Agrociencia

NATIVE BACTERIA IN RASPBERRY CROWN GALL REDUCE THE SEVERITY OF *Agrobacterium tumefaciens*

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Citation: Sánchez-Jiménez E, Aranda-Ocampo S, Ochoa-Martínez DL, Mejía-Sánchez D. 2022. Native bacteria in raspberry crown gall reduce the severity of *Agrobacterium tumefaciens*. **Agrociencia.** https://doi.org/ 10.47163/agrociencia.v56i8.2871

Editor in Chief:
Dr. Fernando C. Gómez Merino

Received: July 01, 2022.
Approved: October 03, 2022.
Published in Agrociencia: December 15, 2022.

ABSTRACT

Native bacterial populations in crown galls caused by *Agrobacterium tumefaciens* may harbour bacteria of interest for biocontrol of this pathogen. In this study, we explored the density of native bacterial populations in crown galls of raspberry (*Rubus ideaus*) and evaluated their *in vitro* and *in vivo* antagonism against *A. tumefaciens*. Bacteria morphologically similar to *A. tumefaciens* were isolated from six gall samples and identified by *virD2* gene sequencing. Bacterial population density was calculated by direct plate count on nutrient agar and R2A media. The *in vitro* antagonism efficiency index against *A. tumefaciens* of the most frequent bacteria was evaluated by dual confrontation on nutrient agar medium, and *in vivo* by inoculation of 1.5 x 10^8 UFC mL^{-1} in the root of tomato (*Solanum lycopersiucm*) plants under greenhouse conditions. By direct sequencing and biovar characterization, it was identified as *A. tumefaciens* biovar 1 in raspberry galls. Native bacterial populations in galls have variable density and their diversity is limited. By partial amplification of the *16S rRNA gene*, 13 strains were identified with the highest frequency in the genera *Pseudomonas* (61.5 %), *Bacillus* (15.3 %), *Alcaligenes* (15.3 %) and *Delftia* (7.6 %). Among these, *Alcaligenes faecalis* showed the highest *in vitro* antagonism index ($p \leq 0.05$) against *A. tumefaciens*, followed by *Delftia* sp. and *Pseudomonas citronellolis*. *In vivo* inoculation of tomato plants with these antagonists against *Agrobacterium tumefaciens* did not prevent infection; however, *Alcaligenes faecalis* significantly reduced ($p \leq 0.05$) the severity of plant stem tumours. *A. faecalis* is the most efficient antagonist *in vitro* and *in vivo* against *A. tumefaciens*.

Keywords: antagonism, biocontrol, *Rubus* sp., tumour.

INTRODUCTION

Agrobacterium tumefaciens causes tumours (also known as "crown gall") in a wide range of dicotyledonous plants; infection occurs by transfer and insertion of the tumour-inducing (Ti) plasmid into the host plant genome.

During tumour development oncogenic cells synthesize opines, which are specific compounds used as a source of carbon, nitrogen and energy by *A. tumefaciens* populations (Gelvin, 2018; Wang *et al*., 2019). Tumours caused by *A. tumefaciens* are

considered specific ecological niches in which approximately 40 types of opines of different chemical composition have been identified (Meyer *et al*., 2019).

Agrobacterium species harbouring the Ti plasmid produce opines in the tumour that can be chemoattractants for other bacterial populations within the same genus, creating a highly specific ecological niche, reducing competition from other microorganisms that are not able to use these opines as an energy source (Quispe-Huamanquispe *et al*., 2017). However, other studies report that efficient long-term tumour colonization by *Agrobacterium* depends largely on competition for the availability of opines and other nutrients among *Agrobacterium* populations, as well as interaction with the host's native microbiota with the ability to transform these same compounds and colonize the tumour tissue (Meyer *et al*., 2019).

The use of antagonistic microorganisms in plant pathogen control can be an efficient tool to reduce agrochemical application and integrate ecologically sustainable disease management alternatives (van Lenteren *et al*., 2018). Research on native microbial antagonists in specific ecological niches has shown that they can be an important source of new bacterial isolates for antibiosis-mediated biological control, nutrient and space competition against phytopathogenic microorganisms (Saikia *et al*., 2022).

In recent years, the incidence and severity of *A. tumefaciens* has increased in raspberry (*Rubus idaeus*) crops, mainly in nursery-grown seedlings. This is due to the use of grafting or pruning techniques, and the inefficient chemical control of this pathogen. Therefore, it is necessary to generate alternatives that contribute to the efficient management of this pathogen. In this research, it is assumed that raspberry tumour tissue harbours culturable bacterial populations antagonistic against *A. tumefaciens*. The objectives were i) to identify tumorigenic *A. tumefaciens* in raspberry crown gall, ii) to determine the density of native bacterial populations in the crown gall, and iii) to evaluate the *in vitro* and *in vivo* antagonism against *A. tumefaciens* of the bacteria most frequently isolated from the crown gall.

MATERIALS AND METHODS

Location and duration of the study

This research was carried out in the laboratory of Phytopathogenic Bacteria of the Phytosanitary postgraduate program, and the greenhouse area of the Colegio de Postgraduados, Campus Montecillo, State of Mexico. The first stage involved the isolation of bacterial populations associated with raspberry crown gall tissue and *Agrobacterium tumefaciens*. The most frequently isolated bacteria were molecularly identified by amplification and partial sequencing of the *16S rRNA* gene. *A. tumefaciens* was biochemically characterized and identified by direct sequencing of the *virD2* plasmid region and *in vitro* pathogenicity tests. In the second stage, the *in vitro* antagonism of the bacteria most frequently isolated from the crown gall was evaluated, among which the strains with the highest antagonism index against *A. tumefaciens* were selected. In the third stage, the most efficient antagonists were evaluated in

an *in vivo* model on tomato plants inoculated with *A. tumefaciens* under greenhouse conditions, with relative humidity > 70 %, and temperature between 30 and 33 °C. The research process began in March 2020 and ended in October 2021.

Plant material

In March and August 2020, six samples of raspberry var. Elvira plants were collected from Driscoll's nurseries in Tlaxcala, Mexico (19° 19′ 03.9″ N, 97° 55′ 44.7″ W) with crown gall symptoms. The galls collected were of different size and colour (non-lignified tissue). The galls were kept in a cooler with plastic bags for transfer to the laboratory, where they were disinfected with 2 % sodium hypochlorite for 2 min; then, they were washed twice with sterile distilled water and dried on absorbent paper for 5 minutes.

Isolation, pathogenicity and determination of *Agrobacterium tumefaciens* biovar

Isolation of *A. tumefaciens* was performed from 1 g of tissue from the periphery of the gall in 100 mL of sterile distilled water that was kept in agitation at 90 rpm for 30 min, to allow bacterial diffusion. From this suspension, 20 µL were plated on D1-M culture medium (Schaad *et al*., 2001; Alippi *et al*., 2011) and incubated at 28 °C for 72 h. Six colonies (one per gall from each raspberry plant) were purified from the bacterial growth with the morphology described for *A. tumefaciens* on D1-M medium (Alippi *et al*., 2011). Pathogenicity was determined by inoculation of carrot slices and tomato (*Solanum lycopersicum*) and sunflower (*Helianthus annuus*) plants. Biovar determination was carried out by the method described by Cubero and López (2001).

Isolation of bacterial populations from crown gall

From each gall (n = 6), 1 g of tissue was used in 10 mL of sterile water that was kept at rest for 30 min. From this suspension, serial dilutions were made from 10^{-1} to 10^{-3}, and 100 µL of each dilution were plated with three replicates on nutrient agar (AN) and R2A agar culture medium. Plates were incubated at 28 °C for 48 h. Total bacterial density (UFC g^{-1} of tissue) was estimated by direct plate count. From the bacterial growth, 13 bacterial colonies with the highest frequency of isolation among the six gall samples were selected by morphology.

Molecular identification of *A. tumefaciens* and native bacteria in the gall

Genomic DNA was obtained with the commercial kit Qiagen® Germany. DNA concentration and quality were checked in a NanoDrop 2000 (Thermo Fisher Scientific, USA). The identification of *A. tumefaciens* was performed by direct sequencing of the *virD2* gene with primers A (59-ATG CCC GAT CGA GCT CAA GT) and C (59-TCG TCT GGC TGA CTT TCG TCA TAA), and PCR conditions described by Haas *et al*. (1995). The *virD2* gene sequences were compared with homologous sequences in the National Center for Biotechnology Information (NCBI) database.

The most frequently isolated native bacteria (n = 13) were identified by partial amplification of the *16S rRNA* gene with primers 8F (5′-AGA GTT TGA TCC TGG

CTC AG-3′) and 1492R (5′-CGG TTA CCT TGT TAC GAC TT-3′), and PCR conditions described by Baker *et al*. (2003). Amplification was performed on a thermal cycler (C1000 Touch TM Thermal Cycler). The amplified fragments were sequenced at Macrogen Inc. (Korea) and compared at the National Center for Biotechnology Information (NCBI) gene bank (http://www.ncbi.nlm.nih.gov/Blast) using the Blastn algorithm.

In vitro antagonism of crown gall bacteria against *A. tumefaciens*

In vitro antagonism against *A. tumefaciens* of 13 most frequently isolated strains was performed by dual culture on AN medium. Plates were inoculated with 250 µL of a suspension of *A. tumefaciens* with $1x10^7$ UFC mL^{-1} adjusted with the McFarland scale and confirmed by plate count on AN medium. Then, the bacteria were inoculated by puncture using a sterile toothpick with bacterial mass and incubated at 28 °C for 72 h. Antagonism was evaluated with four replicates. With the inhibition halo data (mm), an analysis of variance and comparison of means by the Tukey's test ($p \leq 0.05$) were performed with the R program version 3.6.1. Likewise, the Antagonist Efficiency Index (IEA) was calculated by the method described by El-Yazeid *et al*. (2007), which consists of the area of the halo produced by the antagonist divided by the area of colony growth, as it follows:

$$IEA = \frac{\text{Halo area}}{\text{Colony area}}$$

In vivo antagonism of crown gall bacteria against *A. tumefaciens*

Three bacterial strains that showed *in vitro* antagonism against *A. tumefaciens* were inoculated individually; likewise, eight strains identified as *Pseudomonas* sp. were selected for consortium inoculation from a 48-h pure culture incubated at 28 °C on AN. Individual and consortium inoculation was performed by immersion of root and stem collar of tomato var. Ramses plants 20 d after germination in a suspension containing 1.5×10^8 UFC mL^{-1} for 30 min. The inoculated plants were transplanted in black plastic bags (2 kg) with sterile soil-Agrolita® substrate (75:25), and kept in a greenhouse for seven days with relative humidity > 70 %, and temperature between 30 and 33 °C.

Inoculation of *A. tumefaciens*

Tomato plants were inoculated with *A. tumefaciens* directly on the substrate for seven days with 150 mL of a bacterial suspension of $1x10^7$ UFC mL^{-1}. As controls, the following were used: 1) plants inoculated with *A. tumefaciens* without antagonists, and 2) plants irrigated with 150 mL of sterile water on the substrate. The plants were maintained in the greenhouse under the environmental conditions described above for 40 d; the variable analysed was stem length (mm) with tumour development, which was measured with a vernier caliper (Mitutoyo series 530 standard model).

Statistical analysis

The *in vivo* experiment was set up in a greenhouse as a completely randomized design with five replicates. With the data of stem length (mm) with tumours, a descriptive statistical analysis was performed; normality and homogeneity of the data were performed with Shapiro-Wilks and Levene tests respectively, analysis of variance and comparison of means by Tukey's test ($p \leq 0.05$) in R-UCA 4.1.1 (Proyecto R Universidad de Cádiz, https://knuth.uca.es).

RESULTS AND DISCUSSION

Six strains (A1-A6) with morphology similar to *A. tumefaciens* were isolated from raspberry galls; out of these, strain A1 caused abnormal tissue proliferation in carrot slices and tumours in tomato and sunflower plants. The observed symptoms confirm the presence of tumorigenic strains of *A. tumefaciens* and *A. rhizogenes* (Schaad *et al*., 2001). Strain A1 metabolized sucrose, melezitose, but not dulcitol, erythritol, L-tartaric acid or citrate, and was included in biovar 1, according to the description of biovars by Cubero and López (2001). These results are consistent with the relationship of tumorigenic strains of *A. tumefaciens* with biovar 1, which have high adaptive capacity to colonize niches such as soil, water, and plants (Gelvin, 2018). By PCR, strain A1 amplified an approximate 450 bp fragment reported by Haas *et al*. (1995) for the *virD2* gene.

Sequence comparison at the National Center of Biotechnology Information (NCBI) gene bank showed 98 % similarity to the *Agrobacterium tumefaciens virD2* gene sequence (Accession number LC05233232.1). The *virD2* gene is part of the Ti plasmid sequence and is considered essential for the initiation of the infection process, colonization and insertion of the plasmid into the host plant genome. *Agrobacterium* species lacking the *virD2* gene lose their tumorigenic capacity (Gelvin, 2018); therefore, the results confirm that strain A1 used in this research corresponds to tumorigenic *A. tumefaciens*. The results of this research demonstrated that different densities (UFC g^{-1} of tissue) of native bacterial populations outside the genus *Agrobacterium* colonize raspberry gall tissue. However, the results of the statistical analysis showed no significant difference in population density between the galls in both culture media (AN and R2A). Among the bacterial populations isolated from the six galls, diversity was limited, identifying 13 different bacterial morphotypes with the highest frequency of isolation.

Bacteria colonizing the same niche can establish beneficial or competitive relationships (Platt *et al*., 2012). It is known that *A. tumefaciens* can coexist with a large number of bacteria in the soil and rhizosphere; however, coexistence in the tumour with other microorganisms has been little explored. During gall development, *A. tumefaciens* can induce the production of various kinds of opines such as nopaline, agrocipine and octopine, which *A. tumefaciens* uses as carbon and energy sources, creating a highly specific ecological niche for its life cycle (Quispe-Huamanquispe *et al*., 2017). However, the specific opines used by *A. tumefaciens* could favour the establishment of other bacterial species (Nester, 2015), able to use these same compounds as carbon sources,

colonize the gall tissue and possibly establish a competitive relationship (Lacroix and Citovsky, 2019).

In other plant species, approximately 40 different opine types have been characterized in tumours caused by *A. tumefaciens* (Meyer *et al*., 2019); therefore, the results of this research would suggest that the types of opine synthesized in the raspberry tumour and other organic and mineral compounds defined by the host, influence the density and limit the bacterial diversity that colonize the same tumour tissue. Among the bacterial populations in raspberry tumours, 83 bacteria were isolated, among which 13 morphotypes (16 %) were identified with the highest frequency of isolation.

Comparison of *16S rRNA* gene sequences in the National Center for Biotechnology Information (NCBI) gene bank identified 8 strains (61.5 %) in the genus *Pseudomonas*, with a percentage of similarity between 95.9 and 100 %: *P. putida*, *P. citronellolis*, and *P. plecoglossicida*; 2 strains (15.3 %) in the genus *Bacillus*: *B. subtilis* and *B. halotolerans*; and 3 strains (23 %) as *Delftia* sp. and *Alcaligenes faecalis*. *Pseudomonas* spp. were the most abundant colonizers in the tumour tissue (Table 1).

Pseudomonas is the genus with the highest abundance and adaptation in numerous ecological niches; they are identified as microorganisms with great competitive ability due to their capacity to metabolize a wide range of organic compounds as energy sources (Hesse *et al*., 2018). The results of this research are congruent with other studies demonstrating that *Pseudomonas* and *Agrobacterium* species can establish close communication in tumour tissue. Some *Pseudomonas* species have been found to coexist efficiently with *Agrobacterium* due to the expression of genes for the biotransformation of opines produced in the tumour by *A. tumefaciens* (An *et al*., 2006).

In this study, *P. putida* (15.3 % frequency) was identified (Table 1) as the most frequent *Pseudomonas* species within crown gall tissue. Some strains of *P. putida* can metabolize specific opines such as mannopine in tumours caused by *A. tumefaciens* (Meyer *et*

Table 1. Molecular identification by partial amplification of the 16S rRNA gene of 13 strains most frequently isolated from raspberry gall tissue.

ID strain	Gall sample	Identification	Accession number
AAC2	A2	*Delftia* sp.	OP740740
AAC12	A3	*Pseudomonas putida*	OP763643
AAC8	A5	*Pseudomonas* sp.	OP740746
AAC3	A6	*Pseudomonas* sp.	OP740741
AAC11	A1	*Pseudomonas putida*	OP776662
AAC9	A4	*Pseudomonas citronellolis*	OP740745
AAC1	A4	*Alcaligenes faecalis*	OP776337
AAC4	A2	*Pseudomonas* sp.	OP740742
AAC13	A3	*Pseudomonas plecoglossicida*	OP740747
AAC10	A1	*Bacillus subtilis*	OP776663
AAC6	A2	*Bacillus halotolerans*	OP740743
AAC5	A6	*Alcaligenes faecalis*	OP776661
AAC7	A5	*Pseudomonas* sp.	OP740744

al., 2019). Also, a study by Bell *et al*. (1990) showed that some *Pseudomonas* species were more efficient at metabolizing octopine *in vitro* in co-culture with *A. tumefaciens*. *Pseudomonas* spp. are natural root colonizers, mainly inhabiting the epidermis and exudation sites in plants (An *et al*., 2006). In tumours caused by *A. tumefaciens*, abundant exudation of water and nutrients occur, which may also explain the more frequent success in colonizing these tissues.

In vitro antagonism against *A. tumefaciens* of the 13 bacterial strains most frequently isolated from raspberry tumours showed that strains *Delftia* sp. (AAC2), *Alcaligenes faecalis* (AAC5), and *Pseudomonas citronellolis* (AAC9) were antagonistic against *A. tumefaciens*. Results of the IEA statistical analysis showed differences ($p \leq 0.05$) among the strains. The highest degree of antagonism was observed with *Alcaligenes faecalis* (AAC5), followed by *Delftia* sp. (AAC2). With *Pseudomonas citronellolis* (AAC9) there was no significant difference (Figure 1).

In the *in vivo* antagonism assay, tomato var. Ramses plants were susceptible to *A. tumefaciens* infection. With the interaction of antagonists + *A. tumefaciens*, no treatment suppressed infection and tumour development; however, with the data of length (mm) of infected stem (tumour development), the results of the statistical analysis showed differences ($p \leq 0.05$) between treatments. With the inoculation of *Alcaligenes faecalis* (AAC5), stem length with tumours was less than the rest of the antagonists, followed by *Delftia* sp. (AAC2) and *Pseudomonas citronellolis* (AAC9). With the inoculation of *Pseudomonas* spp. consortium (CPS) there was no difference with the control (Table 2). At 40 d after inoculation (ddi), tumours of different sizes developed in the neck and stem of the plants in all treatments. Inoculation with *Alcaligenes faecalis* (AAC5) was less severe than the rest of the treatments (Figure 2).

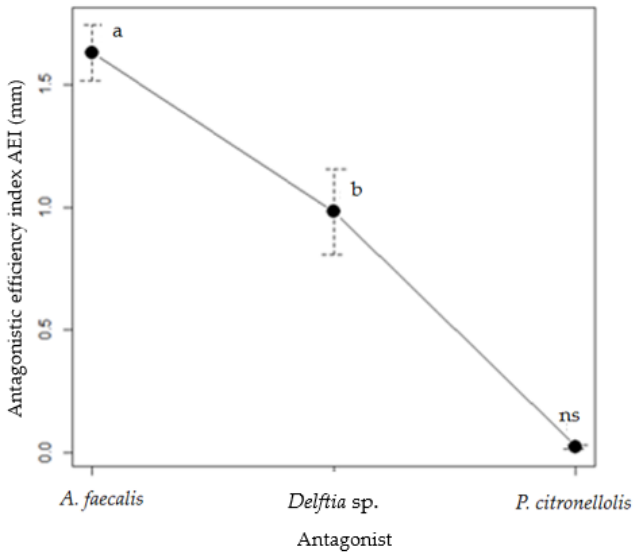

Figure 1. Antagonism Efficiency Index (mm) of native bacteria on raspberry gall against *A. tumefaciens*. According to Tukey's test, a and b are statistically significant; ns, non-significant.

Table 2. Stem length infected (mm) with tumours on tomato plants with the interaction of antagonists + *A. tumefaciens*. According to Tukey's test, different letters indicate significant variables ($p \leq 0.05$); ns, non-significant.

A. tumefaciens inoculation	Treatment	Average length of infected stems (mm)
Soil-Agrolita® (75:25) sterile substrate	Positive control	106
	Alcaligenes faecalis	28.92a
	Delftia sp.	39.06b
	P. citronellolis	47.7c
	CPS (*Pseudomonas* spp. consortium)	66.7ns

With *Delftia* sp. (AAC2), tumours of different sizes developed, dispersed, and a widening of the stem was observed, which may be a response to infection by *A. tumefaciens*. With *Pseudomonas citronellolis* (AAC9) the tumours were larger and longer on the stem. With the *Pseudomonas* spp. consortium, greater severity was observed. In some areas of the stem, the smaller tumours coalesced to form large areas with tissue protrusions (Figure 2). Control plants watered with sterile water in the substrate did not develop any symptoms.

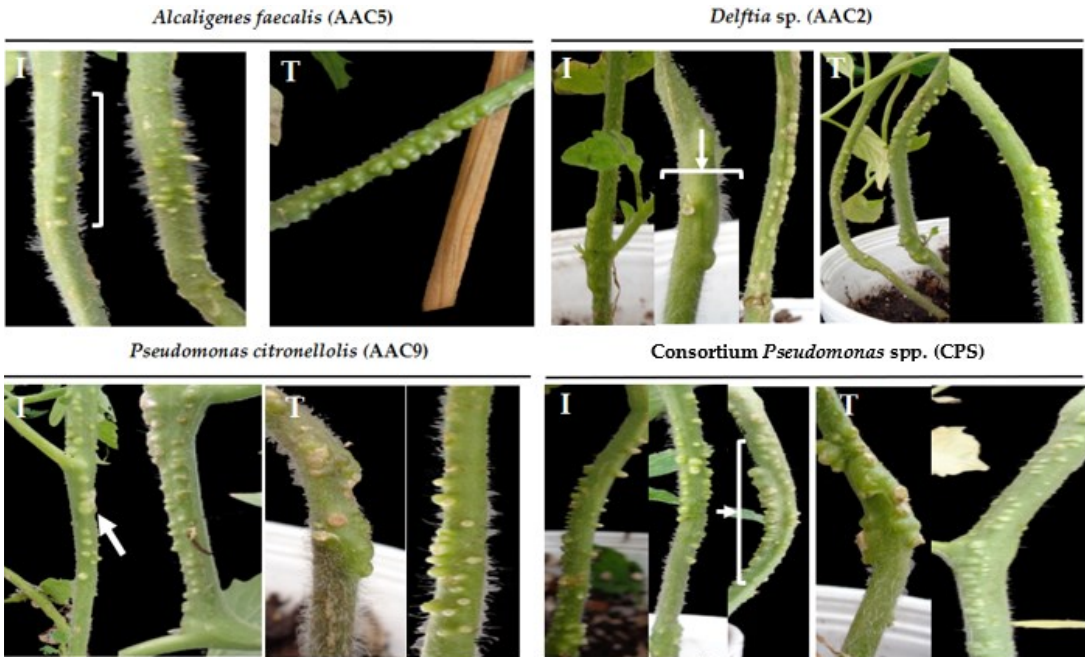

Figure 2. Severity of tumours in the stem of tomato plants var. Ramses after 40 d of antagonistic interaction with *A. tumefaciens*. I: root inoculation 1.5 x 10^8 UFC mL^{-1} of the antagonist + 1x10^7 UFC mL^{-1} of *A. tumefaciens* for 7 d on substrate; T: inoculation of *A. tumefaciens* on substrate without antagonist.

In this research, *Alcaligenes faecalis* (strain AAC5) showed the highest degree of *in vitro* antagonism and *in vivo* protection in tomato plants against *A. tumefaciens*. Other *A. faecalis* strains used as biocontrol agents showed that their genome harbours genes to produce proteins, antimicrobial peptides and siderophores. *A. faecalis* is considered an ecologically important species and its potential for use in agriculture is broad and functional (Lahlali *et al*., 2020; Saikia *et al*., 2022). The *A. faecalis* strain (AAC5) did not suppress *A. tumefaciens* infection in tomato plants; however, it significantly (27 %) reduced tumour severity. Yokoyama *et al*. (2013) related the antagonism of *A. faecalis* to its bacteriostatic activity, which may explain why in this study it only reduced tumour severity without showing bactericidal activity. The high frequency of *A. faecalis* isolation in raspberry tumours may be related to the efficiency to metabolize the type of opines produced in that tissue; other strains of *A. faecalis* have been characterized for their metabolization of phenolic and amino acid compounds (Ray *et al*., 2020).

The interaction between *A. faecalis* and *A. tumefaciens* in other plant species was reported in the study by Limanska *et al*. (2019), who analysed the bacterial community in flowers and grape berries of *Agrobacterium* sp. infected plants. They showed that *A. faecalis* and *Agrobacterium* coexist in this niche, highlighting the ability of the latter to coexist with antagonistic bacteria. This suggests that the presence of *A. faecalis* in raspberry tumours may be related to the interaction with *A. tumefaciens*. Therefore, further studies should focus on elucidating the factors that determine such a relationship, in order to better understand *A. faecalis* potential as a biological control agent against *A. tumefaciens*.

Species within the genus *Delftia* are considered efficient colonizers in different types of plant tissue due to their higher efficiency to metabolize various carbon and amino acid sources (Braña *et al*., 2016) and to biotransform organic and inorganic compounds, which is why their application in agriculture has been of interest (Cagide *et al*., 2018). These metabolic characteristics may explain their higher prevalence in raspberry tumour tissue. There are currently no reports of *Delftia* spp. antagonizing *A. tumefaciens* in *in vitro* and *in vivo* assays; however, other research has reported its biocontrol efficiency against phytopathogenic bacteria and as a plant growth promoter mediated by nitrogen fixation and the production of phytohormones, such as gibberellins and auxins that promote root development. Likewise, some species stand out for the production of siderophores, establishing competition for space and nutrients in different niches (Cagide *et al*., 2018) which can be related to reduced severity and protection in the development of tumours in this research.

Delftia tsuruhatensis, *D. lacustris*, and *D. acidovorans* have been identified as antagonists and biocontrol agents (Han *et al*., 2005). In this study, identification by partial sequencing of the *16S rRNA* gene evidenced the presence of bacteria of the genus *Delftia* in raspberry gall tissue. Phylogenetic analysis showed that this strain is related to *D. acidovorans*, which is identified as a plant growth promoter (Khalifa and Almalki, 2019).

Pseudomonas citronellolis (AAC9) caused less protection against *A. tumefaciens*. However, strains of *P. citronellolis* are considered efficient colonizers of diverse

niches such as root and phyllosphere in plants. Where this bacterium establishes a competitive interaction mainly by the production of siderophores, displacing other bacteria (Remus-Emsermann *et al*., 2016). The efficient root colonization of this species may explain the protection against *A. tumefaciens*. As a biocontrol agent, strains of *P. citronellolis* are identified as efficient antagonists in the suppression of bacterial diseases in agricultural soils. As well as with *Delftia* sp. there are no reports to our knowledge on the antagonism and protection against *A. tumefaciens* by *P. citronellolis.* Therefore, this study provides the first information on *P. citronellolis* potential as a biocontrol agent against *A. tumefaciens*.

Inoculation of the consortium (strains AAC8, AAC3, AAC4, AAC12, AAC11, AAC9, AAC7, AAC13) of *Pseudomonas* spp. showed no differences with the control. In this regard, the cooperative dynamics between bacterial species for the colonization of a particular niche is known to demand a high competition for nutrients; thus, an optimal population density among bacteria is essential for the functionality of a bacterial consortium (Platt *et al*., 2012). Due to this, it is possible that the bacterial density of the *Pseudomonas* spp. consortium inoculated in this study (1.5×10^8 UFC mL^{-1}) could be below the appropriate cell density threshold for optimal consortium functionality in protection against *A. tumefaciens*.

In this research, *A. faecalis* (AA5), *Delftia* sp. (AAC2) and *P. citronellolis* (AAC9) strains reduced the severity of tumours caused by *A. tumefaciens*. Other studies highlighted that the efficiency of *A. faecalis* antagonism against bacteria depends on the synthesis of antimicrobial compounds of protein and enzymatic origin, produced mainly in the exponential growth stage (Lahlali *et al*., 2020). Likewise, Remus and Emsermann *et al*. (2016) demonstrated that efficient protection against plant pathogens by *P. citronellolis* depends on high inoculum densities. On this account, in this study, the antagonistic strains were inoculated at a density of 1.5×10^8 UFC mL^{-1} from a pure culture with 48 h of growth. Which requires further study of different inoculum densities, mainly at concentrations over the threshold of 1.5×10^8 UFC mL^{-1}, and growth stages of the antagonists evaluated here onto a plausible increased protection against *A. tumefaciens*.

CONCLUSIONS

Pathogenic *Agrobacterium tumefaciens* biovar 1 was found in raspberry crown gall tissue with the presence of the *virD2* gene of the tumour-inducing (Ti) plasmid. Different densities of other bacterial populations were found to colonize these galls with limited diversity, *Pseudomonas*, *Bacillus*, *Alcaligenes* and *Delftia* were the most frequent genera. Among these, *Pseudomonas* were the most abundant and frequent colonizers.

Raspberry crown galls harbour bacteria antagonistic against *A. tumefaciens*. *Alcaligenes faecalis*, *Delftia* sp., and *Pseudomonas citronellolis* proved to be *in vitro* antagonists against *A. tumefaciens*. *In vivo* inoculation of these antagonists did not inhibit *A. tumefaciens* infection but did reduce tumour severity. *A. faecalis* isolated from raspberry crown gall was the most efficient *in vitro* and *in vivo* antagonist against *A. tumefaciens*.

ACKNOWLEDGEMENTS

To Driscoll's Inc., Tlaxcala for providing the raspberry plant material with crown gall. To the Phytosanitary-Phytopathology Postgraduate Program under the Colegio de Postgraduados Campus Montecillo and the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the support to conduct this research.

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