 2023.
3. Hooykaas, M.J.G.; Hooykaas, P.J.J. Complete genomic sequence and phylogenomics analysis of *Agrobacterium* strain AB2/73: A new *Rhizobium* species with a unique mega-Ti plasmid. *BMC Microbiol.* **2021**, *21*, 295. [CrossRef] [PubMed]
4. Tekiner, N.; Kotan, R. Pathogenicity of Different *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) Isolates and Their Diagnosis with Classical Methods. *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Derg.* **2022**, *25*, 149–157. [CrossRef]
5. Portier, P.; Fischer-Le Saux, M.; Mougel, C.; Lerondelle, C.; Chapulliot, D.; Thioulouse, J.; Nesme, X. Identification of genomic species in *Agrobacterium* biovar 1 by AFLP genomic markers. *Appl. Environ. Microbiol.* **2006**, *72*, 7123–7131. [CrossRef]
6. Tiwari, M.; Mishra, A.K.; Chakrabarty, D. *Agrobacterium*-mediated gene transfer: Recent advancements and layered immunity in plants. *Planta* **2022**, *256*, 37. [CrossRef]
7. Nabi, N.; Hafsa, A.B.; Zellama, M.S.; Chaouachi, M. Pathogenicity, Phylogenetic relationship and NGS based identifcation and assembly of tumorigenic *Agrobacterium radiabacter* plasmidic and chromosomic reads isolated from *Prunus duclcis*. *Genomics* **2019**, *111*, 1423–1430. [CrossRef] [PubMed]
8. Kumar, N.; Vijayanand, K.G.; Reddy, M.P.; Singh, A.S.; Naraynan, S. *Agrobacterium tumefaciens*-Mediated Genetic Transformation: Mechanism and Factors. *J. For. Environ. Sci.* **2009**, *25*, 195–204.
9. Hooykaas, P.J. The Ti plasmid, driver of *Agrobacterium* pathogenesis. *Phytopathology* **2023**, *113*, 594–604. [CrossRef]
10. Mougel, C.; Thioulouse, J.; Perrie're, G.; Nesme, X. A mathematical method for determining genome divergence and species delineation using AFLP. *Int. J. Syst. Evol. Microbiol.* **2002**, *52*, 573–586. [CrossRef]
11. Costechareyre, D.; Bertolla, F.; Nesme, X. Homologous recombination in *Agrobacterium*: Potential implications for the genomic species concept in bacteria. *Mol. Biol. Evol.* **2009**, *26*, 167–176. [CrossRef]
12. Lassalle, F.; Campillo, T.; Vial, L.; Baude, J.; Costechareyre, D.; Chapulliot, D.; Shams, M.; Abrouk, D.; Lavirre, C.; Oger-Desfeux, C.; et al. Genomic species are ecological species as revealed by comparative genomics in *Agrobacterium tumefaciens*. *Genome Biol. Evol.* **2011**, *3*, 762–781. [CrossRef]
13. Mafakheri, H.; Taghavi, S.M.; Puławska, J.; De Lajudie, P.; Lassalle, F.; Osdaghi, E. Two novel genomospecies in the *Agrobacterium tumefaciens* species complex associated with rose crown gall. *Phytopathology* **2019**, *109*, 1859–1868. [CrossRef] [PubMed]
14. Flores-Félix, J.D.; Menéndez, E.; Peix, A.; García-Fraile, P.; Velázquez, E. History and current taxonomic status of genus *Agrobacterium*. *Syst. Appl. Microbiol.* **2020**, *43*, 126046. [CrossRef]
15. Singh, N.K.; Lavire, C.; Nesme, J.; Vial, L.; Nesme, X.; Mason, C.E.; Lassalle, F.; Venkateswaran, K. Comparative Genomics of Novel *Agrobacterium* G3 Strains Isolated From the International Space Station and Description of *Agrobacterium tomkonis* sp. nov. *Front. Microbiol.* **2021**, *12*, 765943. [CrossRef] [PubMed]
16. Weisberg, A.J.; Wu, Y.; Chang, J.H.; Lai, E.M.; Kuo, C.H. Virulence and Ecology of Agrobacteria in the Context of Evolutionary Genomics. *Annu. Rev. Phytopathol.* **2023**, *61*, 1–23. [CrossRef]

17. Furuya, N.; Shimokusuzono, F.; Nakamura, Y.; Nishimura, K.; Takeshita, M.; Matsuyama, N.; Manabe, K.; Takanami, Y. Crown gall of tobacco caused by *Agrobacterium tumefaciens* biovar 1 in tobacco fields. *J. Gen. Pant Pathol.* **2004**, *70*, 39–44. [CrossRef]
18. Kado, C.I.; Heskett, M.G. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. *Phytopathology* **1970**, *60*, 969–976. [CrossRef] [PubMed]
19. Puławska, J.; Willems, A.; Sobiczewski, P. Rapid and specific identification of four *Agrobacterium* species and biovars using multiplex PCR. *Syst. Appl. Microbiol.* **2006**, *29*, 470–479. [CrossRef] [PubMed]
20. Haas, J.H.; Moore, L.W.; Ream, W. Universal PCR primers for detection of phytopathogenic *Agrobacterium* strains. *Appl. Environ. Microbiol.* **1995**, *61*, 2879–2884. [CrossRef] [PubMed]
21. Szegedi, E.; Bottka, S. Detection of *Agrobacterium vitis* by polymerase chain reaction in grapevine bleeding sap after isolation on a semiselective medium. *Vitis* **2002**, *41*, 37–42.
22. Suzaki, K.; Yoshida, K.; Sawada, H. Detection of tumorigenic *Agrobacterium* strains from infected apple saplings by colony PCR with improved PCR primers. *J. Gen. Plant Pathol.* **2004**, *70*, 342–347. [CrossRef]
23. Kuzmanović, N.; Ivanović, M.; Ćalić, A.; Gašić, K.; Obradović, A. Differentiation of *Phytopathogenic agrobacterium* spp. *Pestic. Phytomed.* **2011**, *26*, 245–253. [CrossRef]
24. Aujoulat, F.; Jumas-Bilak, E.; Masnou, A.; Sallé, F.; Faure, D.; Segonds, C.; Marchandin, H.; Teyssier, C. Multilocus sequence-based analysis delineates a clonal population of *Agrobacterium* (*Rhizobium*) *radiobacter* (*Agrobacterium tumefaciens*) of human origin. *J. Bacteriol.* **2011**, *193*, 2608–2618. [CrossRef] [PubMed]
25. Caccamo, D.; Di Cello, F.; Fani, R.; Gugliandolo, C.; Maugeri, T.L. Polyphasic approach to the characterisation of marine luminous bacteria. *Res. Microbiol.* **1999**, *150*, 221–230. [CrossRef] [PubMed]
26. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]
27. Ryder, M.H.; Tate, M.E.; Kerr, A. Virulence Properties of Strains of *Agrobacterium* on the Apical and Basal Surfaces of Carrot Root Discs. *Plant Physiol.* **1985**, *77*, 215–221. [CrossRef] [PubMed]
28. Tolba, I.H.; Soliman, M.A. Efficacy of Native Antagonistic Bacterial Isolates in Biological Control of Crown Gall Disease in Egypt. *Ann. Agric. Sci.* **2013**, *58*, 43–49. [CrossRef]
29. Schaad, N.W.; Jones, J.B.; Chun, W. *Laboratory Guide for Identification of Plant Pathogenic Bacteria*; The American Phytopathological Society: St. Paul, MN, USA, 2001.
30. Puławska, J. Crown gall of stone fruits and nuts, economic significance and diversity of its causal agents: Tumorigenic *Agrobacterium* spp. *J. Plant Pathol.* **2010**, *92*, S87–S98.
31. Holmes, J.E.; Sanghera, H.; Punja, Z.K. Crown gall development on cannabis (*Cannabis sativa* L., marijuana) plants caused by *Agrobacterium tumefaciens* species-complex. *Can. J. Plant Pathol.* **2023**, *45*, 433–445. [CrossRef]
32. Alexandre, A.; Laranjo, M.; Young, J.P.; Oliveira, S. dnaJ is a useful phylogenetic marker for *Alphaproteobacteria*. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 2839–2849. [CrossRef] [PubMed]
33. Puławska, J.; Kałuzna, M. Phylogenetic relationship and genetic diversity of Agrobacterium spp. isolated in Poland based on gyrB gene sequence analysis and RAPD. *Eur. J. Plant Pathol.* **2012**, *133*, 379–390. [CrossRef]
34. Pérez-Yépez, J.; Armas-Capote, N.; Velázquez, E.; Pérez-Galdona, R.; Rivas, R.; León-Barrios, M. Evaluation of seven housekeeping genes for multilocus sequence analysis of the genus *Mesorhizobium*: Resolving the taxonomic affiliation of the Cicer canariense rhizobia. *Syst. Appl. Microbiol.* **2014**, *37*, 553–559. [CrossRef] [PubMed]
35. Kuzmanović, N.; Puławska, J.; Smalla, K.; Nesme, X. *Agrobacterium rosae* sp. nov., isolated from galls on different agricultural crops. *Syst. Appl. Microbiol.* **2018**, *41*, 191–197. [CrossRef] [PubMed]
36. Kuzmanović, N.; Behrens, P.; Idczak, E.; Wagner, S.; Götz, M.; Spröer, C.; Smalla, K. A novel group of *Rhizobium* tumorigenes-like agrobacteria associated with crown gall disease of rhododendron and blueberry. *Phytopathology* **2019**, *109*, 1840–1848. [CrossRef] [PubMed]
37. Mysore, K.S.; Bassuner, B.; Deng, X.B.; Darbinian, N.S.; Motchoulski, A.; Ream, W.; Gelvin, S.B. Role of the *Agrobacterium tumefaciens VirD2* protein in T-DNA transfer and integration. *Mol. Plant Microbe Interact.* **1998**, *11*, 668–683. [CrossRef] [PubMed]
38. Zoina, A.; Raio, A.; Peluso, R.; Spasiano, A. Characterisation of agrobacteria from weeping fig (*Ficus benjamina*). *Plant Pathol.* **2001**, *50*, 620–627. [CrossRef]
39. Peluso, R.; Raio, A.; Morra, F.; Zoina, A. Physiological, biochemical and molecular analyses of an Italian collection of *Agrobacterium tumefaciens* strains. *Eur. J. Plant Pathol.* **2003**, *109*, 291–300. [CrossRef]