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RESEARCH PAPERS

Isolation of non-pathogenic *Agrobacterium* spp. biovar 1 from agricultural soils in Slovenia

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Summary. The disease crown gall, which causes damage on perennial agricultural crops, is economically important in many countries. We therefore explored the presence of *Agrobacterium* spp. in a variety of agricultural soils where fruit trees are grown. Most samples were collected in four regions of Slovenia where a majority of fruit tree plantations are situated. Crown gall-affected plants were not observed on any of the sampling sites. Members of *Agrobacterium* spp. biovar 1 were isolated from 63 of the 72 soil samples (88%), but none from forest soil. All isolates of this *Agrobacterium* species complex were determined to be non-pathogenic by biotests and were assigned to genomic species based on a *rec*A allele sequence analysis. Fourty-three isolates were allocated into genomic species G1 and 26 isolates into G4. Among both genomic species, new alleles of the *rec*A gene were recognized: seven new alleles in G1 (denominated G1-15 to G1-21) and four in G4 (G4-7 to G4-10). Two alleles predominated among the Slovenian strains, *rec*A-G1-15 and *rec*A-G4-2. Different colony morphologies were observed between strains of G1 and G4 on KB medium.

Key words: crown gall, *rec*A, genomic species.

Introduction

The disease crown gall, which can affect nursery and mature plants, is regarded as one of the most economically important diseases of crops such as fruit and nut trees, cranberries, grapes and ornamental plants (De Cleene and De Ley, 1976; Kennedy and Alcorn, 1980). The causative agents are the widespread soil and rhizospheric, Gram-negative bacteria, *Agrobacterium tumefaciens* (Smith and Townsend, 1907; Conn, 1942). Most are found as non-pathogen soilborne bacteria but some are plant pathogens which can produce tumours on a large variety of plants. Tumour formation is the result of uncontrolled plant cell division stimulated by bacterial tumour genes expressing inside plant cells. A conjugative tumour-inducing plasmid (Ti) of the pathogenic strains enables the bacteria to transfer and incorporate tumour genes (T-DNA segment on Ti-plasmid) into the genomes of host plants. A tumour which is usually formed on a plant stem just above ground level is characteristic for crown gall. The disease is rarely fatal to plants, unless it occurs on young or stressed plants found mainly in nurseries of fruit trees and ornamental plants. The infections can manifest as loss of plant vigour, reduction in crop yield, or as plant death (Poncet *et al.*, 1996; Epstein *et al.*, 2008).

In Europe, high levels of disease incidence have been recorded, mainly in nurseries, in countries where fruit production is important. This includes many Mediterranean countries, but the disease is also problematic in continental climates as well (Pionnat *et al.*, 1999; Pulawska, 2010). The diverse climate conditions of Slovenia, which include Mediterranean and continental environments, lead to the many different fruit tree varieties grown in Slovenia. However, crown gall has never been of economical concern in pome and stone fruit production in this country.

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The highly diverse group of *Agrobacterium* biovar 1 (*A. tumefaciens* / *A. radiobacter*) at present includes at least 11 genomic species or genomovars designated as G1 to G9 (Mougel *et al.,* 2002), G13 (Portier *et al.*, 2006) and G14 (Pulawska *et al.*, 2012). The term genomic species is applied to bacteria that are genetically so closely related that they can be classified as one species yet possess enough infraspecies diversity to cluster into discrete groups of strains. All biovar 1 genomic species are due to receive Latin binomial names, except for genomic species G4 which is already designated as *A. radiobacter* (Young *et al*., 2006; Lindström and Young, 2011). For the genomic species G2, G8 and G14 the proposed denominations are *R. pusense* (Panday *et al.,* 2011), *A. fabrum* (Lassalle *et al.,* 2011) and *R. nepotum* (Pulawska *et al*., 2012), respectively. Classification of *Agrobacterium* strains into biovars can be achieved by a multiplex PCR reactions, which target biovar-unique chromosomal genes (Pulawska *et al*., 2006). On the other hand, determination of genomic species can be achieved with genome-wide methodologies of amplified fragment length polymorphism (AFLP) (Mougel *et al.,* 2002; Portier *et al.,* 2006) and a multilocus sequence analysis (MLSA) (Martens *et al.,* 2008) as well as with *rec*A sequence analysis (Costechareyre *et al.,* 2010).

The difference in the crown gall incidence between Slovenia and other fruit-producing countries (Al-Karablieh and Khlaif, 2002; Epstein *et al*., 2008; Pulawska, 2010) lead us to explore the presence of *A. tumefaciens* strains in agricultural soils. We focused on soils of pome fruit tree orchards that represent almost 80% of the Slovenian fruit growing area (Statistical Office, 2011) to provide an insight into the presence of the *A. tumefaciens* genomic species in Slovenia.

Materials and methods

Soil sampling

Soil samples were collected from agricultural lands in October 2010, at 66 locations across Slovenia's regions of Savinjska, Gorenjska, Spodnjeposavska and Podravje. From the regions of Osrednjeslovenska and Dolenjska one sample per location was collected. We analysed 72 soil samples from 53 orchards (apple, peach, pear and plum tree plantations), seven meadows, five fields, three vineyards and four forests (Table 1). Samples were taken from the superficial soil layer (10–15 cm depth) using a 10 mL soil probe. At each site we randomly selected an area (20 × 20 m) where the soil sample was collected from ten points. After each sampling, the probe was washed with water and disinfected with 70% ethanol. All soil samples were transferred in cooling boxes to the laboratory and stored at 10°C until further processing. Crown gall symptoms were not found on any of the fruit trees at any of the sampling sites.

Bacterial isolation and identification

Isolation of soil agrobacteria was carried out according to standard microbiological techniques and started within 1 week after soil samples were collected. Ten grams of thoroughly mixed soil from each sample were suspended in 90 mL of sterile deionized water and shaken on a laboratory mixer at 300 rpm for 30 min. The suspension was left for a few minutes to let large soil particles settle. Ten-fold dilutions were prepared from the soil suspension in sterile deionized water, and were streaked onto plates of medium 1A (Brisbane and Kerr, 1983). This medium is selective for *A. tumefaciens* biovar 1 strains. The plates were incubated at 27°C for 3–4 d. They were visually inspected for colonies with specific colouring and morphology of *A. tumefaciens* (Lippincott *et al*., 1983), which were then directly counted for soil population density evaluation (cfu g^{-1}). The selected colonies were subcultured on a King's B medium (KB) to obtain pure cultures and tested with multiplex PCR, as described by Pulawska *et al*. (2006) with a modification. PCR was carried out in a total volume of 20 μL in a Verity 96 Well Thermal Cycler (Applied Biosystems), applying the protocol for fast DNA amplification. The PCR reactions contained the GeneAmp Fast PCR Master Mix (2×) (Applied Biosystems) with 1 μM of each primer (Pulawska *et al*., 2006) and deionized water to adjust the reaction volume. The amplification conditions comprised an initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 67°C for 15 s, extension at 72°C for 30 s and a final extension step at 72°C for 10 min. The amplified PCR fragments were visualized on a 2% agarose gel. One or two *Agrobacterium* biovar 1 strains from each soil sample were selected for long term preservation in meat peptone broth with glycerol (1:3), and stored at –80°C till further use.

Pathogenicity of *Agrobacterium* strains

Biological tests for assessing pathogenicity of *A. tumefaciens* strains were performed on tomato, sunflower and *Kalanchoe daigremontiana* plants. Four-week-old seedlings were each punctured three times in the stem using a sterile entomological needle dipped in pure culture colonies of the bacteria grown on KB medium at 27°C for 24 h. Bio-tests were performed in triplicates. Inoculated seedlings were maintained in a glasshouse at 20–30°C with natural lighting conditions. In the period of 3 to 6 weeks post inoculation, the plants were visually inspected for tumour formation every 5 days. The strain C58 was used as a positive control, and water inoculation was used as a negative control.

The *rec*A gene sequence amplification and analysis

All confirmed strains of biovar 1 *A. tumefaciens* were selected for classification to genomic species by applying the *rec*A sequence analysis according to Costechareyre *et al.* (2010) with a modification. Bacterial DNA was isolated using the standard alkaline lysis method (Sambrook *et al.*, 1989) from a loop-full of each bacterial strain grown on KB medium at 27°C for 24 h. Dilutions (1:1000) were prepared in a sterile TE buffer (pH 8.0). For the *rec*A gene amplification, a forward primer (FwdrecA2 5′-ATG CTG GCT SMG CCT TGC GA-3′) was constructed according to the genome sequence of the C58 strain (acc. no. L07902.1). The reverse primer used was F2899 with T3 tail (5′-ATT AAC CCT CAC TAA AGG GAT GCA GGA AGC GGT CGG CRA TSA-3′) (Costechareyre *et al.*, 2010). PCR assays were carried out in a Verity 96 Well Thermal Cycler (Applied Biosystems) in a final volume of 50 μL. The reaction mixture contained GoTaq PCR buffer (Promega), 0.7 mM $MgCl_2$ (Promega), 125 μM of each dNTP, 1 μM of each primer and 2.5 U of GoTaq DNA polymerase (Promega), and 2.5 μL of diluted bacterial DNA. Cycling conditions comprised an initial denaturing step of 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s of annealing at 55°C, followed by an elongation at 72°C for 2 min and a final elongation step of 7 min at 72°C. The amplified PCR fragments were visualized on a 1% agarose gel.

The PCR fragments were sequenced (Macrogen) with the FwdA2 primer and a universal T3 primer. Computer software BioEdit v. 7.0.5.3 (Hall, 1999) was used for assembly, alignment, identity matrix calculations and *in silico* translation of the sequences. Similarity of the 969 bp long *rec*A sequence to all known sequences was determined by the BlastN algorithm on a NCBI server (Altschul *et al.*, 1990). Sequence polymorphism was analyzed with DnaSP v. 5.10.01 (Librado and Rozas, 2009).

Colony morphology and temperature effects

The colony morphology of all strains was observed on the KB medium after 4 d of incubation at 27°C. Further, the colony morphology of seven randomly selected G1 strains (3-2, 10-1, 11-1, 24-1, 30-1, 52-1 and 72-3) and two G4 strains (29-1 and 44-1) was inspected on 1A medium after 4 d at 27 °C, and on the KB medium after 4 d incubation at 21°C or 1 week at 12°C.

Results

Agrobacterium tumefaciens detection and pathogenicity assessment

Agrobacterium tumefaciens strains were isolated from 63 of the 72 soil samples (88%) on selective medium 1A. Forest soil samples did not contain *A. tumefaciens* biovar 1 isolates. The population density of *A. tumefaciens* in soil was evaluated by a direct counting of colonies on 1A plates. Populations varied from 5×10^2 to 3×10^6 cfu g^{-1}, with an average population for all samples from 10^3 to 10^4 cfu g^{-1}. All the strains were determined to be non-pathogenic when inoculated into different hosts, whereas the pathogenic control strain developed tumours on inoculated plants (data not shown).

Our G1 strains had orange-brown to red-orange colony pigmentation and the G4 strains had light purple colonies with dark centres after 4 d incubation at 27°C (Figure 1). The colony morphology of the G1 strains was convex, glistening and circular, while we noticed greater mucus production in colonies of the G4 strains. The differences in colony morphology between genomic species were exhibited also on the KB medium at 27 and 21°C. The G1 strains had rough, wrinkly surfaces at the colony centres and the G4 strains had smooth colony surfaces and produced greater higher amounts of mucus. The surfaces of the G1 colonies were slightly smoother at 12°C as observed on seven randomly selected G1 strains (Figure 2). Both selected G4 strains had a mucoid phenotype at 12°C.

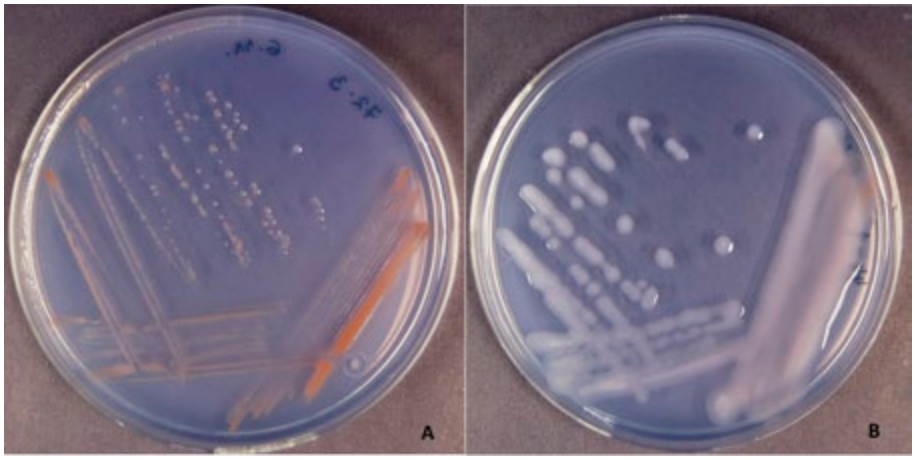

Figure 1. Colony morphologies of *Agrobacterium tumefaciens* genomic species G1 and G4 on 1A medium. (A) Orange-brown pigmentation of genomic species G1 (strain 72-3). (B) Lavender pigmentation and mucoid phenotype of genomic species G4 (strain 44-1). Cultures were incubated at 27°C for 4 d.

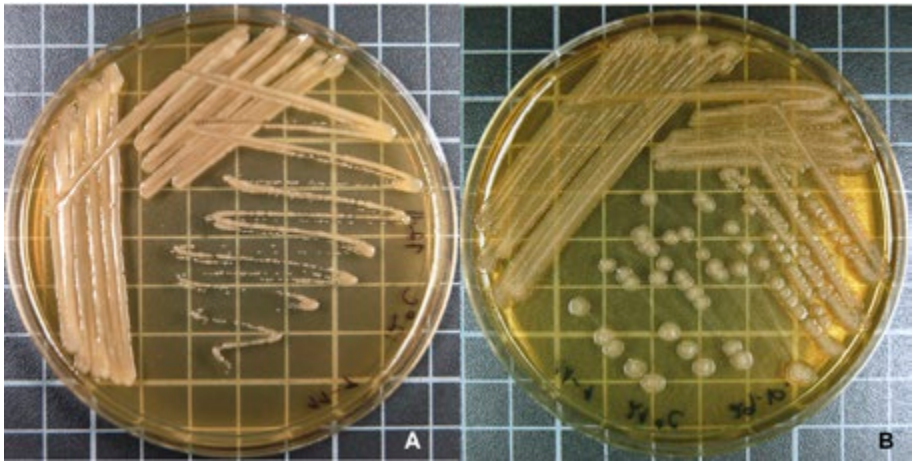

Figure 2. Colony morphologies of G1 strains (11-1) differed at 12 or 21°C on KB medium. (A) Smooth colonies at 12°C after 1 week. (B) Rough colonies at 21°C after 4 d.

Diversity of *rec*A gene allele sequence

The sequences determined in this study have been deposited in the GenBank with accession numbers KC291939 to KC292007.

The amplification of *rec*A genes using primer set FwdrecA2 - F2899 resulted in a 1138-bp long amplified fragment. Based on identity matrix calculations, we allocated the 69 determined *rec*A gene sequences into 25 groups of identical sequences representing the 25 *rec*A alleles (Tables 1 and 2). Allocation to genomic species was based on sequence similarity to all previously deposited sequences (BlastN algorithm). Eleven groups were identified as genomic species G1 and seven as G4 (*A. radiobacter*). Fourty-one *rec*A sequences were identical to the known *rec*A alleles. According to the BlastN algorithm calculations, the remaining 28 sequences had four to 12 mismatches and 99% similarity to one of the known *rec*A alleles (E value = 0.0). Each of the new *rec*A sequences was given a new allele number. Allele sequences that differed from a known allele by one mismatch were

not considered as new. Star-coding (*) was applied to distinguish them from reference allele sequences (Tables 1 and 2).

Two alleles dominated in the soil samples, and these were the allele *rec*A-G1-15 from G1 and the allele *rec*A-G4-2* from G4 (Tables 1 and 2). The allele *rec*A-G1-15 had high sequence similarity to the known allele *rec*A-G1-4 (*A. tumefaciens* strain CFBP 5622, acc. no. FM164291.1), yet differed by 10 mismatches. The allele *rec*A-G4-2* differed from the most similar allele *rec*A-G4-2 (*A. tumefaciens* strain ATCC 4718, acc. no. FM164308.1) by one mismatch and is therefore marked with a star. Although high diversity of the *rec*A gene sequence was documented in this study, it turned out that all G1 alleles resulted in one identical protein sequence while all G4 alleles resulted in a second protein differentiated by five amino acids (I/V_{40}, N/G_{113}, I/V_{140}, M/T_{315}, and S/N_{317}) when translated *in silico*. The exception among G1 alleles was strain 51-1 that had a unique protein sequence and was differentiated from the rest of the G1 strains by one amino acid (S/N_{317}).

To test whether the *rec*A locus was under selective pressure, we estimated the degree of selection by calculating the d_N/d_S ratio representing the ratio of nonsymonymous substitutions to synonymous substitutions. The d_N/d_S ratio for the *rec*A gene of our strains was significantly below 1 that which renders this locus absent from strong positive selective pressure (Table 3).The genomic species G1 and G4 were found in all agricultural lands except in forest soil. There was an almost uniform distribution of the *A. tumefaciens* genomic species G1 and G4 within the Slovenian regions of Savinjska (G1/G4 = 19/13), Spodnjeposavska (G1/G4 = 4/6) and Podravje (G1/G4 = 6/4). However, G1 was predominant in the Gorenjska region (G1/G4 = 10/1). In fruit tree orchards, 32 of the 53 isolates (60%) were found to be members of genomic species G1. In some soil samples (n = 3) we determined isolates of both genomic species. Further, we found no obvious correlation in *rec*A allele distribution and agricultural habitats. From six samples we retrieved isolates with different *rec*A alleles of the same genomic species.

Discussion

This was the first survey for the presence of the *Agrobacterium* spp. biovar 1 in agricultural lands in Slovenia. Agrobacteria are ubiquitous soil bacteria that can be found in virtually every habitat, including water and sediments (D'Hondt *et al.*, 2004; Süss *et al.*, 2006).

According to our results, non-pathogenic members of the *A. tumefaciens* biovar 1 are widely occurring on agricultural lands in Slovenia, except in forest soil where they are either absent or present in numbers below the threshold of detection. The populations of *A. tumefaciens* biovar 1 in soil samples determined in this study is similar to those reported from other studies on soils with previous histories of crown gall (Alconero, 1980), or on soils without crown gall-affected plants (Schroth *et al.*, 1971; Bouzar *et al.*, 1993; Mougel *et al.*, 2001). The most frequently isolated genomic species in soil samples from Slovenia was G1 (62%), followed by G4 (38%) which represents the *A. radiobacter* species according to recent changes in nomenclature (Lindström and Young, 2011). This result agrees with a previous report where the population of G1 was one of the most frequently isolated genomic species from non-contaminated soil from corn fields (Vogel *et al.*, 2003). As we have shown, both populations of both genomic species can be present at the same location at the same time, but it has been suggested that they very likely inhabit different microhabitats (Lassalle *et al.*, 2011). We have not observed any obvious correlations between geographic location, agricultural land and genomic species, but analysis of more samples could provide a more reliable insight. Similarly, previous extensive studies of Agrobacteria diversity on plasmid and chromosomal levels found no correlation between genetic diversity, geographic origin, or host plants (Nesme *et al.*, 1987; Michel *et al.*, 1990; Pulawska *et al.*, 1998; Raio *et al.*, 2004; Rhouma *et al.*, 2006; Pulawska and Kalužna, 2012).

Even though *rec*A sequences differed within one genomic species but resulted in the same RecA protein sequence, we have identified one strain which did not comply with this rule and had one amino acid changed compared to the rest of the known strains of the same genomic species. We identified 11 new *rec*A alleles that were found exclusively in Slovenian strains. The remaining ten *rec*A alleles were identical to those determined by other authors in strains originating from the USA, Germany, France, Tunisia, Italy and Belgium (Costechareyre *et al.*, 2010; De Meyer *et al.*, 2011; Shams *et al.*, 2013). A greater number of different *rec*A allele sequences were found in the genomic species G1 than in G4, indicat-

Table 1. Origin, observed colony morphology, and determined *rec*A alleles with sequence accession numbers of strains of *Agrobacterium tumefaciens* biovar 1 soil populations from agricultural lands in Slovenia.

Strain name	Region	Collection site	Colony morphology [a]	Allele code	Accession No.
11-1	Gorenjska	Orchard	Rough	recA-G1-1	KC291939
46-1	Savinjska	Orchard	Rough	*rec*A-G1-1	KC291940
1-1	Gorenjska	Orchard	Rough	*rec*A-G1-2	KC291941
6-1	Gorenjska	Orchard	Rough	*rec*A-G1-2	KC291942
24-1	Savinjska	Orchard	Rough	*rec*A-G1-2	KC291943
43-1	Savinjska	Orchard	Rough	*rec*A-G1-2	KC291944
75-1	Podravje	Vineyard	Rough	*rec*A-G1-2	KC291945
3-2	Gorenjska	Orchard	Rough	*rec*A-G1-6	KC291946
5-3	Gorenjska	Orchard	Rough	*rec*A-G1-6	KC291947
22-3	Savinjska	Orchard	Rough	*rec*A-G1-6	KC291948
33-1	Savinjska	Orchard	Rough	*rec*A-G1-6	KC291950
62-1	Savinjska	Orchard	Rough	*rec*A-G1-6	KC291952
25-1	Savinjska	Orchard (nursery)	Rough	*rec*A-G1-6	KC291949
41-2	Savinjska	Orchard (nursery)	Rough	*rec*A-G1-6	KC291951
39-1	Savinjska	Orchard	Rough	*rec*A-G1-6*	KC291978
7-2	Gorenjska	Orchard	Rough	*rec*A-G1-7	KC291953
63-1	Savinjska	Orchard	Rough	*rec*A-G1-7	KC291955
34-1	Savinjska	Vineyard	Rough	*rec*A-G1-7	KC291954
23-1	Savinjska	Orchard	Rough	*rec*A-G1-7*	KC291977
10-1	Gorenjska	Orchard	Rough	*rec*A-G1-10	KC291975
9-2	Gorenjska	Orchard	Rough	*rec*A-G1-12	KC291971
20-3	Savinjska	Orchard	Rough	*rec*A-G1-12	KC291972
8-2	Gorenjska	Orchard	Rough	*rec*A-G1-14	KC291964
43-5	Savinjska	Orchard	Rough	*rec*A-G1-14	KC291965
52-2	Spodnjeposavska	Meadow	Rough	*rec*A-G1-14	KC291966
Z2	Spodnjeposavska	Field	Rough	*rec*A-G1-14	KC291967
8-3	Gorenjska	Orchard	Rough	*rec*A-G1-15[b]	KC291956
9-3	Gorenjska	Orchard	Rough	*rec*A-G1-15[b]	KC291957
49-2	Spodnjeposavska	Orchard	Rough	*rec*A-G1-15[b]	KC291960
76-1	Podravje	Orchard	Rough	*rec*A-G1-15[b]	KC291963
15-1	Savinjska	Field	Rough	*rec*A-G1-15[b]	KC291958
30-1	Osrednjeslovenska	Meadow	Rough	*rec*A-G1-15[b]	KC291959
45-1	Savinjska	Meadow	Rough	*rec*A-G1-15[b]	KC291961
52-1	Spodnjeposavska	Meadow	Rough	*rec*A-G1-15[b]	KC291962
4-1	Gorenjska	Orchard	Rough	*rec*A-G1-16[b]	KC291968

(Continued)

Table 1. (Continued)

Strain name	Region	Collection site	Colony morphology [a]	Allele code	Accession No.
16-1	Savinjska	Orchard	Rough	*rec*A-G1-16[b]	KC291969
70-1	Podravje	Orchard	Rough	*rec*A-G1-16[b]	KC291970
26-1	Savinjska	Meadow	Rough	*rec*A-G1-17[b]	KC291973
31-1	Savinjska	Orchard	Rough	*rec*A-G1-17[b]	KC291974
13-1	Savinjska	Meadow	Rough	*rec*A-G1-18[b]	KC291976
51-1	Spodnjeposavska	Orchard	Rough	*rec*A-G1-19[b]	KC291979
55-2	Spodnjeposavska	Orchard	Rough	*rec*A-G1-20[b]	KC291980
72-3	Podravje	Field	Rough	*rec*A-G1-21[b]	KC291981
56-1	Podravje	Field	Smooth	*rec*A-G4-1	KC291982
2-1	Gorenjska	Orchard	Smooth	*rec*A-G4-2*	KC291987
17-1	Savinjska	Orchard	Smooth	*rec*A-G4-2*	KC291988
28-1	Savinjska	Orchard	Smooth	*rec*A-G4-2*	KC291990
40-5	Savinjska	Orchard	Smooth	*rec*A-G4-2*	KC291991
44-1	Savinjska	Orchard	Smooth	*rec*A-G4-2*	KC291992
53-1	Spodnjeposavska	Orchard	Smooth	*rec*A-G4-2*	KC291994
58-1	Spodnjeposavska	Orchard	Smooth	*rec*A-G4-2*	KC291995
67-3	Podravje	Orchard	Smooth	*rec*A-G4-2*	KC291996
21-A	Savinjska	Orchard (nursery)	Smooth	*rec*A-G4-2*	KC291989
48-1	Dolenjska	Vineyard (nursery)	Smooth	*rec*A-G4-2*	KC291993
57-1	Spodnjeposavska	Orchard	Smooth	*rec*A-G4-5	KC291984
65-1	Podravje	Orchard	Smooth	*rec*A-G4-5	KC291985
74-1	Podravje	Orchard	Smooth	*rec*A-G4-5	KC291986
29-1	Savinjska	Orchard	Smooth	*rec*A-G4-5	KC291983
48-2	Spodnjeposavska	Vineyard (nursery)	Smooth	*rec*A-G4-7[b]	KC292007
27-1	Savinjska	Meadow	Smooth	*rec*A-G4-8[b]	KC292002
34-2	Savinjska	Vineyard	Smooth	*rec*A-G4-8[b]	KC292003
35-1	Savinjska	Orchard	Smooth	*rec*A-G4-8[b]	KC292004
60-1	Savinjska	Orchard	Smooth	*rec*A-G4-8[b]	KC292005
68-1	Podravje	Orchard	Smooth	*rec*A-G4-8[b]	KC292006
20-2	Savinjska	Orchard	Smooth	*rec*A-G4-9[b]	KC291997
54-2	Spodnjeposavska	Orchard	Smooth	*rec*A-G4-9[b]	KC291998
69-1	Podravje	Orchard	Smooth	*rec*A-G4-9[b]	KC291999
25-2	Savinjska	Orchard (nursery)	Smooth	*rec*A-G4-10[b]	KC292000
61-1	Savinjska	Orchard	Smooth	*rec*A-G4-10[b]	KC292001

[a] Growth on KB medium at 27°C.
[b] New *rec*A alleles determined in this study.
*Indicates allele with one mismatch to reference allele.

Table 2. The diversity and frequency of *rec*A alleles of the *Agrobacterium tumefaciens* biovar 1 soil populations from agricultural habitats in Slovenia.

Genomic species	Allele status	No. of alleles	Allele code	No. of isolates
G1	Known	1	*recA-G1-1*	2
		2	*recA-G1-2*	5
		3	*recA-G1-6*	7
			*recA-G1-6**	1
		4	*recA-G1-7*	3
			*recA-G1-7**	1
		5	*recA-G1-10*	1
		6	*recA-G1-12*	2
		7	*recA-G1-14*	4
	New	8	*recA-G1-15*	8
		9	*recA-G1-16*	3
		10	*recA-G1-17*	2
		11	*recA-G1-18*	1
		12	*recA-G1-19*	1
		10	*recA-G1-20*	1
		11	*recA-G1-21*	1
Total G1				**43**
G4	Known	1	*recA-G4-1*	1
		2	*recA-G4-2**	10
		3	*recA-G4-5*	4
	New	4	*recA-G4-7*	1
		5	*recA-G4-8*	5
		6	*recA-G4-9*	3
		7	*recA-G4-10*	2
Total G4				**26**
Total G1+ G4				**69**

Table 3. Analysis of the *rec*A gene fragment in the *Agrobacterium tumefaciens* biovar 1 soil isolates.

Genomic species	Size of the fragment (bp)	No. of alleles	No. of polymorphic sites	*dN/dS*
G1	969	11	40	0.022
G4	969	7	22	0.024
G1+G4	969	18	109	0.023

ing larger diversity within G1, while isolates of other known genomic species were not found. The *rec*A sequences which differed from the known alleles were given new allele numbers when they differed from known alleles at more than one site. High *rec*A allelic diversity of our strains showed no positive selection pressures, with the ratio d_N/d_S significantly less than 1.00. This favours the use of *rec*A sequence as a marker in taxonomic analyses.

According to Pulawska (2010), crown gall symptoms are regularly observed on stone fruit trees and nuts in Greece, Tunisia, Hungary, Belgium, Holland, Poland, Italy, Spain and France. On the other hand, low crown gall incidence was noted in the Czech Republic, Lithuania and Serbia. The situation in Slovenia is similar and most likely associated with efficient disease management in nurseries that employ eradication of young symptomatic plants. Furthermore, we suspect that the presence of only non-pathogenic Agrobacteria found in agricultural soils could be linked to low disease incidence. It was proposed that the ratio between non-pathogenic and pathogenic Agrobacteria affects incidence and the extent of crown gall (New and Kerr, 1972). The more abundant non-pathogenic strains could physically block the infection sites (Lippincott *et al*., 1977). Therefore the abundance of non-pathogenic Agrobacteria could act as natural protection against crown gall.

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Literature cited

Alconero R. 1980. Crown gall of peaches from Maryland, South Carolina, and Tennessee and problems with biological control. *Plant Disease* 64, 835–838.

Al-Karablieh N. and H. Khlaif, 2002. Occurrence and distribution of crown gall disease in Jordan. *Phytopathologia Mediterranea* 41, 226–234.

Altschul S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215, 403-410.

Bouzar H., D. Ouadah, Z. Krimi, J.B. Jones, M. Trovato, A. Petit and Y. Dessaux, 1993. Correlative association between resident plasmids and the host chromosome in a diverse *Agrobacterium* soil population. *Applied Environmental Microbiology* 59, 1310–1317.

Brisbane P.G. and A. Kerr, 1983. Selective media for the three biovars of *Agrobacterium*. *Journal of Applied Bacteriology* 54, 3 425–431.

Conn H.J., 1942. Validity of the genus *Alcaligenes*. *Journal of Bacteriology* 44, 353–360.

Costechareyre D., A. Rhouma, C. Lavire, P. Portier, D. Chapulliot, F. Bertolla, A. Boubaker, Y. Dessaux and X. Nesme, 2010. Rapid and efficient identification of *Agrobacterium* species by *rec*A allele analysis. *Microbial Ecology* 60 (4), 862–872.

D'Hondt S., B.B. Jorgensen, D.J. Miller, A. Batzke, R. Blake and B.A. Cragg, 2004. Distributions of microbial activities in deep subseafloor sediments. *Science* 306, 2216–2221.

De Cleene M. and J. De Ley, 1976. The host range of crown gall. *Botanical Review* 42, 389–466.

De Meyer S.E., K. Van Hoorde de, B. Vekeman, T. Braeckman and A. Willems, 2011. Genetic diversity of rhizobia-associated with indigenous legumes in 496 different regions of Flanders (Belgium). *Soil Biology and Biochemistry* 43, 2384–2396.

Epstein L., S. Kaur, J.R. McKenna, J.A. Grant, W. Olson and W.O. Reil, 2008. Crown gall can spread between walnut trees in nurseries and reduce future fields. *California Agriculture* 62, 111–115.

Hall A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95–98.

Kennedy B.W. and S.M. Alcorn, 1980. Estimates of U.S. crop losses to prokaryote plant pathogens. *Plant Disease* 64, 674–676.

Lassalle F., T. Campillo, L. Vial, J. Baude, D. Costechareyre, D. Chapulliot, M. Shams, D. Abrouk, C. Lavire, C. Oger-Desfeux, F. Hommais, L. Gueguen, V. Daubin, D. Muller and X. Nesme, 2011. Genomic species are ecological species as revealed by comparative genomics in *Agrobacterium tumefaciens*. *Genome Biology and Evolution* 3, 762–781.

Librado P. and J. Rozas, 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452.

Lindström K. and J.P.W. Young, 2011. International Committee on Systematics of Prokaryotes- Minutes: Subcommittee on the taxonomy of *Agrobacterium* and *Rhizobium*. *International Journal of Systematic and Evolutionary Microbiology* 61, 3089–3093.

Lippincott B.B., M. H. Whatley and J.A. Lippincott, 1977. Tumor induction by *Agrobacterium* involves attachment of the bacterium to a site on the host plant cell wall. *Plant Physiology* 59 (3), 388–390.

Lippincott J.A., B.B. Lippincott and M.P. Starr, 1983. The genus *Agrobacterium*. In: *The procaryotes: a Handbook of Habitats, Isolation and Identification of Bacteria* (M.P. Starr, ed.), Springer-Verlag, New York, NY, USA, 842–845.

Martens M., P. Dawyndt, R. Coopman, M. Gillis, P. De Vos and A. Willems, 2008. Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). *International Journal of Systematic and*

Evolutionary Microbiology 58, 200–214.

Michel M.F., A.C. Brasileiro, C. Depierreux, L. Otten, F. Delmotte and L. Jouanin, 1990. Identification of different *Agrobacterium* strains isolated from the same forest nursery. *Applied Environmental Microbiology* 56, 3537–3545.

Mougel C., B. Cournoyer and X. Nesme, 2001. Novel tellurite-amended media and specific chromosomal and Ti plasmid probes for direct analysis of soil populations of *Agrobacterium* biovars 1 and 2. *Applied Environmental Microbiology* 67, 65–74.

Mougel C., J. Thioulouse, G. Perrière and X. Nesme, 2002. A mathematical method for determining genome divergence and species delineation using AFLP. *International Journal of Systematic and Evolutionary Microbiology* 52 (2), 573–586.

Nesme X., M.F. Michel and B. Digat, 1987. Population heterogeneity of *Agrobacterium tumefaciens* in galls of *Populus* L. from a single nursery. *Applied Environmental Microbiology* 53, 655–659.

New P.B. and A. Kerr, 1972. Biological control of crown gall: field measurements and glasshouse experiments. *Journal of Applied Bacteriology* 35, 279–287.

Panday D., P. Schumann and S.K. Das, 2011. *Rhizobium pusense* sp. nov., isolated from the rhizosphere of chickpea (*Cicer arietinum* L.). *International Journal of Systematic and Evolutionary Microbiology* 61, 2632–2639.

Pionnat S., H. Keller, D. Héricher, A. Bettachini, Y. Dessaux, X. Nesme and C. Poncet, 1999. Ti plasmids from *Agrobacterium* characterize rootstock clones that initiated a spread of crown gall disease in Mediterranean countries. *Applied and Environmental Microbiology* 65 (9), 4197–4206.

Poncet C., C. Antonini, A. Bettachini, D. Hericher, S. Pionnat, L. Simonini, Y. Dessaux Y and X. Nesme, 1996. Impact of the crown gall disease on vigour and yield of rose trees. *Acta Horticulturae* 424, 221–225.

Portier P., M. Fisher-Le Saux, C. Mougel, C. Lerondelle, D. Chapulliot, J. Thioulouse and X. Nesme, 2006. Identification of genomic species of *Agrobacterium* biovar 1 by AFLP genomic markers. *Applied Environmental Microbiology* 72, 7123–7131.

Pulawska J., 2010. Crown gall of stone fruits and nuts – economic significance and diversity of its causal agent tumorigenic *Agrobacterium* spp. *Journal of Plant Pathology* 92, 87–98.

Pulawska J. and M. Kalužna, 2012. Phylogenetic relationship and genetic diversity of *Agrobacterium* spp. isolated in Poland based on *gyr*B gene sequence analysis and RAPD. *European Journal of Plant Pathology* 133, 379–390.

Pulawska J., T. Malinowski and P. Sobiczewski, 1998. Diversity of plasmids of *Agrobacterium tumefaciens* isolated from fruit trees in Poland. *Journal of Phytopathology* 146, 465–468.

Pulawska J., A. Willems and P. Sobiczewski, 2006. Rapid and specific identification of four *Agrobacterium* species and biovars using multiplex PCR. *Systematic and Applied Microbiology* 29, 470–479.

Puławska J, A. Willems, S.E. De Meyer and S. Süle, 2012. *Rhizobium nepotum* sp. nov. isolated from tumors on different plant species. *Systematic and Applied Microbiology* 35 (4), 215–220.

Raio A., R. Peluso, X. Nesme and A. Zoina, 2004. Chromosomal and plasmid diversity of *Agrobacterium* strains isolated from *Ficus benjamina* tumors. *European Journal of Plant Pathology* 110, 163–174.

Rhouma A., A. Boubaker, X. Nesme and Y. Dessaux, 2006. Plasmid and chromosomal diversity of a Tunisian collection of *Agrobacterium tumefaciens* strains. *Tunisian Journal of Plant Protection* 1, 73–84.

Sambrook J., E.F. Fritsch and T. Maniatis (ed.), 1989. *Molecular Cloning, A laboratory Manual*. 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schroth M.N., A.R. Weinhold, A.H. McCain, D.C. Hildebrand and N. Ross, 1971. Biology and control of *Agrobacterium tumefaciens*. *Hilgardia* 40, 537–552.

Shams M., L. Vial, D. Chapulliot, X. Nesme and C. Lavire, 2013. Rapid and accurate species and genomic species identification and exhaustive population diversity assessment of *Agrobacterium* spp. using *rec*A-based PCR. *Systematic and Applied Microbiology*, http://dx.doi.org/10.1016/j.syapm.2013.03.002 (in press).

Smith E.F. and C.O. Townsend, 1907. A plant-tumor of bacterial origin. *Science* 25, 671–673.

Statistical Office of the Republic of Slovenia, 2011. Statistical Yearbook: [Online: http://www.stat.si/letopis/Letopis-PrvaStran.aspx?lang=en]

Süss J., K. Schubert, H. Sass, H. Cypionka, J. Overmann and B. Engelen, 2006. Widespread distribution and high abundance of *Rhizobium radiobacter* within Mediterranean subsurface sediments. *Environmental Microbiology* 8, 1753–1763.

Van Berkum P., P.Elia and B.D. Eardly, 2006. Multilocus sequence typing as an approach for analysis of *Medicago*-nodulating rhizobia. *Journal of Bacteriology* 188 (15), 5570–5577.

Vogel J., P. Normand, J. Thioulouse, X. Nesme and G.L. Grundmann, 2003. Relationship between spatial and genetic distance in *Agrobacterium* spp. in one cubic centimeter of soil. *Applied Environmental Microbiology* 69, 1482–1487.

Young J.M., S.R. Pennycook and D.R.W. Watson, 2006. Proposal that *Agrobacterium radiobacter* has priority over *Agrobacterium tumefaciens*. Request for an Opinion. *International Journal of Systematic and Evolutionary Microbiology* 56 (2), 491–493.

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