Tumorigenic bacteria from blueberry stem galls are related to *Rhizobium tumorigenes*-like Agrobacteria found in Rhododendron crown gall in Germany.

By

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ABSTRACT

Blueberry stem galls in Pacific Northwest are often attributed to *Agrobacterium tumefaciens*. The disease also is associated with indole-3-acetic-acid (IAA) producing bacteria, as described on cranberry stem gall. Lack of studies makes it difficult to conclude whether these two types of bacteria could be the causal agent of stem gall of blueberry. Isolation methods in other studies failed to recover consistently tumorigenic bacteria from blueberry stem galls. In this study, two isolation methods were initiated and an effective one for recovering tumorigenic bacteria from blueberry stems was discovered. Micropropagating the infected blueberry plants enabled the isolation of the tumorigenic bacteria. When the isolates were tested with *virD2* gene specific primers in PCR analysis, positives results obtained. Moreover, gall symptoms were obtained on blueberry 'Draper' experimentally with the tumorigenic isolates, using a revised inoculation procedure described in this paper. Sequencing results of the partial *virD2* gene in this study suggest that the bacterium causing stem galls on blueberry is tumorigenic. It is similar in identity to the bacteria that cause crown gall disease on Rhododendron in Germany, which is known as a novel group of *Rhizobium tumorigenes*-like Agrobacteria.

INTRODUCTION

In 2017, a severe outbreak of stem gall on stems of mature 'Draper' blueberry plants was observed in several fields in Oregon (V. Stockwell, *personal communication*). Growers must decide if they should remove the plants immediately or wait until plants are no longer producing fruit. Blueberry is a very important crop in the state (Lies, 2018) but there are no effective methods

to cure infected plants with stem gall and it is unknown if other blueberry cultivars are as susceptible to the pathogen responsible for inducing the galls in 'Draper' cultivar.

The bacterium *A. tumefaciens* was hypothesized to be the causal agent of stem gall disease on blueberry in Pacific Northwest. However, there is insufficient evidence to prove this bacterium is responsible for the disease because tumorigenic bacteria are rarely isolated from galls on blueberry stems. *A. tumefaciens* is a plant pathogen that causes crown gall tumors after infection on wounded plants. Crown gall tumors contain a DNA segment from the bacterium called T-DNA. The origin of the T-DNA is from the bacterial Ti-plasmid present in the tumor-inducing bacterium. When plants get infected, the T-DNA of the Ti-plasmid is integrated with the plant genome causing disruption of hormones such as auxin and cytokinin, and the infected tissue forms a tumor (Chilton *et al.,* 1980 and Anand *et al.*, 2006). Sizes of galls can vary from plant to plant and can easily be confused with other gall-like growths caused by auxin indole-3-acetic-acid (IAA) producing bacteria or fungi (Canfield *et al.,* 1995). PCR analysis to detect the conserved tumorigenic *virD2* gene in bacteria isolated from blueberry galls is often negative. McManus *et al.* (2004) previously described a causal agent of cranberry stem gall as IAA-producing bacteria. The morphology of stem galls on cranberry is similar to the ones observed on blueberry. Possibly, IAA-producing bacteria also could be a causal agent of blueberry galls. However, further studies were needed to verify this.

Crown gall disease is easily spread in the fields and is able to survive in soil or in gall tissue in the soil for at least 2 years (Burr *et al.*, 1995). Water and contaminated soils are common sources of crown gall infections. Blueberry fields are pruned each winter to remove old, spent stems and encourage growth of new stems. During pruning of the fields, a worker may inadvertently cut through gall tissue and may spread the bacterium to new stems or plants with subsequent pruning

cuts. Additionally, cut stems are generally left to decompose in fields between the plant rows, but they may also be a source of the pathogen for new infections. *A. tumefaciens* enters plants through wounds on the stems. Damage on plant tissues caused by frost, insect injury, and human activities can be entry points for the pathogen and infection sites. After a plant has symptoms of stem gall, fruit yields decrease (Cubero *et al.*, 2001). For blueberries in Oregon, yields were reduced on galled plants by up to 30% (Yang and Stockwell, unpublished data). Diseased canes generally produced smaller and fewer berries and some cane with severe galling do not produce any berries. There also is a positive correlation between machine harvesting and an increased incidence of stem gall in the field. (Yang and Stockwell, unpublished data). Blueberry stem gall disease is rarely seen in hand-picked blueberry fields (V. Stockwell, *personal communication*).

Stem gall is not considered a common disease of blueberry, but when an outbreak occurs, the results can be devastating (Bristow *et al*., 2017). Currently, growers completely remove the stems observed to have stem gall symptoms. A question arises on the effectiveness of this method, as researchers are uncertain whether the pathogen spreads internally through blueberry tissue or is only localized to the gall. If the pathogen is 'systemic', such as with *Agrobacterium vitus*, then removing infected stems will not control the disease. If the pathogen is 'non-systemic' or solely caused by IAA-producing bacteria, then stem removal and application of antibacterial compounds, like copper ions, may control the disease.

It is important to isolate the pathogen and test its pathogenicity experimentally to be able to proceed with ways to control it. By developing reliable pathogenicity assays specifically for blueberry, researchers can test possible control methods. Isolating pathogenic *Agrobacterium* from blueberry galls is challenging, as galls are often colonized by other genera of bacteria, yeasts and

fungi. By using a revised isolation method that includes Koch's Postulates, isolation of blueberry stem gall pathogens can be improved.

The objectives of this study were to (i) identify the bacteria that are associated with blueberry stem gall and are capable of causing symptoms on healthy blueberry plants and (ii) develop pathogenicity assays for stem gall on blueberry. The main method to achieve these goals used Koch's Postulates to confirm the causal agent of the disease.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used as controls in this study are listed in Table 1. In the laboratory, all bacterial strains and isolates were cultured routinely on solidified potato dextrose agar amended with 0.5% $CaCO_3$ (PDA-Ca) (Moore *et al*., 2001), mannitol-glutamate-yeast extract (MGY) (Moore *et al*., 2001), or King's medium B (KB) (King *et al*., 1954). All bacterial isolates were stored at -80°C in cryovials containing nutrient broth amended with 15% glycerol and nutrient broth amended with 7% DMSO.

Table 1. Previously described bacteria used in this study

Strain	Bacterial species	Source	Pathogenicity assays (this study)				PCR analysis		
			Tomato	*Bryophyllum*	Sunflower	*'Draper'*	*virD2*	*tms2*	*flaA*
A506	*Pseudomonas fluorescens*	Pear	-[a]	NT[b]	NT	-	-	NT	NT
B49C	*Agrobacterium tumefaciens*	Apple	+	+	NT	-	+	+	NT
C58	*Agrobacterium tumefaciens*	Cherry	+	+	NT	-	+	+	NT
K84	*Agrobacterium radiobacter*	A. Kerr	-	-	NT	-	-	-	-
JL5150	*Agrobacterium tumefaciens*	Blue-berry	+	NT	+	-	+	NT	-
JL5198	*Agrobacterium tumefaciens*	Grape	+	NT	+	-	+	NT	-

[a] "-" denotes that galls were not observed on inoculated tissues; "+" denotes that galls formed at the site of inoculation.
[b] NT = not tested

Isolation of bacteria from galls on blueberry stems in commercial blueberry fields. Galled blueberry stems were collected from symptomatic plants in blueberry fields in Oregon. Galls were small and numerous with a cream-to-light brown color and spongy texture.

Stem segments with galls were submerged in 10% bleach for 10 min, then in 70% alcohol for 2 min, followed by 2 rinses with sterile distilled water. The galls were cut off of the stem with a flame-sterilized, single-edge razor blade and chopped into small pieces. The diced tissue was suspended in sterile 10 mM phosphate buffer and incubated for 10 min. The tissue suspension was streaked onto the following semi-selective media for isolation of agrobacteria from environmental samples, media IA and 2E (Brisbane and Kerr, 1983) and MGYT amended with cycloheximide (50 µg/ml) and thallium nitrate (80 µg/ml) (Mougel *et al*., 2001). Colonies that developed on the semi-selective media were transferred to KB, MGY, and PDA-Ca to differentiate *Agrobacterium* spp. from other environmental bacterial genera such as *Pseudomonas* spp. and *Pantoea* spp. by colony morphology and pigmentation. Representative colonies of predominant morphological types of bacteria were transferred twice and stored at -80°C.

Bacterial DNA extraction and PCR assays. The colonies from field isolates were grown for 16 to 24 h at 27°C on a rotary shaker at 200 r.p.m. in nutrient broth (Difco Laboratories, Detroit, MI). Total genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD), according to the manufacturer's instructions for Gram-negative bacteria. Genomic DNA also was isolated from two control strains; a known crown gall pathogen *A. tumefaciens* strain C58 and the non-pathogenic *Agrobacterium radiobacter* strain K84.

The PCR primer pair A/C' were used to detect the gene *virD2* carried on the pTi of gall-forming agrobacteria (Table 2) (Haas *et al*., 1995). Each reaction tube contained 15.5 µl MilliQ H2O; 2.5 µl Buffer (10X); 1.5 µl MgSO4 (25 mM); 2 mM each dATP, dCTP, dGTP, and dTTP;

0.5µl of Taq (KOD Hot Start polymerase, EMD Millipore, Burlington, MA); and 10 µM of each primer, and 1 µl of template DNA at a concentration between 40 to 100 ng/µl. The amplification conditions were: initial denaturation at 95°C for 2 min, followed by 29 cycles of denaturation at 95°C for 20 sec, annealing at 55°C for 20 sec, extension at 68°C for 20 sec and then a final extension at 68°C for 5 min. PCR products were separated with gel electrophoresis with 1% agarose containing ethidium bromide and visualized with UV transillumination.

Genomic DNA of isolates that were PCR-positive for *virD2* was used for PCR assays to amplify 16S rRNA (Weisburg *et al.*, 1991) and *fla*A (Kuzmanovic *et al*., 2019) (Table 2). Amplicons of *virD2* and 16S rRNA were sequenced using Sanger sequencing by the Core Laboratory at the Center for Genomic Research and Bioinformatics at Oregon State University.

Table 2. PCR primers used in this study

Primer names	Target	Amplicon Size (bp)	Sequence (5'-3')	Annealing temperature (ºC)	Source
fD1	16S		AGAGTTTGATCCTGGCTCAG		Weisburg *et*
rP2	rRNA	~1400	ACGGCTACCTTGTTACGACTT	55	*al*. 1991
tms2F1			TTTCAGCTGCTAGGGCCACATCAG		Puławska *et*
tms2R2	*tms2*	458	TCGCCATGGAAACGCCGGAGTAGG	60	*al.* 2004
A'			ATGCCCGATCGAGCTCAAGT		Haas *et al.*
C'	*virD2*	224	TCGTCTGGCTGACTTTCGTCATAA	55	1995
			GTTTGAAAGTCGCTTCCGCATCC		Kuzmanović,
TFP_R	*flaA*	304	CTCAGCATGTTGACGCCGTTG	62	*et al.* 2019

Indole-3-acetic-acid production by bacterial isolates. Isolates from field galls that caused gall-like callus growth in inoculation sites on tomato were tested for production of IAA. The isolates were cultured in three replicate test tubes containing KB broth amended with 0.2 mg ml^{-1} L-tryptophan at 200 r.p.m. for 48 hr at 27°C. A 1.4 ml sample of the broth culture was placed in an eppendorf tube and cells were pelleted by centrifugation (5 min at 14,000 rpm). A 1 ml sample of

the culture supernatant was transferred to a glass test tube and combined with 2 ml of reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) (Gordon and Weber, 1951). The samples were incubated at room temperature for 30 min, and OD_{530nm} was measured using a spectrophotometer (Thermo Spectronic 20+). Non-inoculated broth plus reagent was used as a reference. Cultures of a known IAA-producer (*Pantoea vagans* C9-1) and a known non-producer of IAA (*Pseudomonas fluorescens* A506) were included as controls (Smits *et al*., 2011). Each isolate was tested twice with similar results.

Pathogenicity assays. The pathogenicity of bacteria isolated from blueberry galls was tested on herbaceous plants (tomato *Lycopersicon escultentum* 'Bonnie Best', *Bryophyllum daigremontianum*, sunflower *Helianthus annuus*) and blueberry *Vaccinium corymbosum* 'Draper' grown in a greenhouse. Tomatoes and sunflowers were inoculated at 4 to 5 weeks old, whereas the *Bryophyllum* were inoculated on the young emerging leaves. For blueberry plants, a mixture of matured and young 'Draper' were used. Herbaceous plants were inoculated by creating a wound by stabbing or pricking the stems with toothpicks or needles, followed by smearing bacteria on the wounds (Moore *et al.,* 2001). On blueberry plants, the surface of the stem was gently abraded with a fine metal file prior to applying bacteria to the wounded surface. The inoculation sites were left uncovered and observation was made after 3 to 4 weeks for symptoms. Occasionally, the inoculation sites were covered with dampened cotton followed by wrapping with aluminum foil or parafilm. This was attempted on a small number of matured blueberry 'Draper' as a trial because of the difficulty for the isolates to cause symptoms. Known pathogenic strains of *A. tumefaciens* were used as positive controls (Table 1). Inoculation tests were repeated at least twice for each isolate. In carrot assay, organic carrots were sterilized in bleach and alcohol similar to the procedures done on gall tissues. Carrots were sliced into discs shape (~5mm) and placed on

moistened filter papers on clean petri dishes with adaxial parts facing down. Five discs were placed on each petri dish with the center being the controls, which were discs inoculated with sterile water. Using sterile technique, 5µl sterile water was dispensed on each carrot disc to moisten the surface followed by inoculation of field gall isolates growing on media. Only several isolates field gall isolates were tested in carrot assay. Symptoms were observed after 3 to 4 weeks of inoculation. A modified inoculation method was performed with three isolates from blueberry explants, in which each isolate was suspended in sterile water amended with Break-thru (Plant Health Technologies, Lathrop, CA) and the cell concentration was adjusted to 10^9 colony forming units per ml. The negative control consisted of sterile water amended with Break-thru. The stems of young blueberry 'Draper' were dipped in the suspension for five seconds and incubated in a Percival model LED-30HL1 (Percival Scientific, Inc., Perry, IA) growth chambers with an IntellusUltra real time controller and LED lighting. Growth chambers were programmed for a 16-h photoperiod (60 µmol/m^2s) with daytime temperatures of 24 °C (± 0.5 °C) and nighttime temperatures of 18 °C (± 0.5 °C), and constant relative humidity 50% (±10%). Each treatment had four replicate plants. Plants were bottom-watered every two days and symptom development was monitored over a month.

Tissue culture of asymptomatic stems from plants with symptoms of stem gall. In August 2019, canes from blueberry mother plants cultivar 'Draper' with and without symptoms of stem gall were collected from the field. Asymptomatic shoot segments were cut from each cane and disinfected by submersion in 0.02% Tween for 20 to 30 min, then 25% sodium hypochlorite for 10 min, and then followed by three rinses with sterile deionized water. Single node explants, about 1-2 cm in length, were transferred into culture tubes. Media in culture tubes contained original woody plant media (WPM) amended with sucrose (20 g/L), agar (6 g/L), and trans-Zeatin (2

mg/L). pH value was adjusted to 5.3 before autoclaving (Gonzalez *et al*. 2000, Lloyd *et al*. 1980, Ostroluka *et al*. 2007, Reed *et al*. 1991, Smanhotto *et al.* 2019, and Zhang *et al.* 2006). Explants were incubated in a Percival model LED-30HL1 (Percival Scientific, Inc., Perry, IA) growth chambers with an IntellusUltra real time controller and LED lighting. Growth chambers were programmed for a 16-h photoperiod with daytime temperatures of 24 °C and nighttime temperatures of 18 °C, and constant relative humidity 50%. After two months, stems of clean explants were transferred to new culture tubes containing WPM. After 5 weeks, galls were observed on 12 explants. The galls were removed, surface disinfested (10% bleach for 1 min, 70% alcohol for 30 sec, and 2 rinses with sterile distilled water), diced, suspended in 1 ml 10 mM phosphate buffer, pH 7, and spread on PDA-Ca. Colonies were transferred twice, tested with PCR for presence of *virD2* and *fla*A (Table 2). Three isolates were tested for pathogenicity on tomato, sunflower, and blueberry.

RESULTS

Direct isolation of bacteria from galls collected from blueberry fields.

Bacteria were isolated from multiple samples of galled stems collected from Oregon blueberry fields, but only 50 isolates were further analyzed (data not presented). Bacteria showing the morphology typical of Rhizobiaceae species were isolated from blueberry galls on media 1A, 2E, and MGYT and transferred to PDA-Ca. Less than 30% of the total field isolates had *agrobacterium*-like colonies. These colonies were white to cream colored, circular, domed and mucoid. This isolation technique mostly recovered other types of bacteria such as *Pseudomonas* spp. and *Pantoea* spp. Among the *agrobacterium*-like colonies, only one isolate was clearly positive for *virD2* with the PCR assay (Table 3, Figure 1). The rest of the *agrobacterium*-like isolates were non-tumorigenic and also tested negative for *virD2* with the PCR assay (Figure 1).

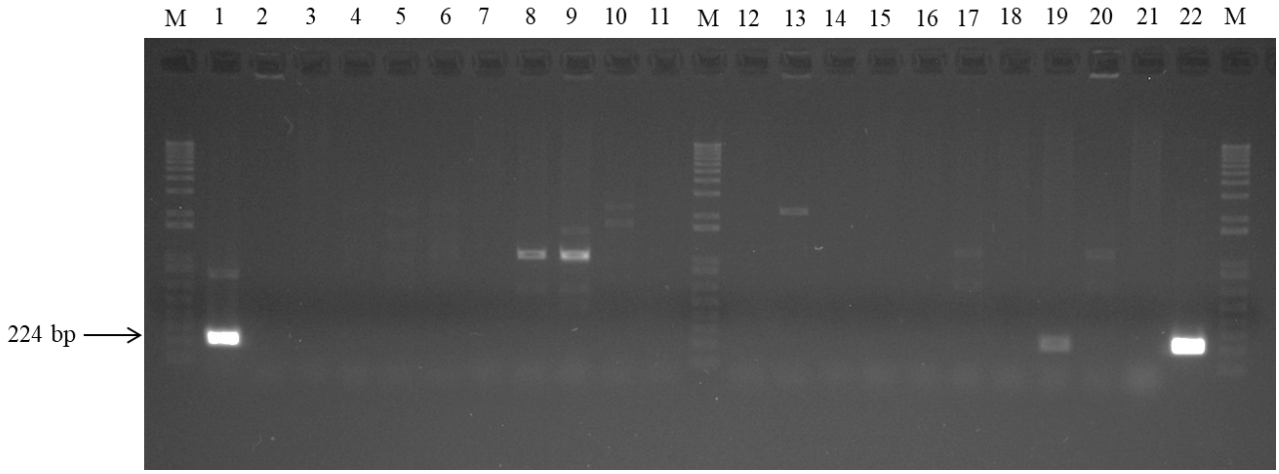

Fig 1. Electrophoresis gel showing PCR products obtained in *virD2* (A/C) PCR reaction with isolates from field galls; Lane 1 – A5A2 Red 1 (positive control); Lanes 2-18 – field isolates; Lane 19 – 6b*2e; Lanes 20-21 – field isolates; Lane 22 – C58 (*A. tumefaciens* positive control); M – 1 Kb+ marker.

***virD2* was rarely detected in bacteria isolated from galls collected from blueberry fields.**

Table 3 lists the bacteria isolated directly from galls from "Draper' blueberry plants in the field and from galls that developed on 'Draper' blueberry explants that were positive in the *virD2* PCR assay. All isolates analyzed were tested for the presence of Ti plasmid using *virD2* gene specific primers (A and C') yielding a 224 bp amplicon to confirm their tumorigenic characteristic. Other primers were also tested on isolates from previous isolation method such as tms 2F1/R2 and 16s rRNA; assist with differentiating between closely related bacterial species.

All isolates from the field galls were negative with *virD2* primers except for two isolates which are included in Table 3. However, the appearance of the band of isolate 6b*2e was faint in PCR gel analysis.

Pathogenicity assay with bacteria isolated from galls collected from blueberry fields. Pathogenicity assays were performed on 50 isolates gathered directly from field sites. Generally, field isolates did not reproduce symptoms of stem gall on tomatoes, *Bryophyllum* and blueberry

'Draper'. Field isolates were not tested for inducing stem galls on sunflowers. However, 29 isolates from the field were found to cause callus on tomato stems, but these isolates were confirmed to be non-tumorigenic based on PCR tests for *virD2*. Representative isolates were subsequently tested for production of IAA.

BLASTN analysis of partial gene sequences of 16S rRNA of *virD2*-negative isolates found matches with a variety of bacteria in the Genbank collection of the NCBI. The most common identity of the *virD2*-negative bacteria from field galls were to the genus *Rhizobium*, *Pseudominobacter* and *Agrobacterium*. Table 4 provides partial 16S rRNA sequence of two representative isolates.

Table 3. Tumorigenic bacterial strains isolated from blueberry 'Draper' galls in this study

		Pathogenicity assay results			**PCR analysis**	
Sample[a]	**Year of isolation**	**Tomato**	**Sunflower**	***'Draper'***	***virD2***	***flaA***
A5A2 Red1	2019	-[b]	-	-	(+)	-
6b*2e	2019	-	-	-	(+)	-
Tcg-1	2020	NT[c]	NT	NT	+	+
Tcg-2s	2020	NT	NT	NT	+	+
Tcg-3s	2020	+	+	+	+	+
Tcg-4s	2020	NT	NT	NT	+	+
Tcg-5s	2020	NT	NT	NT	+	+
Tcg-7s	2020	+	+	+	+	+
Tcg-8s	2020	NT	NT	NT	+	+
Tcg-9s	2020	NT	NT	NT	+	+
Tcg-10s	2020	NT	NT	NT	+	+
Tcg-11s	2020	NT	NT	NT	+	+

Tcg-13s	2020	NT	NT	NT	+	+
Tcg-14s	2020	+	+	+	+	+

[a] Isolation source. Samples in 2019 were isolated directly from symptomatic gall tissue from commercial blueberry fields near Jefferson, Oregon and Dayton, Oregon. Samples from 2020, designated 'tsg', were isolated from basal galls on micropropagation of blueberry stems that were originally collected from commercial blueberry fields near Jefferson, Oregon

[b] "-" indicates that no amplicon of the appropriate size was visible on the gel, "+" indicates that an application of the expected size was visible on the gel, and "(+)" indicates a faint band of a size similar to the amplicon specific for tumor inducing plasmids was observed

[C]NT = not tested

Table 4. Partial 16S rRNA sequence of two *virD2*-negative isolates from galls in commercial fields

Isolate	NCBI Results	Top Hit Accession Number	Consensus Sequence
2Bb	*Agrobacterium* sp.	AY776241.1	CAGTCGAACGCATCGCAAGATGAAGTGG CAGACGGGTGAGTAACGCGTGGGAATCT ACCGTACCCTACGGAATAGCTCCGGGAA ACTGGAATTAATACCGTATACGCCCTTT
2Ca	*Phyllobacterium* sp.	MK589716.1	TGCAGTCGAACGCCCCGCAAGGGGAGTG GCAGACGGGTGAGTAACGCGTGGGAATC TACCCAATTCTTCGGAACAACACATGGA AACGT

Analysis of IAA-production by bacteria from galls collected from blueberry fields.

Six isolates from field galls were tested for production of IAA. Five of the six isolates produced a significant amount of auxin in the IAA assays (Table 5). The amount of IAA produced was similar to the strain that is known to produce IAA and greater than the non-producer control strain. Since auxin can initiate the formation of stem callus, these isolates were tested on their ability to induce callus on carrots discs (Vasanthakumar *et al*., 2004). Inoculation of carrot discs with these isolates resulted in the formation of white to brown spongy-textured callus and roots on the surface of the carrot discs (Figure 2). Similar gall-like growths with roots were observed on tomato stems inoculated with IAA-producing isolates from galls collected from the field (Figure 3).

Table 5. Indole-3-acetic acid (IAA) production of field gall isolates measured using colorimetric assay of Gordon and Weber (1951). Data are averages from at least two assays for each isolate

Sample ID	Average quantity(± 1 standard deviation) of IAA (µg/ml) produced in culture
A506[a]	0.13 ± 0.03
C9-1[b]	0.44 ± 0.03
1Ab	0.25 ± 0.08
1Bb	0.27 ± 0.04
1Da	0.30 ± 0.06
2A	0.35 ± 0.05
2Bb	0.10 ± 0.05
2D	0.19 ± 0.05

[a] Non-producing IAA bacteria as a negative control. Values equal or below the value measured on this isolate are considered non-significant.
[b] IAA producing bacteria as a positive control. Values below the value measured on [a] are considered significant.

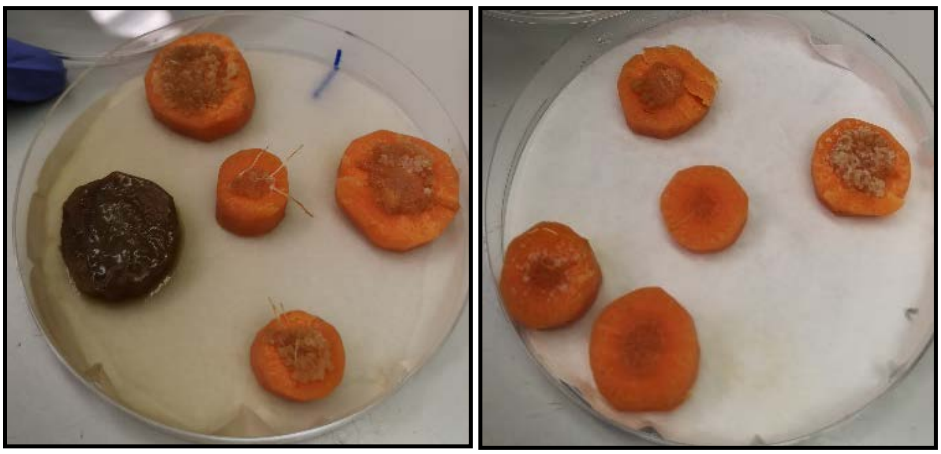

Fig 2. IAA producing bacteria obtained from blueberry stems; callus and root growth was observed on carrot discs inoculated on the basal surfaces with *virD2*-negative bacteria from field galls.

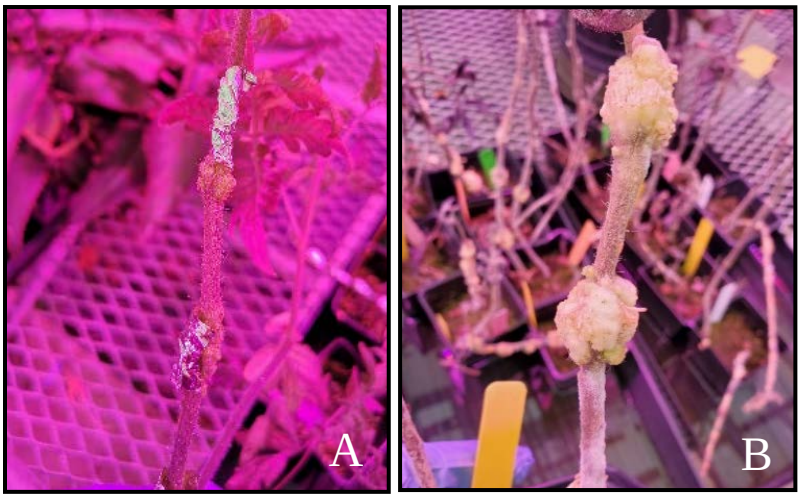

Fig 3. A, Galls on tomato stem in the greenhouse caused by IAA producing bacteria (obtained from field galls) two weeks after inoculation. **B**, Galls caused by IAA producing bacteria (obtained from field galls) on tomato stem four weeks after inoculation.

Isolation of bacteria from galls that formed on micropropagated blueberry stems.

A total of 216 explants were started in nutrient containing medium. After two months, 117 explants were lost due to fungal contamination. In the following transfer, a total of 97 viable explants were obtained. Twelve of the explants from micropropagated blueberry stems developed basal galls four months after initiation in media. The galls were tan and eventually darkened to a brown color.

Bacteria with a uniform colony morphology were isolated from each of the galls on PDA-Ca after seven days of incubation at 27ºC. The colonies were small, circular, white to cream-colored, and slightly mucoid. The isolates were transferred and grew well on PDA-Ca, but did not grow on other media developed for culturing agrobacteria such as IA, 2E and MGY (Figure 4). Acid production on PDA-Ca was observed as clearing zones in the agar beneath the bacterial growth (Schaad *et al.*, 2001).

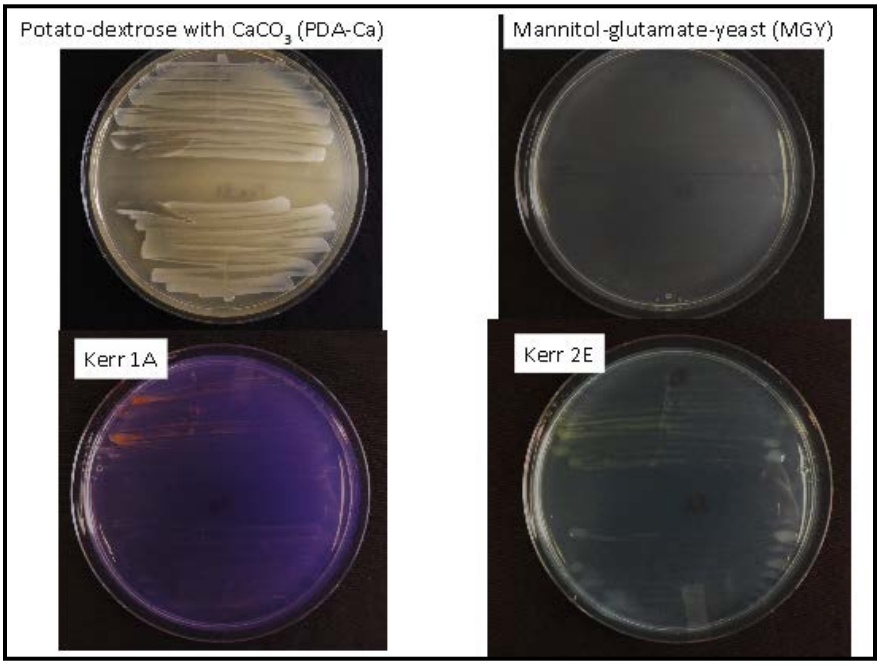

Fig 4. Growth of two isolates from tissue culture galls, Tcg-2s (top half of plate) and Tcg-3s (bottom half of plate), on media PDA-Ca, MGY, IA and 2E, six days after streaking and incubation at 27°C.

virD2 was detected in each bacterial isolate from galls that formed on cultured blueberry explants.

All isolates from blueberry explants were positive in the PCR assay with *virD2* gene specific primers (Table 3, Figure 5). The *virD2* amplicon was sequenced for the 12 bacterial isolates (Table 6). Among the 12 isolates, the *virD2* sequence of nine of the isolates were an identical match with *virD2* sequence of a novel *Rhizobium tumorigenes*-like agrobacteria strain recently isolated from stem galls on Rhododendron (Kuzmanovic *et al.* 2019). The *virD2* sequence of the remaining four strains did not match sequence in GenBank.

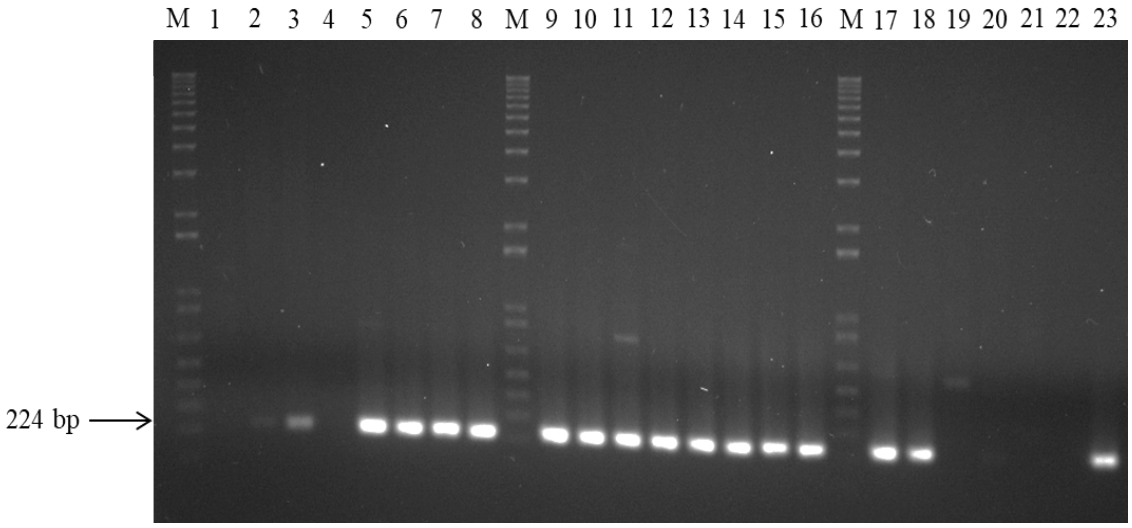

Fig 5. Electrophoresis gel showing PCR products obtained in *virD2* A/C' primers with isolates from galls on tissue culture explants; Lane 1 – K84 (*A. radiobacter* as negative control); Lane 2 – A5A2 Red 1; Lane 3 – 6b*2e; Lane 4 – 8b2e; Lane 5 – Tcg-1s; Lane 6 – Tcg-2s; Lane 7 – Tcg-3s; Lane 8 – Tcg-4s; Lane 9 – Tcg-5s; Lane 10 – Tcg-7s; Lane 11 –Tcg-8s; Lane 12 – Tcg-9s; Lane 13 – Tcg-10s; Lane 14 –Tcg-11s; Lane 15 –Tcg-13s; Lane 16 – Tcg-14s; Lane 17 – C58 (*A. tumefaciens* as positive control); Lane 18 – JL5150 (*A. tumefaciens* as positive control); 19 – JL5178; 20 – JL5184; 21 – JL5189; 22 – JL5193; 23 – JL5198 (*A. tumefaciens* as positive controls).

Table 6. Partial *virD2* amplicon sequence of isolates from galls on 'Draper' blueberry explants and NCBI BLASTN results

Isolate	NCBI Results	Top Hit Accession Number	Consensus Sequence
Tcg-1s	Uncultured *Rhizobium* sp.	MK423929.1	ATCAATCAGTTGGAGTACTTATCTCGTA AGGGCAAGCTGGAGCTACAGCGCTCTGC ACGGCACCTCGATGTTTTCGTACCTCCA GCTGAAATTCGCGATCTCGCCCGTAGTT GGGTTCAAGAGACCGGGAGTTATGACG AAAG
Tcg-2s	Uncultured *Rhizobium* sp.	MK423929.1	CAATAATCTCAATAGTTGGATATTCTCT CGTATGGCAGCCGAGCTAAGCGCTCCCA CGGCACCTCGATTTTTCGTACCTCAGCC AAATTCGCGATCTCGCCCGTAGTTGGGT TCAAGAGACCGGGAGTTATGACGAAAG CAGCCAGACGC
Tcg-3s	Uncultured *Rhizobium* sp.	MK423929.1	ATCAATCAGTTGGAGTACTTATCTCGTA AGGGCAAGCTGGAGCTACAGCGCTCTGC ACGGCACCTCGATGTTTTCGTACCTCCA GCTGAAATTCGCGATCTCGCCCGTAGTT GGGTTCAAGAGACCGGGAGTTATGACG AAAG
Tcg-4s	No significant similarity found	-	ATCNTCGGNTACTATCTCTAGNGCAGCT AAGTCCAGCGCNCCGACGGCATTCGACT CTNCGCCNTCAGCCGAATCGCGATCTCG CCCGTAGTCGGTCAGAGACCGGGAGTAC ACG
Tcg-5s	Uncultured *Rhizobium* sp.	MK423929.1	ATCAATCAGTTGGAGTACTTATCTCGTA AGGGCAAGCTGGAGCTACAGCGCTCTGC ACGGCACCTCGATGTTTTCGTACCTCCA GCTGAAATTCGCGATCTCGCCCGTAGTT GGGTTCAAGAGACCGGGAGTTATGACG AAAG
Tcg-7s	Uncultured *Rhizobium* sp.	MK423929.1	ATCAATCAGTTGGAGTACTTATCTCGTA AGGGCAAGCTGGAGCTACAGCGCTCTGC ACGGCACCTCGATGTTTTCGTACCTCCA GCTGAAATTCGCGATCTCGCCCGTAGTT GGGTTCAAGAGACCGGGAGTTATGACG AAAG
Tcg-8s	Uncultured *Rhizobium* sp.	MK423929.1	ATCAATCAGTTGGAGTACTTATCTCGTA AGGGCAAGCTGGAGCTACAGCGCTCTGC ACGGCACCTCGATGTTTTCGTACCTCCA GATGAAATTCGCGATCTCGCCCGTAGTT GGGTTCAAGAGACCGGGAGTTATGACG AAAG
Tcg-9s	Uncultured *Rhizobium* sp.	MK423929.1	ATCAATCAGTTGGAGTACTTATCTCGTA AGGGCAAGCTGGAGCTACATCGCTCTGC ACGGCACCTCGATGTTTTCTACCTCCAG ATCAAATTCCCGATCTTGCCCGTAGTTG GGTTCAAGAGACCGGGAGTTATGACGA AAG

Tcg-10s	No significant similarity found	-	CATAATAGTCGGNTACTATCTCTAGAGC AGCTANGCTATGCGCTCCGACTGCATTC GACTTCTNNNCCTTAGCCANATTCGCGA NTCGCCCGTAGTNGGTTCAGCGACGGGA GTTACACGATCAG
Tcg-11s	Uncultured *Rhizobium* sp.	MK423929.1	ATCAATCAGTTGGAGTACTTATCTCGTA GGGCAAGCTGGAGCTACAGCGCTCTGCA CGGCACCTCGATGTTTTCGTACCTCCAG CTGAAATTCGCGATCTCGCCCGTAGTTG GGTTCAAGAGACCGGGAGTTATGACGA AAG
Tcg-13s	No significant similarity found	-	ATTAAACTCAATCATCGGCTACTATCTC TAGNTACGCTAGCTTAAGCTTCTTACTG CTTTCGACTTTTNNNCCTTAGCCAAATN GCGATTCGTCGTAGTCGGTCAGAGACGG GAGTNCACGATG
Tcg-14s	Uncultured *Rhizobium* sp.	MK423929.1	ATCAATCAGTTGGAGTACTTATCTCGTA AGGGCAAGCTGGAGCTACAGCGCTCTGC ACGGCACCTCGATGTTTTCGTACCTCCA GCTGAAATTCGCGATCTCGCCCGTAGTT GGGTTCAAGAGACCGGGAGTTATGACG AAAG

Kuzmanovic *et al.* (2019) developed primers targeting a unique region of the gene encoding for flagellin A to detect a *Rhizobium tumorigenes*-like agrobacterium isolated from stem galls of Rhododendron. All 12 of the isolates from the galls on the tissue culture explants were positive for the *FlaA* marker for the *Rhizobium tumorigenes*-like agrobacteria (Figure 6, Table 3).

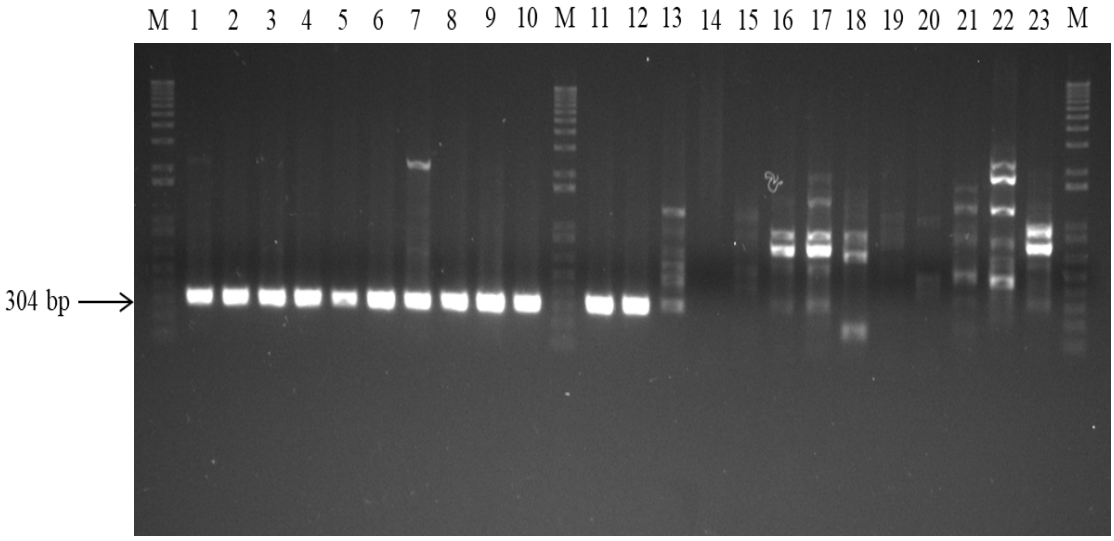

Fig 6. Electrophoresis gel showing PCR products obtained in *flaA* PCR with isolates from galls from blueberry tissue culture explants; Lane 1 – Tcg-1s; Lane 2 – Tcg-2s; Lane 3 – Tcg-3s; Lane 4 – Tcg-4s; Lane 5 – Tcg-5s; Lane 6 – Tcg-7s; Lane 7 – Tcg-8s; Lane 8 – Tcg-9s; Lane 9 – Tcg-10s; Lane 10 – Tcg-11s; Lane 11 – Tcg-13s; Lane 12 – Tcg-14s; Lane 13 – A5A2 Red 1; Lane 14 – 6b*2e; Lane 15 – 8b2e; Lane 16 – K84 (*A. radiobacter*); Lane 17 – JL5150 (*A. tumefaciens)*; Lane 18 – JL5178; Lane 19 – JL5184; Lane 20 –JL5189; Lane 21 – JL5193; Lane 22 – wfJ; Lane 23 – JL5198 (*A. tumefaciens)*; M – 1 KB+ marker.

Pathogenicity of isolates from galls on blueberry explants.

Three isolates (tcg-3S, tcg-7S, and tcg-14S) were tested for pathogenicity on stems of tomato and sunflower. These isolates from blueberry explants caused galling symptoms on tomatoes and sunflowers within 3 to 4 weeks after inoculation (Figure 7). The isolates also formed small galls on wound-inoculated ‘Draper’ blueberry plants 10 weeks after inoculation, but not every plant developed galls. The greenhouse wound-inoculation method for blueberry was slow and galling was inconsistent. We used a novel inoculation method of dipping plants in an aqueous suspension of the pathogen amended with an organosilicon surfactant called Breakthru. Stem gall symptoms were successfully obtained on each ‘Draper’ blueberry plant a month after inoculation. Galls were not observed on water-Breakthru treated controls. On tomatoes and sunflowers, the galls were slow-growing and appeared smaller than the galls forming with control isolates described in Table

1. On young ‘Draper’ plants, galls were small sizes and forming consistently along the length of the stem (Figure 8).

Fig 7. **A**, Small galls forming on young sunflower stem treated with gall isolate of blueberry explants. **B**, Galls forming on young tomato stem treated with gall isolate of blueberry explants.

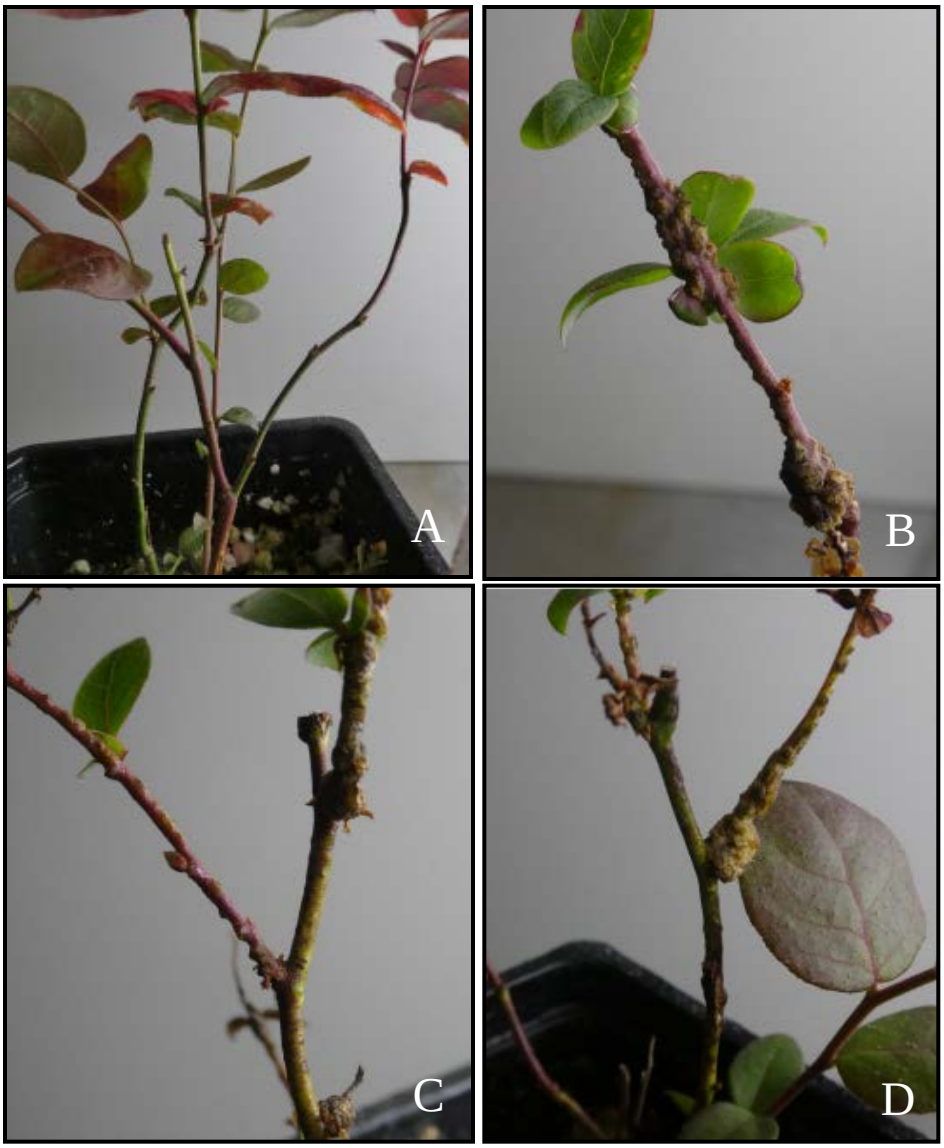

Fig 8. **A**, ‘Draper’ plant treated with water. **B**, ‘Draper’ plant treated with suspension of isolate Tcg-3s. **C**, ‘Draper’ plant treated with suspension of isolate Tcg-7s. **D**, ‘Draper’ plant treated with suspension of isolate Tcg-14s.

DISCUSSION

Past studies were not able to isolate tumorigenic bacteria from blueberry stems. Here, we successfully identified bacteria isolated from blueberry stem galls. Isolates of field galls described in methods section (see above) did not produce galling symptoms in the greenhouse tests except for unusual callus growth on tomato stems which was caused by IAA producing bacteria. Therefore, they are considered to be non-tumorigenic. In this paper, we found that different genera

of bacteria are capable of producing IAA and they may cause small callus growths on wounded stems. Nonetheless, these isolates were *virD2*-negative and did not cause galls that looked like though seen on blueberry plants. Consequently, we did not dwell on the details of the non-pathogenic bacteria that we isolated from field galls because our focus was on isolating and identifying tumorigenic bacteria from blueberry galls. Isolates from galls of blueberry explants are considered tumorigenic as galling symptoms were observed in the greenhouse tests. While the identity of these bacteria is understudy, our preliminary results are significant because the potential tumorigenic ability of these isolates was shown with PCR assays using *virD2* gene specific primers. Looking at different results obtained in greenhouse tests and PCR assays from these two different isolation techniques, micropropagating tissues of infected blueberry plant is a better technique to recover tumorigenic bacteria from blueberry stem galls than isolating bacteria from field galls.

Consistent results for isolation of tumorigenic isolates from field galls were not realized, as most isolates were negative for the *virD2* gene using specific primers and galling symptoms were not observed on plants inoculated with isolates obtained from field samples. This could have been because we recovered the non-tumorigenic bacteria on our culture media more easily than the tumorigenic ones. The growth of other bacteria might have overgrown or inhibited the growth of the bacteria of interest when they were directly grown on solid medium. Although one isolate was positive with *virD2* primers, the amplified band was not strong (Table 2). Sequencing the *virD2* amplicon of that isolate would provide needed data to determine if the amplicon was sequence from virD2 and if it was similar to the other strains. Overall, there are many reasons why we isolated non-tumorigenic isolates from galls collected in the field. Understanding the characteristics of the pathogen that causes stem gall of blueberry may lead to the development of

media that selects for the pathogen in environmental samples or incubation conditions that favor pathogen growth over growth of non-pathogenic environmental bacterial.

In contrast, bacteria with a uniform morphology were obtained from galls that developed on blueberry explants. The morphology of bacteria colonies growing on a medium such as PDA, was similar for isolates from tissue culture galls and some of the colonies isolated from field galls. Although some of the isolates from field galls and explant galls looked similar, only the isolates from explant galls were positive for *virD2.*

The additional steps in tissue culture technique could be the reason for this difference in observation. The tissue culture environment of young blueberry stems could have provided a suitable growing environment for the bacteria of interest. While on field galls there were several genera of bacteria cohabiting, the surface disinfestation process and succulent blueberry stem tissue may have selected for and encouraged the flourishing of tumorigenic bacteria. Previous studies of blueberry gall were not able to recover tumorigenic bacteria consistently from the stems but only from the roots (M. Putnam, *personal communication*) when tested with the same primer sets (Haas *et al*., 1995). To our knowledge, this study is the first to utilize tissue culture as a method to isolate tumorigenic bacteria from field infected plants (G. Sanahuja, *personal communication*).

Another difficulty with isolating the tumorigenic bacterium from field galls is that we found that the bacteria isolated from blueberry explants do not grow well on the semi-selective media, IA, 2E and MGYT. . The tumorigenic isolates would be missed if galled tissue were spread on these media for isolation of agrobacteria. While the gall pathogens from explants grew well on PDA-Ca, numerous other bacteria also grow well on the general medium. The slow-growing pathogen may be overgrown by environmental bacteria when galled tissue suspensions from field plants are spread on this medium or overlooked because the pathogen colony size is small and

unremarkable. Suspensions of surface disinfected galls from tissue cultured explants from galled blueberry stems had low populations of environmental bacteria. Predominate bacteria from explant galls that grew on PDA-Ca was the slow-growing pathogen. This process took a long time, but the pathogen was isolated easily from each of the sampled tissue culture galls.

We used two methods to determine pathogenicity of the bacterial isolates from blueberry explant galls. Because our PCR tests gave positives when targeting the conserved *virD2* gene on these isolates, we were suspected that they are tumorigenic. The first assay, involved wounding plants and applying bacterial masses from solidified culture media. Consistent galling symptoms were observed on inoculated tomatoes and sunflower stems. The galls took over a month to develop on wound-inoculated 'Draper' blueberry stems and they were limited to just the site of inoculation. The inability to mimic a natural environment of an actual blueberry field could be a limitation in gall formation on artificially inoculated blueberry stems. The second pathogenicity test evaluated relied on the addition of Break-thru to a bacterial suspension to allow the bacteria to penetrate the cuticle and infect cells without wounding the plant. Break-thru is a commercial surfactant that has been used to introduce endophytic bacteria into grape plants without physically wounding the plants (Baccari *et al.* (2018).

This method of using tissue culture to recover the blueberry stem galls bacterial pathogen is time-consuming and requires special skills. This method is not suitable for rapid diagnosis of blueberry stem gall but this study made progress in answering the question of the actual causal agent of blueberry stem gall. Future plans include whole genome sequencing of representative isolates and additional pathogenicity tests on 'Draper' and other blueberry cultivars to determine their susceptibility.

In summary, this study described two isolation techniques of blueberry stem gall bacteria and one was found to be more efficient in isolation of tumorigenic bacteria than the method of direct isolation from field galls. The traditional wound-inoculation method to test bacterial isolates for their ability to cause galling of tomato and sunflower worked well. The inoculation method where the blueberry plant was dipped in a bacterial suspension amended with Break-Thru worked well for the more woody tissues of blueberry. In addition to the recent report (Kuzmanovic *et al.*, 2019), our results suggest that the tumorigenic bacteria of blueberry stem galls could be similar to the bacterial causal agent of crown gall disease on rhododendron found in Germany.

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