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Distribution Patterns of Allorhizobium Vitis in Missouri Vineyards and Non-Vineyard Soils

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DISTRIBUTION PATTERNS OF *ALLORHIZOBIUM VITIS* IN MISSOURI VINEYARDS AND NON-VINEYARD SOILS

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Master of Science, Plant Science

By

Jacquelyn M. Wray

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ABSTRACT

Crown gall disease causes significant economic loss to the grape and wine industry. Preventive strategies are most effective for mitigating the loss of grapevines in vineyards, as there is no known cure for this disease. The bacterium *Allorhizobium vitis* carrying a tumor-inducing (Ti) plasmid is the causative agent. *A. vitis* bacteria live systemically in the grapevine before causing visible symptoms and can survive in residual plant tissues and soil for more than two years. Diagnostic methods have been developed to detect *A. vitis* bacteria in grapevines and soil. However, more reliable, specific, and high-throughput diagnostics are still needed for screening nursery stocks and soil. We have found primer sets unique to *A. vitis* on the Ti plasmid's origin of replication and virulence regions. We have developed procedures and protocols for reliably detecting *A. vitis* bacteria. We surveyed the incidence of *A. vitis* in five vineyards and six non-vineyard sites, covering three Missouri AVAs (American Viticultural Areas). We report the distribution of *A. vitis* in canes and soils within these sites. Our results show that *A. vitis* bacteria are present near and within the sampled vineyard soil and soils taken from non-vineyard areas across the state. Whether they are tumorigenic requires further investigation. The outcomes from this research will help the grape and wine industry to develop effective strategies for preventing and managing crown gall disease in vineyards.

KEYWORDS: *Allorhizobium vitis,* Tumor inducing (Ti) plasmid, *RepABC* operon, virulence, *vir* genes, crown gall, grapevine, soil, soil microbes, diagnostics, and detection

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LITERATURE REVIEW

The Causal Pathogen: Discovery and Proof of Tumorigenicity

Allorhizobium vitis belongs to the α-proteobacteria class of the family *Rhizobiaceae* and is a tumor-inducing phytopathogen. This gram-negative bacterium has a rod shape with peritrichous flagella, enabling it to move freely in the soil (Kuzmanovic et al. 2022). Genetically diverse in chromosomal and plasmid DNA, *A. vitis* has been classified and reclassified based on whole genome sequencing, phylogenetic analysis, and plasmid type. Both tumorigenic and non-tumorigenic strains carry a variety of ecologically essential plasmids, including tartrate and opine-catabolic plasmids. However, pathogenicity is determined by a sizeable conjugal tumor-inducing (Ti) plasmid. Ti plasmids have conserved and variable regions. The diversity of the variable region has a high correlation with different genomic strains of the bacteria (Kuzmanovic et al. 2018).

In 1907, researchers Smith and Townsend conducted a groundbreaking study demonstrating that the causative agent of crown gall was bacterial. This finding contradicted the previous theory that the spores of *Dendrophagus globosus*, a fungus, caused the disease. Smith and Townsend isolated the organisms in gall tissue and inoculated them onto healthy plants, reproducing the disease symptoms with a 100% success rate. After some characterization experiments, they proposed *Bacterium tumefaciens* (Smith and Townsend 1907).

Nearly forty years later, in 1941, Armin C. Braun focused his research on crown gall and its causative bacterium. Braun demonstrated that these tumors could persist without *Agrobacterium*. This revelation reshaped researchers' approach to studying this disease, shifting their attention to understanding how the bacterium induced permanent changes in plant cells, leading to tumor formation (White and Braun 1941). Braun named the *Agrobacterium*-mediated

tumor formation the Tumor Inducing Principle (TIP) (Braun and Mandle 1948). By 1975, researchers had suggested that specific plasmid genes determined the Tumor Inducing Principle. Advancements in DNA purification technology allowed for the ultracentrifugation of agrobacterial DNA, revealing peaks of super-coiled DNA forming a very large plasmid. This discovery prompted investigations into establishing a correlation between the discovered plasmid and oncogenicity (Van Larebeke et al. 1975).

Over the last four decades, various biotypes of *Agrobacteria* have been characterized. The bacteria of biotype 3 were found to be specific to *Vitis* spp. In 2015, this species was reclassified as *Allorhizobium vitis* using multilocus sequence analysis (MLSA) (Mousavi et al. 2015). Studying *Agrobacteria* strains has provided many new tools for genomic and biotechnological studies. The most notable contribution is the Ti-plasmid, which opened the door for the field of plant genetic transformation (Hooykaas 2023).

The Life Cycle of *A. vitis* and the Mechanism of Pathogenicity

After plants infected with crown gall are introduced into vineyard soil, *A. vitis* disseminates into the soil and moves freely. This bacterium can then find neighboring plants to infect or can remain in the ground among remnant grapevine tissues, awaiting a new vine (Arya et al. 2018). Nematodes have been observed to be a vector of *Agrobacterium tumefaciens* in their host roots, but it has not yet been determined if they serve as a vector for *A. vitis*. However, it has been observed that nematodes and the root injury they cause can enhance the infection from soil (Kuzmanovic et al. 2018). The bacterium can enter through the roots or an injury and live systemically throughout the vine. There are four phases of infection. First, damage to the plant initiates the plant's wounding response, sending out chemical signals. One chemical signal is

syringic acid methyl ester, a phenolic compound that induces the expression of proteins involved in virulence. Bacteria recognize this signal and attach to the cells on the plant's injured surface. The virulence proteins initiated begin to process and transfer the T-DNA region of the Ti plasmid, containing the oncogenic genes. This region is then integrated into the plant nuclear genome. Once expressed, the T-DNA region encodes oncogenes for auxin and cytokinin biosynthesis. Unchecked growth hormones lead to dedifferentiated cell division or tumor growth (Vizitiu et al. 2011).

Ti plasmids are typically maintained at one copy per chromosome. However, copy number is regulated by external factors. Conjugation is induced on the surface of a gall formation when *A. vitis* detects the population density through quorum sensing. Plant growth chemicals can also cause conjugation. This leads to an increase of immature T-complex to be transferred into the plant nuclear genome. Once inside the nucleus, T-DNA is inserted into the chromosome during DNA replication. Since T-DNA encodes proteins for making phytohormones, the transformed plant cell starts multiplying and forms tumors. These tumor cells carry the T-DNA that also encodes proteins for synthesizing opines, a nitrogenous source for the bacterium. The opines are transported into the intercellular spaces for stable maintenance of the bacterial population. Once the tumor is established, the plant begins producing salicylic acid in response to the bacterial infection to contain and limit the further spread of bacteria (Cevallos et al. 2008).

Ecology and Epidemiology of *A. vitis*

For centuries, crown gall has challenged grape growers. These agriculturalists recognized different susceptibility between cultivars and their cold hardiness. To manage the disease, growers have implemented cultural practices such as trunk protection and cold-hardy rootstocks. Studies have evaluated these susceptibility differences among *Vitis* germplasms. It was generally found

that *V. riparia* and *V. rupestris* parentage had more resistance than cultivars of *V. vinifera* (Burr and Otten 1999).

The family *Rhizobiaceae* are plant-associated, soil-inhabiting bacteria. *A. vitis* strains have thus far been detected in soil exclusively in association with grapevine plants and have never been isolated from non-vineyard soils. Soil colonies are a significant inoculum source (Kuzmanovic et al. 2018). Tumorigenic strains are almost exclusively detected in gall tissue or plant residues within the soil. *A. vitis* has been detected for up to two years in colonized roots and cane remnants in soil. This suggests they can maintain their Ti plasmid while existing in the saprophytic state within the decaying grape tissues. Tumorigenic strains may not persist in soil without grapevine tissues (Burr et al. 1987), and the primary introduction into vineyard soils is through contaminated grapevines. Further investigations of soil survival are necessary by more sensitive detection methods to find if *A. vitis* live freely in soils (Burr and Otten 1999).

Crown gall is frequently observed at graft unions, where wound-induced signaling hormones, like auxin, trigger cell differentiation and callus formation. This is particularly detrimental to nurseries and young vineyards (Hao et al. 2018; Kuzmanovic et al. 2018). Wounding also commonly occurs by freeze injuries and cultural practices. However, high temperatures and humidity in warmer climates can also injure and initiate infection (Kuzmanovic et al. 2018).

A. vitis lives endophytically and epiphytically on and in grapevines, randomly distributed with varying cell densities (Hao et al. 2018). Dr. Lehoczky hypothesized that bacterial cells are concentrated in the roots of grapevines during the winter months and migrate throughout the vine during sap flow. He demonstrated the endophytic survival of *A. vitis* in asymptomatic grapevines by observing the initiation of gall formation at injury sites. Studies have identified the presence of

A. vitis in the xylem and phloem tissues and the rind layer of dormant cuttings (Sule 1986). Another study reported that the freezing of canes facilitated the systemic movement of the bacterium, enhancing its dissemination throughout the vine. As one of the most freeze-sensitive tissues in dormant vines, cambial tissue is where most crown galls are initiated. This creates a repository for *A. vitis,* making it available to enter the vascular system when initiated by freeze damage. The implications are that a freeze injury triggers virulence (*vir*) gene responses and facilitates the bacterium's internal movement (Burr and Otten 1999; Stover et al. 1997).

Minimizing the threat of crown gall and biological control. Because of the need for standardized protocols and detection methods, grapevine crown gall is not considered for quarantine in many countries. Low population numbers and asymptomatic vines limit the detection of this pathogen. Global distribution of crown gall is spread from nurseries due to latent infection of the propagation materials (Kuzmanovic et al. 2018). Using pathogen-free propagation material is one way to help minimize the threat of crown gall. In the 1980s, it was thought that submersion of dormant grape cutting in a 50-55°C water bath for 30 minutes would eradicate the bacteria. A set of experiments showed a low bacterium level near the gall tissue, but it was still detectable after treatment. Another side effect is that hot water can injure bud tissues (Burr and Otten 1999).

A few strains within *A. vitis* are nonpathogenic and antagonistic to the tumorigenic strains. Some of these studied are VAR03-1, ARK-1, and F2/5. These strains have sequences with a high level of dissimilarity in the "housekeeping" genes *pyrG, recA,* and *rpoD.* Pre-treating nursery-vine roots in a cell suspension of the antagonistic strains before planting in Ti-contaminated soils resulted in a lower incidence of crown gall (Kawaguchi et al. 2021). The strain F2/5 will inhibit infection if applied before exposure to the Ti-strains; however, it will not inhibit colonization and

growth of tumorigenic *A. vitis* at a wound site. Also, strain F2/5 elicits a hypersensitive response after infiltrating and will cause root necrosis.

Preventative methods remain the most effective way to mitigate the effects of crown gall. While incidence can be reduced, this disease still has no cure. Increased sensitivity in detection methods is necessary for effective prevention (Burr and Otten 1999).

Wild *Vitis* spp. as a source of tumorigenic *A. vitis*. The presence of tumorigenic *A. vitis* has been observed in stems and dormant canes of symptomless wild grapevines, indicating that these wild *Vitis* spp. may be a significant reservoir for this pathogen. In 2016, a survey was conducted in 59 wild *V. riparia,* and 19 tested positive for tumorigenic *A. vitis.* These vines were all asymptomatic and were located adjacent to vineyards and far-removed areas (Kuzmanovic et al. 2015).

Microbial diversity in gall formations. It is commonly accepted that the vast majority of microbial life is not readily cultured and, as a result, has yet to be extensively studied. There needs to be more understanding of how microbial life is established and maintained at an appropriate balance. The mechanisms and factors for establishing and supporting plant microbial communities must be better understood, including species diversity and richness in specific microenvironments. One such microenvironment is within a gall formation. A rich, diverse microbial community can exist within the gall due to an expanded range of nutrients available. Studies have shown that the microenvironment associated with crown gall has a core microbiota sampling. The abundance is positively correlated to the presence of *A. vitis* (Gan et al. 2019).

The microbiota within healthy versus infected grapevines have differing diversities and richness within the colonies. Within the gall tissue microenvironment, other opportunistic bacteria adapt and thrive. Soil microbiota diversity, richness, composition, and structure varied with

distance from the vine. Site-specific grapevine-associated microbiota consists of *Pseudomonas* in the aboveground colonies, while *Nitrososphaera, Methylobacterium,* and *Agrobacteria* compose the soil and root colonies. Soil, root, cane, and graft unions each harbor a distinct microbiota, with soil bearing the most diversity. *A. vitis* promotes colonization of endophytic microbes at the infection site. Aboveground microbiota is influenced by seasonal conditions, while there seems to be no significant influence on root or soil microbes. In graft unions infected with crown gall, the microbiota richness stayed consistent throughout the seasons and contained more diversity. Graft unions without infection had a significant decrease in richness during autumn. Soil and root microbes share operational taxonomic units (OTUs) with those living in a gall formation. This suggests that soil microbes are pathogenic microbial seed banks. Growth in gall formation is significantly reduced during drought stress, leading to a reduction in population density. This makes detection and isolation difficult during summer (Faist et al. 2016).

Although *A. vitis* is known for its tumorigenicity, it can cause root necrosis. This provides a competitive niche for the bacterium, as it can persist in the soil, residing in grapevine root debris. This suggests that grapevine root systems serve as a reservoir for *A. vitis.* Both tumorigenic and non-tumorigenic strains can cause root necrosis. Therefore, *A. vitis,* regardless of tumorigenicity, can be detrimental to an establishing vineyard. (Kuzmanovic et al. 2018).

Genome Structure and Genetic Diversity of *A. vitis*

A. vitis contains multiple non-homogeneous genomic species. DNA fingerprinting of *A. vitis* chromosomes shows that strain diversity is highly correlated to the type of Ti plasmid carried by the strain (Burr and Otten 1999). Understanding this genetic variation provides essential insight into this pathogen's epidemiology, ecology, and evolution (Kuzmanovic et al. 2022).

The selective pressure imposed by hosts, environment, soil reservoirs, and disease severity significantly influences diversity. As an endophytic pathogen of grapevine gall tissues, the diversity within the bacterial community is shaped by host defense mechanisms, inter and intra-specific competition, and the evolution of pathogenic function across various niches. The ability to produce opines, supplied by transformed plant cells, and the subsequent catabolism, gives *A. vitis* a competitive nutritional advantage during infections. *Agrobacteria* containing differing Ti plasmids compete for opines. Other bacterial and fungal taxa have evolved the ability to utilize these opines, creating inter-specific competition. An additional boost in competitiveness is given to *A. vitis* populations harboring a tartrate utilization plasmid, allowing them to gain nutrition from an abundant grapevine molecule (Barton et al. 2018).

A. vitis has two circular chromosomes. The larger of the two has an origin of replication (ORI) similar to other α-proteobacteria. Gene content and order are more often conserved in this chromosome. The smaller chromosome, classified as a chromid, has a *RepABC* origin of replication, typical of the family *Rhizobiaceae.* Comparing genomes of *Rhizobiales* suggests intragenomic gene transfer of the primary chromosome to plasmids, resulting in the chromid. Both contain rRNA operons essential for prototrophic growth (Slater et al. 2009).

A vitis has undergone several reclassifications based on new technologies, allowing for a more in-depth analysis of genetic diversity. Initially lumped into *Agrobacterium tumefaciens,* they were later reclassified as *Agrobacterium* biovar 3 (or biotype 3). DNA-DNA hybridization, phenotypic, and serological tests have led to a reclassification into a new species, *Agrobacterium vitis.* Improvement in technology, multilocus sequencing analysis (MLSA), and subsequent genome-wide phylogenies proved this group is phylogenetically distinct from the genus

Agrobacterium. This led to another reclassification into the newly created genus *Allorhizobium* under the family *Rhizobiaceae* (Kuzmanovic et al. 2022).

Various subclades of *A. vitis* have been recognized, and their species relativeness is determined using calculations of overall genome relatedness indices (OGRIs) and estimation of genome-based phylogenies. These related genes' predicted functions are stress response, aromatic compound degradation response, secondary metabolite biosynthesis, and environmental signal perception. Differences between the subspecies are their consumption of different nutrient sources, polyamines, nickel ions, or phenolic compounds. Genes for specificity between subspecies *A. vitis sensu stricto* and *Allorhizobium ampelinum* sp. are found on their chromids or plasmids (Kuzmanovic et al. 2022).

In the natural biology of the species, high recombination in the *A. vitis* genome occurs. However, this is also facilitated through agricultural practices associated with grapevine propagation material production. Another major factor in recombination is the globalization of agriculture, where diverse rootstocks and scions are distributed, providing opportunities for gene exchange between different genetic lineages of *A. vitis* (Kuzmanovic et al. 2015).

Genome Structure and Genetic Diversity of Ti Plasmids

A typical Ti plasmid is approximately 200 kbp with 155 open reading frames (ORFs), able to encode a functional protein for replication and maintenance, conjugative transfer, virulence, opine catabolism, sensory perception of plant host signals, and quorum sensing (Gordon and Christie 2014). Pathogenicity functions are carried on this self-transmissible plasmid and are shared amongst the bacterial population. Tight regulation of plasmid maintenance limits the

cellular burden, allowing them to colonize a bacterial population and transmit genes and strategies to provide that population with a competitive advantage (Barton et al. 2018).

There is a strong correlation between the Ti plasmid type and chromosome within specific *Agrobacterium* and *Allorhizobium* species. Specific pathosystems of *A. vitis* may influence this interaction (Kuzmanovic et al. 2018). Tumor-inducing plasmids are an amalgamation of highly conserved regions and divergent sequences due to frequent horizontal transfer, deletions, insertions, and recombination events in their evolutionary history. These large-scale genomic events can narrow the host range of the plasmid and, consequently, variability within that range (Barton et al. 2018). This evolutionary recombination makes it challenging to understand the evolutionary pathways and to construct phylogenic trees. Knowing this evolutionary history is crucial in predicting oncogenic properties and the plasmids' evolving capacity (Burr and Otten 1999). Contributing to this evolution, bacterial cells restrict the uptake of similar plasmids, unable to coexist within the same cell line. This is known as plasmid incompatibility and promotes the uptake of a broader range of plasmid types (Cevallos et al. 2008).

Ti plasmids are regulated by population density and chemical signals generated by the host plant, which induce the conjugation system and increase the plasmid copy number. Regulators of copy number include the transmembrane sensor kinase, *VirA*, and the response regulator, *VirG*. These genes modulate the transcriptional activity of the *RepABC* operon (Cevallos et al. 2008).

***RepABC* plasmid replication.** The *RepABC* operon is responsible for plasmid replication and stable maintenance. This replicon family is distributed among many α-proteobacteria and is found on extrachromosomal plasmids and some secondary chromosomes, known as chromids (Gordon and Christie 2014). *RepABC* operons have many similarities; however, several

differences lend to the host specificity, including sequence diversity within this locus (Cevallos et al. 2008).

RepABC plasmids exist in low copy numbers. Therefore, stable replication and propagation mechanisms must be efficient. Typical for a low-copy number plasmid, the regulatory genes for replication and partitioning map at different *loci* and are controlled by other regulatory mechanisms. However, the *RepABC* operon has all the regulatory elements required for replication and segregation within a single transcription unit. This operon has three primary transcriptional genes, *repA, repB,* and *repC,* with *repA* being the most upstream. Negatively auto-regulating rep genes, *repA* encodes proteins to bind to upstream promoter sites. *RepA* and *repB* proteins are involved in the active segregation of plasmids, while *repC* encodes for a replication initiator protein. On a sizeable intergenic region between *repB* and *repC,* a small antisense RNA, known as the counter-transcribed RNA (ctRNA), is transcribed. The ctRNA binds to the mRNA of *repB* to inhibit translation. The predicted secondary structure is a single stem-loop rich in uracil at the 3' tail.

To increase diversity in gene uptake, plasmids belong to incompatibility groups. Plasmids with the exact replication and partitioning system will not be stably maintained over multiple generations within the same bacterial line. However, researchers have observed replicons harboring two functional *RepABC* operons (Cevallos et al. 2008).

Quorum sensing and chemical signals from the host will positively or negatively impact *RepABC's* regulatory mechanisms. In general, bacteria uptake several plasmids, allowing the transfer of genes between these cells. It is assumed that free-living *Allorhizobium* maintains one Ti plasmid per chromosome, with that number increasing on the surface of the grapevine gall tissue due to the increased perception of population density (Cevallos et al. 2008). Conjugative

transfer occurs under the regulation of *Tra/Trb* genes. These genes are part of a type IV secretion system that uses biochemical pathways to process the chemical signals of quorum sensing (Wetzel et al. 2015). The *RepABC* operon found on Ti plasmids has four promoter sites upstream from *repA.* The transcription factor positively influences these promoters, TraR, used in quorum sensing in many gram-negative bacteria. A complex signaling cascade regulates the transcription of TraR. Without the signaling molecules opines, this gene will be repressed (Cevallos et al. 2008). Increased transcription of TraR induces conjugative transfer in response to opine signals produced by plant neoplasias (Wetzel et al. 2015).

Virulence region and transfer DNA. The virulence region, or *vir* genes, is responsible for the processing, transferring, and integrating the transfer DNA (T-DNA) into the host cell genome. The T-DNA houses oncogenic genes that, when integrated into the plant nuclear genome, cause unregulated cellular division for gall formation. The T-DNA also houses opine synthesis genes that produce a nutrient source for the bacteria when integrated into the plant nuclear genome. The virulence region is located between the origin of replication and the transfer DNA regions.

Of the *vir* genes, *virG* is the regulatory element responsible for gene expression. Within the promoter region, a 'vir box' encodes a sequence for *virG* to recognize and bind to for repression of *vir* expression. The virulence-essential operons are *virA, virB, virG,* and *virD.* Operons *virC, virE, virF,* and *virH* are involved in plant-bacterium interactions. However, these operons have variability in their organization, leading researchers to classify them based on these organizational groupings. These plasmid groupings are differentiated into five vir phylogenies (vir I-V). Ti plasmids specific to *A. vitis* are organized in the vir III phylogeny, except for one type of opine synthesis region. Ti plasmids with a vitopine synthesis region fall into the

phylogenic group vir IV (Nabi et al. 2022). Of the vir proteins, some are responsible for external sensing and regulation. *VirA* is a chemoreceptor kinase that encodes sensory proteins, detecting monosaccharides and phenolic compounds secreted by wounded plants, such as acetosyringone. In the presence of these molecules, *virA* auto-phosphorylates, transferring a phosphate group to *virG,* a transcriptional regulator (Burr and Otten 1999).

A virulence protein involved in processing the transfer DNA region is virD2. This protein encodes an endonuclease, cleaving the T-DNA borders and releasing the ssDNA, known as the T-strand. The ssDNA-binding protein virE2 protects this strand following transportation to the plant cell (Burr and Otten 1999). T-DNA's right and left borders comprise a consensus sequence (3'- YGRCAGGATDTATNNNNDGTMDN- 5'). Four types of T-DNA structures have been discovered. A Ti plasmid can contain one, two, or three T-DNA regions within these structures. The organization of the vir region is determined by the number of T-DNAs present (Nabi et al. 2022). It is suggested that transfer is initiated at the right border proceeding left, as the right border is more intrinsically active and essential for T-DNA transfer (Zhu et al. 2000).

Genes for tumorigenesis are located on the T-DNA. Encoding for the biosynthesis of phytohormones, *iaaM* and *iaaH* auxin oncogenes and *ipt* cytokinin oncogene, are highly conserved in Ti plasmids (Nabi et al. 2022). The oncogenes *iaaM* and *iaaH* direct the conversion of tryptophan into indoleacetic acid within the auxin class of growth-regulating phytohormones. The *ipt* oncogene condenses isopentenyl pyrophosphate and adenosine monophosphate (AMP) into cytokinin zeatin, a phytohormone promoting cell division (Zhu et al. 2000).

The transformed plant cell produces opines, a selective nutrient source for *A. vitis*. Opines are conjugates of amino acids, α-ketoacids, sugars, and sometimes sugar phosphodiesters. Those

genes encoding opine synthesis are located on the T-DNA region of a Ti plasmid (Kuzmanovic et al. 2018).

Opine catabolism region. Opines are essential molecules in the epidemiology of crown gall and the ecology of tumorigenic bacteria. While the transformed plant cell ensures the production of this nutrient source, another region on the Ti plasmid transfers the ability for *A. vitis* to metabolize it. Various types of opines can be synthesized and metabolized in combinations on Ti plasmids (Zhu et al. 2000). *A. vitis* strains have been known to carry the octopine/ cucumopine (O/C), nopaline (N), and vitopine (V) opine types. The number of T-DNA regions varies based on opine type. Ti plasmids are commonly classified according to these opine types. Type O/C plasmid contains two independent T-DNA fragments: TA-DNA and TB-DNA. This variation of the Ti plasmid is the most abundant organization type, according to current studies. N-type plasmids have a single T-DNA, while V-type contains three independent T-DNAs (Kuzmanovic et al. 2018).

Some *Rhizobiaceae* strains carry a small plasmid called the opine-catabolic plasmid (pOC). These plasmids carry genes transferring the ability to catabolize opines. However, they lack the genes needed for pathogenicity- the *vir* genes and T-DNA. pOCs have been identified in tumorigenic and non-tumorigenic strains isolated from tumors and soils near diseased plants. Those bacteria harboring this plasmid gain an advantage over other microbes in the diversity within galls and soils. Because opine regions exist in non-tumorigenic strains, isolating this region to prove pathogenicity cannot be done (Kuzmanovic et al. 2018).

Another advantageous plasmid for microbes within a grapevine gall is the tartrate utilization plasmid (pTrs). Tartaric acid is an organic molecule naturally occurring in many fruits, especially grapes. This acid is crucial in winemaking, contributing to flavor profiles and

textures. Its predominant role lies in maintaining chemical stability and influencing taste and color. Most *A. vitis* strains degrade or utilize tartrate, lending to their grapevine host specificity (Crouzet and Otten 1995). This plasmid's tartrate dehydrogenase genes (*ttuC*) are readily integrated into the chromosome of the acceptor strain. Three varieties of tartrate plasmids have been described with a conserved TAR region (Burr and Otten 1999). pTrs can be carried by tumorigenic and non-tumorigenic strains. These plasmids identified among *A. vitis* strains are diverse in size, transfer frequency, and stability. Using tartrate as a carbon source enhances strain competitiveness within grape plant species (Kuzmanovic et al. 2018).

One study aimed to determine if non-tumorigenic strains can acquire and maintain Ti plasmids. They mated tumorigenic strains (carrying O/C and N Ti plasmid types) with non-tumorigenic strains. Equal concentrations of donor and recipient strains were incubated on grape galls. Ti plasmids given antibiotic resistance to rifampicin with the O/C or N opines were selected. No stable transfers were detected. However, many recipient strains gained tartrate utilization from the donor strains (Burr and Otten 1999).

INTRODUCTION

In crown gall disease, malignant tumors form around wounded tissues on grapevine canes, trunks, and graft unions. Currently, no cure is available. This disease results from dynamic interactions of pathogen, host, and environmental conditions (Burr and Otten 1999), causing significant economic losses and devastating viticulture areas where freeze injuries occur. This disease causes a severe decline in vine viability, eventually leading to vine death, resulting in productivity and profitability losses for these vineyards (Kuzmanovic et al. 2018). Knowing the incidence of the causal pathogen in vineyards and soils is the first step for preventing and reducing crown gall disease.

The primary causal agent of crown gall disease is *Allorhizobium vitis*. Previously, *Agrobacterium vitis* biotype 3 was reclassified into the genus *Allorhizobium* in the family *Rhizobiaceae* (Mousavi et al. 2015). This Gram-negative bacterium is rod-shaped with peritrichous flagella, enabling its free mobility in soil (Kuzmanovic et al. 2022). Strains of tumorigenic and non-tumorigenic *A. vitis* occur, carrying a variety of ecologically important and genetically diverse plasmids. Tumorigenicity relies on a sizeable conjugal tumor-inducing (Ti) plasmid (Kuzmanovic et al. 2018).

The primary introduction of *A. vitis* into vineyard soils is through contaminated grapevines. Worldwide distribution of *A. vitis* in viticulture areas occurs due to the international shipment of propagative materials in which the bacteria reside without causing visible symptoms (Kuzmanovic et al. 2018). *A. vitis* lives endophytically and epiphytically on grapevines with varying densities. Both tumorigenic and non-tumorigenic strains may negatively affect graft unions and overall plant health (Hao et al. 2018). This bacteria has been detected in colonized

roots and cane tissues, remnant in soil for up to two years (Burr and Otten 1999), but may not persist in soils without grape tissues (Kuzmanovic et al. 2018). *A. vitis* has been found in wild *Vitis* species, a living source of inoculum reservoir. However, reports on the distributions of *A. vitis* in native habitat soils, with or without wild grapevines, are scarce and vital in studying these soils as reservoirs (Hao et al. 2018). Increased sensitivity in detection methods is needed to find if *A. vitis* lives freely in soil (Burr and Otten 1999).

Because of their high sensitivity and speed, DNA-based techniques for detecting *A. vitis* are commonly chosen (Kuzmanovic et al. 2018). These methods will detect *A. vitis* strains defined by the primer sets' conserved sequences. One notable method is magnetic capture hybridization to enrich target bacterial DNA. Detection of tumorigenic strains is then accomplished by qPCR. This method can detect *A. vitis* from asymptomatic grapevines (Johnson et al. 2016). In 2018, digital droplet PCR was developed (Voegel and Nelson 2018) in which primer sets, defined by the genes *virA*, *pehA*, and *virD2,* were used to detect *A. vitis* (Eastwell et al. 1995; Kaewnum et al. 2013). In 2020, RT-PCR primers were developed to monitor the *A. vitis* population in grapevine plantlets. These primers were designed according to genes on the *A. vitis* chromosome and Ti plasmid (Nguyen-Huu et al. 2021), targeting conserved chromosomal genes and *virD3,* a conserved gene within the *vir* region (Vogel and Das 1992). While there is great benefit in using these detection methods, extra steps to enrich the bacteria are costly and time-consuming. In contrast, PCR methods are typically limited in sensitivity, leading to false negatives. To provide a sensitive yet cost-effective method, we developed primer sets for PCR that increased detection sensitivity by increasing the specificity.

Crown gall is emerging as a severe disease in Missouri vineyards. Knowing the presence and prevalence of crown gall bacteria in grapevines and soils in vineyards is imperative to

implement preventive strategies. In addition, the status of crown gall bacteria in wild grapevines and native habitats is unknown. In this study, we improved procedures for detecting *A. vitis* bacteria with more specificity and sensitivity. We surveyed the incidence of *A. vitis* in five vineyards and six non-vineyard sites, covering multiple Missouri AVAs (American Viticultural Areas). We also surveyed nursery vines commonly imported into the state. We report the distribution of *A. vitis* within these canes and soils sampled.

MATERIALS AND METHODS

Vineyard Soils and Cane Tissues

A sampling strategy was adopted in a commercial vineyard block to collect samples from 20 grapevines arranged in a "W" pattern. At each point of the "W," samples of both cane and soil were collected, resulting in a total of five sampling locations within each block. Soil and cane tissue samples were collected from four vines at each sampling location. The position of these four vines was two adjacent vines in a row and two adjacent vines directly across the alley. Each cane sample consisted of two-inch cuttings from three shoots of a single vine. Each soil sample consisted of a composite of two cores taken approximately 1 foot from each side of the grapevine trunk, directly under the drip line, to a core depth of 6 inches. Corers were sanitized through immersion in a 10% bleach solution, then two deionized water rinses, and allowed to dry between each point on the "W." Collected samples were stored on ice and transported to the lab. In total, 20 cane and 20 soil samples were collected per commercial vineyard block (Table 1). Additionally, two soil samples were collected from areas outside the sampled vineyard block for qualitative comparison (Table 2).

Soil samples were allowed to dry for 48 hours and then ground to a fine powder using a mortar and pestle. Between processing each sample, the mortar and pestle were sanitized with 10% bleach, rinsed, and dried. Processed soil samples were labeled and stored at room temperature for later use. Cane samples were weighed in triplicates of 100 mg per cane tissue and then labeled and stored at -80°C.

Non-Vineyard Soils

Similarly, a strategy of sampling soils in the "W" pattern was adopted, when possible, in a non-vineyard site. Four soil samples were collected at each location of the five points on the "W" pattern; each sample consisted of two cores to a depth of 6 inches. Twenty soil samples were collected at each non-vineyard site (Table 3). Soil samples were processed as described in the earlier section.

Nursery Cane Tissues

Fifty-two own-rooted and grafted grapevines of different cultivars were ordered from two nurseries commonly used by the Missouri wine and grape community. Upon arrival, a 0.5 to 1-centimeter section was cut from the cane near the shoot-tip. The pruner was washed with 70% ethanol between samples. Each cane was tested individually (Table 4). Cane samples were weighed to 100 mg, labeled, and stored at -80°C.

DNA Extraction, Amplification, and Visualization

Total DNA was extracted from the 200 soil samples using a modular universal DNA extraction protocol (Sellers et al. 2018). Total DNA was extracted from 100 mg of cane tissues using the Synergy™ 2.0 Plant DNA extraction kit with the modification of 500 µl of CTAB added to the homogenized samples, incubated at 55˚C for 30 minutes, and vortexed every 10 minutes. (OPS Diagnostics, Lebanon, NJ, USA). The quality and concentration of each sample were assessed using a NanoDrop One spectrophotometer (Thermofisher Scientific, Delaware, USA).

A novel primer set, *repA*, for detecting *A. vitis* bacteria in soil was designed by aligning multiple reference sequences available in the NCBI database (GenBank accession no. CP056046,

KY000075, CP000637, and AP023283). The repA amplicon is captured by an elongated 35 nt primer set and targets the *RepABC* operon of the Ti-plasmid (Table 5). The second target was in the *vir* region of the Ti-plasmids, *virD* (Nguyen-Huu et al. 2021). The forward and reverse primer of the 'virD3cons' primer set were elongated to 35 nt each to increase specificity to tumorigenic plasmids within soil samples (Table 5). A segment of the *A. vitis* chromosome was targeted using the primer set 'PGF/PGR,' targeting the Polygalacturonase gene. These primers were elongated to 35 nt each (Table 5).

Thermocycler conditions for PCR were as follows: 98°C for 1 minute, followed by 35 cycles of 98°C for 10 seconds, 67°C for 20 seconds, 72°C for 27 seconds, with a final extension of 72°C for 7 minutes. DNA fragments were separated in a 1% agarose gel electrophoresis, and the images were taken under UV light.

Verification of PCR-Amplified DNA Fragments by Sanger Sequencing

DNA fragments in soil samples taken from non-vineyard sites and grapevine gall tissues were amplified by 'repA' and 'virD3cons' primer sets. These were then visualized by UV, and the fragments were cut from the gel and purified using MinElute® Gel Extraction Kit (QIAGEN group). These gel-purified DNA fragments were sent for Sanger Sequencing through the Nevada Genomics Center (University of Nevada, Reno). The sequences were analyzed in CodonCode Aligner and compared to an *A. vitis* reference sequence (accession number: KY000056.1).

Verification of *A. vitis* Detection by Selective Media

To verify the presence of *Allorhizobium* or *Agrobacteria* within the sampled soils, 1 gram of sample was added to 5 ml of sterile water to create a slurry. Three 10^{-1} serial dilutions were

made, and then 100 µl of each undiluted and serial dilution were spread onto Petri dishes containing selective 1A-t media. This selective media was created using an isolation protocol (Finer et al. 2016). The plates were incubated at 28˚C for three days. Colonies were restreaked on YEP media for colony isolation (Finer et al. 2016) and incubated at 28˚C for three more days.

RESULTS

Improving the Specificity and Reliability of Detecting *A vitis*

Complexities arise when testing soil samples using PCR. Target sequence detection can be disrupted by compounds found in plant tissues and soils, known as inhibitors. These inhibitors bind to the primer sequences, amplifying poorly matched DNA fragments. When visualizing under UV, this creates nonspecific banding on the 1% agarose gel. This binding can also produce false negative results, as the target sequence is not amplified appropriately for detection. The extraction of total DNA can also result in false negatives during PCR, as the non-target microbial DNA can dilute the target DNA.

Ti plasmid DNA was targeted and amplified to overcome these challenges using elongated primer sets. A novel primer set targets the Ti plasmid's origin of replication, part of the *RepABC* operon. Another extended primer set targets the virulence region, an indicator of tumorigenicity. These primers were elongated from the standard length of approximately 20 nt to 35 nt (Table 5). This elongation and an increased annealing temperature supplied the conditions to eliminate nonspecific DNA banding caused by the abovementioned complexities (Figure 1).

Theoretically, detection sensitivity should be increased with the elimination of nonspecific binding. A serial dilution of total DNA from grapevine gall tissue was tested by comparing the standard 'virD3cons' (18 nt) and the elongated 'virD3cons' (35 nt) primer sets. Serial dilutions ranged from 1000 pg/µL to 1 pg/µL. The standard 'virD3cons' primer set could detect targeted DNA molecules within 100 pg/µL of total DNA. The elongated 'virD3cons' primer set could detect targeted DNA molecules within 10 pg/µL total DNA, a 10-fold increase in the detection limit (Figure 2).

We theorized that the novel primer set ‘repA’ is specific to Ti plasmids harbored in *A. vitis*. This was tested by PCR, amplifying total DNA from various tree gall tissues. DNA fragments defined by ‘virD3cons’ were amplified in all samples of total DNA from tree gall tissue; however, the ‘repA’ amplicon was not. The total DNA extracted from grapevine gall tissue contained ‘virD3cons’ and ‘repA’ amplicons (Figure 3).

We verified that the PCR-amplified DNA fragments in soil samples from non-vineyard sites and grapevine gall tissues matched the sequences defined by the ‘virD3cons’ and ‘repA’ primer sets. The sequences received from the University of Nevada after Sanger sequencing were aligned to a reference sequence in the NCBI database using CodonCode Aligner (GenBank accession no. KY000056.1). Sequences containing nucleotides with a 100% identity and a Phred score of 19 or greater were counted as ‘matched.’ The DNA fragments from grapevine gall tissue matched 127 nucleotides within the ‘virD3cons’ sequence and 250 in the ‘repA’ sequence. The DNA fragments amplified from a non-vineyard soil sample had 101 nucleotides matched to the ‘virD3cons’ sequence and 247 nucleotides compared to the ‘repA’ sequence.

To verify that an *Allorhizobium* or *Agrobacterium* species was being detected, slurry from multiple soil samples, including soils from non-vineyard sites, was spread on 1A-t selective media. Isolated colonies were then streaked on YEP agar. Colonies grew from all soil and gall samples plated. These colonies were then tested for the targeted DNA fragments ‘virD3cons’ and ‘repA’ by PCR. Chromosomic *A. vitis* DNA, described by the primer set ‘PGF/PGR,’ was also tested by PCR (Table 5). Both gall tissues and some soil samples contained this amplicon (Table 6).

Distribution of *A. vitis* in Vineyards

To assess the spatial distribution of *A. vitis* across commercial Missouri vineyards, we collected 80 vine samples and 90 composite soil samples in the summer of 2022 (Figure 4). Using the newly designed 'repA' primer set in conjunction with the elongated 'virD3cons' primer set in PCR, we examined the presence of the two amplicons in soils and cane tissues within a vineyard block. We grouped these results into four categories: I. Presence of both 'repA' and 'virD3cons' amplicons; II. The presence of only 'repA' amplicon; III. The presence of only 'virD3cons' amplicon; IV. Absence of both 'repA' and 'virD3cons' amplicons (Table 7).

Examining the incidence within cane tissues, both amplicons occurred in 58% of the canes sampled. Either 'repA' or 'virD3cons' were amplified in 1% and 41% of sampled cane tissues. All cane samples tested had either 'repA' or 'virD3cons' or both, resulting in no true negative results (Table 7).

On average, both amplicons were detected in 34% of the sampled soils. Either 'repA' or 'virD3cons' were amplified in 12% and 21% of sampled soils, while 32% of soils tested had neither DNA fragments present, representing true negatives (Table 7).

Having corresponding cane and soil samples allows for more analysis to be conducted at the vineyard level. This can give insight into the distribution within vineyards. However, many variables are unaccounted for, so these findings are to encourage more in-depth studies than provide any conclusions. Vineyard 2 is located in the Hermann AVA and contains the cultivar Chardonel. This is a very established vineyard with grapes growing in these soils for many years. In testing, there was a high incidence of 'virD3cons' or both amplicons within the soil and cane samples. No samples from this vineyard tested negative (Figure 5). Vineyard 3 was also located in the Hermann AVA and contained the cultivar Norton. Interestingly, this vineyard tested negative for either amplicon in most soils sampled. However, 19 out of 20 canes contained either

‘virD3cons’ or both amplicons (Figure 6). Vineyard 4 is not part of an official AVA but is located along the Missouri River. This vineyard contained the cultivar Chardonel. The canes in this vineyard tested 100% for the presence of both amplicons. However, only about half of the soils had ‘virD3cons’ or both amplicons (Figure 7). Finally, vineyard 5, located along the Missouri River, contained the cultivar Vidal Blanc. This vineyard found the most variation in amplicons, with more ‘repA’ or both DNA fragments detected in soil samples. We detected both DNA fragments in most cane samples (Figure 8).

To summarize, we found the incidence of *A. vitis* as defined by the ‘virD3cons’ and ‘repA’ amplicons in approximately 67% of vineyard soil samples, with 34% of that total having the presence of both amplicons within the same sample. We collected corresponding cane and soil samples in four of the vineyards tested. Overall, the canes tested had a 100% incidence rate (Figure 9).

Two soil samples were taken from areas outside the sample vineyard block for qualitative purposes. In each case, either ‘repA’ or ‘virD3cons’ DNA fragments were present, or in two samples, both amplicons were present (Table 2). This unexpected finding led to a sampling of non-vineyard sites for a more comprehensive look into distribution patterns.

Incidence of *A. vitis* in Non-Vineyards

To investigate if *A. vitis* is present in non-vineyard soils in Missouri, we collected 110 composite soil samples from diverse land use locations within the state during the summer of 2023 (Figure 4). Both DNA fragments were amplified from 22.73% of the soil samples. Either ‘repA’ or ‘virD3cons’ were amplified in 4.55% and 22.73%, respectively. Neither DNA fragment was present in 50% of the soil sampled (Table 8).

Every site tested had an occurrence of one or both DNA fragments. Sites 1 and 2, on average, had the highest incidence of 'virD3cons' DNA fragments, amplified from 35% of the samples (Table 8). The land use in these locations is cattle farms and Ozark Forest (Table 3). Site 2 was the only location not tested in a "W" pattern, as the forest was too dense to allow this sampling pattern. Site 3 had only two samples containing either 'repA' or 'virD3cons' DNA fragments. 90% of this site tested negative for either amplicon (Table 8). The land use at this location is crop production (Table 3). Site 4, a cattle farm, also had a higher incidence of 'virD3cons', amplified from 30% of the samples. Sites 5 and 6 had the highest incidence of 'repA' and 'virD3cons' DNA fragments present, amplified from 30% and 90% of the samples tested in these locations (Table 8). The land use of these locations is grain production and cattle farms, respectively (Table 3).

Distribution Pattern of *A. vitis* in Vineyard and Non-Vineyard Soils

Vineyard soils had a higher incidence of both target amplicons than non-vineyard soils. The incidence of 'virD3cons' was elevated in non-vineyard soils, while the incidence of 'repA' DNA fragments was elevated in vineyard soils. Non-vineyard soils had a more significant occurrence of neither DNA fragment being amplified (Figure 10). However, every site contained targeted Ti plasmid DNA, suggesting the presence of *A. vitis.* This shows a distribution pattern of incidence in all surveyed land use areas across Missouri.

Incidence of *A. vitis* in Grapevine Nurseries

To assess the frequency of *A. vitis* in nursery vines being imported into the state, we ordered 52 vines from commercially available nurseries commonly used by the Missouri grape

and wine industry. These were a mixture of own-rooted and grafted vines (Table 4). The presence of ‘repA’ and ‘virD3cons’ DNA fragments was found in 53% of the vines sampled, over half the samples. Containing at least one of the tested amplicons, 25% of the vines contained the ‘virD3cons’ DNA fragment, while another 13% had only the ‘repA’ DNA fragment. Only 6% of the tested vines had neither amplicon present, suggesting a true negative (Figure 11).

DISCUSSION

Knowing the incidence of the causal pathogen, *Allorhizobium vitis,* is an essential first preventative step to reduce the occurrence of crown gall in vineyards. For this reason, we developed primer sets for PCR to increase detection sensitivity while keeping the test cost- and time-efficient. Using these primers, we surveyed the distribution patterns of *A. vitis* within Missouri vineyards, non-vineyard sites, and nursery vines regularly imported into the state. Our findings show the presence of Ti plasmid DNA fragments in 67% of sample vineyard soils and 100% of sampled vineyard cane tissues. Two soil samples were collected in areas immediately outside the sampled vineyard blocks for qualitative comparison. In each of these areas tested, targeted DNA fragments were present. This led to expanding our survey to non-vineyard sites throughout Missouri. Our findings show the presence of Ti plasmid, as indicated by our 'repA' and 'virD3cons' amplicons,' in 50% of the soils sampled from non-vineyard areas. Finally, we assessed the incidence of these DNA fragments within nursery vines and found the presence of our target DNA fragments in 94% of the vines tested.

Improving the Specificity and Reliability of Detecting *A. vitis*

To detect suspected tumorigenic strains, a primer set for the Ti-plasmid's virulence region was selected from previous studies. This primer set targeted virD3 of the conserved *vir* genes. This region encodes proteins that assist in integrating oncogenes into the plant host's genome (Hooykaas 2023). The presence of this gene suggests the occurrence of a tumor-inducing (Ti) plasmid. This study's second set of primers targets the origin of replication on a Ti-plasmid. These plasmids use a *repABC* operon for replication and maintenance. Replicons within

the *repABC* plasmid family have low copy numbers and require high efficiency in their segregation mechanisms. Their regulatory mechanisms include the ability to be positively or negatively impacted by their external environments, most notably by quorum-sensing and chemical signals from the host. For this reason, it is assumed that free-living *A. vitis* maintains one Ti plasmid per chromosome. Copy number increases on the surface of gall tissue due to the increased perception of population density. There are differences within the *repABC* family, including the sequence diversity of this locus (Cevallos et al. 2008). Two conserved sequences have been identified for *A. vitis* strains (Otten 2021). The presence of these sequences suggests the presence of *A. vitis.*

Complexities in testing soil samples with PCR lead to false negatives and nonspecific DNA banding under UV visualization. We elongated our primer sets to 35 nt in length to increase the specificity in annealing to the target amplicons. Longer primers increase the chance of annealing a unique sequence specific to the target region and reduce the likelihood of off-target binding as more base pairs are required for stable hybridization. Longer primers need a higher annealing temperature to create a stable primer-template hybrid. More stringent annealing conditions make it challenging for the primer to anneal with mismatches, increasing the specificity for the target sequence. This elongation and increased annealing temperature eliminate nonspecific DNA banding when visualizing under UV (Figure 1) and increase detection sensitivity in PCR by 10-fold (Figure 2).

Microbial diversity within the soil can also create false negative results as the non-target microbial DNA dilutes the target DNA. The sequence diversity between *A. vitis* strains and other tumor-inducing *Agrobacteria* adds to this complexity of genetic diversity. The genetic diversity of Ti plasmids adds another level of complexity. To detect pathogenicity, we targeted DNA

sequences within the *vir* genes. This region is conserved on Ti plasmids and is involved in tumorigenicity. To specifically detect *A. vitis* Ti plasmids, we targeted DNA sequences in the *repABC* operon. This region has sequences unique to Ti plasmids harbored in differing bacteria. We conducted a preliminary test on the specificity of the 'repA' primer set by testing it on two crown gall tissues taken from trees and one crown gall tissue taken from a grapevine. We also tested the 'virD3cons' primer set on these three samples. All samples contained the 'virD3cons' amplicon, verifying this sequence is within the conserved *vir* region of Ti plasmids, involved in tumorigenicity. The 'repA' amplicon was only present in the grapevine gall tissue. The *RepABC* operon has sequence diversity among the species it is harbored in, and these preliminary results verify that this sequence is within a unique region of the Ti plasmid (Figure 3).

Aligning the Sanger-sequenced DNA fragments from gall tissues and soils to reference sequences within the NCBI database gave us confidence in the sequences we detected. However, there's a possibility of detecting remnant fragments of Ti plasmid instead of Ti plasmids harbored in viable bacteria. Within cane tissues, detecting remnant fragments seems unlikely. However, the answer was still unknown in the soil. Plating our soil samples on selective media allowed us to observe the viability of the *Allorhizobium* or *Agrobacteria* species harbored there, which contained the detected fragments from the Ti plasmid. We tested the colonies present for the 'repA' and 'virD3cons' sequences and a chromosomal sequence defined by 'PGF/PGR' (Table 6). These soil samples contained viable *A. vitis* bacteria harboring a Ti plasmid. Furthermore, viable *A. vitis* was found in non-vineyard soil samples.

Distribution of *A. vitis* in Missouri

Ecological predictions of *A. vitis* can be made by comparing vineyard soils with their corresponding cane tissues. Overall, our target DNA fragments were detected in the cane tissues, even when there was no detection in the soil. Examining distribution patterns of individual vineyards, vineyard 4 was distinct (Figure 7). The topography of this vineyard may contribute to the pattern. Approximately the first half of these samples were located on the side of a hill, while the second half resided in the valley at the bottom. Those soils taken from the hillside tested primarily negative, while those in the valley tested positive for both amplicons and had many vines displaying symptoms of crown gall. A possible explanation is that *A. vitis* resides in only the top layer of soil and can be washed into the valley during rainfall.

Studying the distribution of *A. vitis* within Missouri vineyards, we find a higher frequency of both amplicons being present or 'virD3cons' only in the cane samples. The high incidence of 'virD3cons' detection in grapevine cane tissues suggests this DNA fragment is amplified from a bacterium capable of producing grapevine galls. In comparison, there was a higher frequency for 'repA' only or negative results in soil samples (Figure 9). This suggests the presence of *A. vitis* in soils, tumorigenic or not. The cane and soil results indicate tumorigenic *A. vitis* distributed throughout Missouri vineyards.

Unsurprisingly, *A. vitis* was detected in Missouri vineyards and grapevines. However, their prevalence was more than anticipated. With 100% detection in grapevine canes, propagative materials will likely distribute this pathogen throughout Missouri. Combined with the 94% detection rate of *A. vitis* in the nursery vines tested, this provides significant plausibility. More surprising was the presence of 'virD3cons' and 'repA' in 50% of the soils sampled in non-vineyard sites. If these fragments are determined to be within a single bacterial cell, this will be significant, as it was previously determined that *A. vitis* can only be found in soils with remnant

grapevine materials and has not been isolated from non-vineyard soils (Kuzmanovic, 2018). Moreover, these DNA fragments were detected in every land use location sampled.

The biological implications of these results suggest a diversity in the strains being detected. At least three different strains of *A. vitis* or other *Agrobacterium* species may be detected by these primer sets. The DNA fragment 'virD3cons' detected in cane tissue most likely belongs to a population capable of infecting grapevines. However, in the soil, it is unknown. Detection results with higher rates of samples containing only 'virD3cons' could detect any number of *Agrobacteria* containing a Ti plasmid. The DNA fragment 'repA' has sequence similarity between the chromid and the Ti plasmid origin of replications. While this amplicon has specificity for *A. vitis,* the question of tumorigenicity remains if only detecting this amplicon. However, it is essential to note that *A. vitis,* regardless of tumorigenicity, can be detrimental to an establishing vineyard (Kuzmanovic et al. 2018). Samples containing both 'repA' and 'virD3cons' amplicons are more likely to have tumorigenic capacity within the grapevine. However, there is still a question about whether both fragments belong to one bacterium; further investigation is required.

CONCLUSION

We have developed a diagnostic protocol with a novel primer set for detecting *A. vitis* using standard PCR methods. This study has confirmed the presence of *A. vitis* within Missouri commercial vineyards, both in canes and soils. The high incidence of detection within nursery vines supports the theory of dissemination through propagative material. The incidence of these DNA fragments within non-vineyard soils in variable land-use regions across Missouri was unexpected. It opened the door for future ecological studies on the ubiquitous nature of *A. vitis*.

There are limitations within this study, as we are only detecting DNA fragments. Knowing the entire sequence of the detected organism would give greater insight into pathogenicity. However, this is not feasible for extensive survey testing. Even with knowing the whole genome, the question of tumorigenicity remains, though probable conclusions can be made. Multiple strains have likely been detected, though there is only a slight pattern variation between vineyard and non-vineyard soil results.

Future research should investigate the tumorigenicity of the *A. vitis* found in non-vineyard soils. The next step should be to grow these soils on selective media, isolate them, and send them in for whole genome sequencing. Inoculating model plants with isolated colonies would be the final tumorigenicity test. Clean propagative materials are essential to prevent the distribution and recombination of strains. Future vineyards should be planted with the awareness that *A. vitis* is found in non-vineyard soils, and extra precautions should be taken to prevent injury. More in-depth ecological studies on *A. vitis* in non-vineyard soils would give greater insight into this pathogen.

Table 1. A list of vineyards, number of cane and soil samples, cultivars, and American Viticulture Areas (AVA).

Vineyard	Number of Samples		Cultivar	AVA
	Soil	cane		
1	10	0	Planting site	Hermann
2	20	20	Chardonel	Hermann
3	20	20	Norton	Hermann
4	20	20	Chardonel	N/A
5	20	20	Vidal Blanc	N/A
Total	90	80		

Table 2. Incidence of *A. vitis* strains in soil samples collected immediately outside a vineyard block, as indicated by amplified 'repA,' 'virD3cons', or both DNA fragments.

AVA	Number of Soil Samples				
	repA	virD3cons	Both	Absence	Total
Hermann	0	0	1	1	2
N/A	1	0	0	1	2
N/A	0	1	1	0	2

Table 3. A list of non-vineyard sites, number of soil samples, name location in Missouri, and land use of each sampling site.

Non-vineyard	Number of Soil Samples	Location	Land Use
1	20	Marshfield	Cattle Farm
2	20	Springfield	Ozark Forest
3	20	Republic	Crop Production
4	20	Springfield	Cattle Farm
5	20	Republic	Grain Production
6	10	Rocheport	Cattle Farm
Total	110		

Table 4. A list of cultivars and their root status ordered from two nurseries.

Cultivar	Root status	Number of vines	Nursery source
Vignoles	Own rooted	2	A
	Own rooted	4	B
	3309	2	A
Traminette	Own rooted	2	A
	3309	2	A
	101-14	4	B
Vidal Blanc	Own rooted	2	A
	Own rooted	4	B
	S04	2	A
	1103P	4	B
Norton	Own rooted	2	A
	3309	2	A
Chardonel	Own rooted	2	A
	3309	2	A
	3309	4	B
Chambourcin	Own rooted	2	A
	Own rooted	4	B
	3309	2	A
Cayuga White	Own rooted	4	B
Total		52	

Table 5. A list of primers used in this study.

Primer	Sequence	Region	Source
repA- forward	5’ AGGCAATGTTTCTCAGCCTTGCATTCGAGAAACCA 3’	*RepABC* operon	This study
repA-reverse	5’ CCATCCTGAGTTAGAACTGCGCTAGCGTCTGCACT 3’		
virD3cons- forward	5’AATCCGGAGGTGATGGTTCAGCATCGTGGTGGAAC 3’	VirD operon	(Nguyen-Huu et al. 2021)
virD3cons- reverse	5’ GGCGTCATGTAAGCGTTGTCTGGTGATTTGAGCCA 3’		
PGF	5’ AATAGCTCTTCCACCAAGACCTGCAAGCCGCTGAT 3’	*pehA* gene	(Szegedi 2002)
PGR	5’ GCCCGGGTCGAAACCATCGGTATTCTTGACCGTAT 3’		

Table 6. Incidence of DNA fragments 'virD3cons', 'repA,' 'PGF/PGR' in colonies grown on 1A-t selective media.

Sample	Tested Amplicons		
	virD3cons	repA	PGF/PGR
Gall Tissue 1	+	-	+
Gall Tissue 2	N/A	+	+
Vineyard Soil 1	+	-	-
Vineyard Soil 2	-	-	-
Native Soil 1	-	-	+

Table 7. Incidence of *A. vitis* strains in cane and soil samples collected in vineyards, as indicated by amplified 'repA,' 'virD3cons', or both DNA fragments.

Vineyard	Number of Soil Samples					Number of Cane Samples				
	repA	virD3cons	Both	Absence	Total	repA	virD3cons	both	Absence	Total
1	0	2	5	3	10	Planting site				0
2	0	8	12	0	20	0	18	2	0	20
3	0	4	0	16	20	0	10	10	0	20
4	0	4	8	8	20	0	0	20	0	20
5	11	1	6	2	20	1	5	14	0	20

Table 8. Incidence of *A. vitis* strains in soil samples collected from non-vineyard sites, as indicated by amplified 'repA,' 'virD3cons,' or both DNA fragments.

Non-vineyard Site	Number of Samples				
	RepA	VirD3	Both	Absence	Total
1	1	7	4	8	20
2	1	7	4	8	20
3	1	1	0	18	20
4	1	6	2	11	20
5	0	4	6	10	20
6	1	0	9	0	10

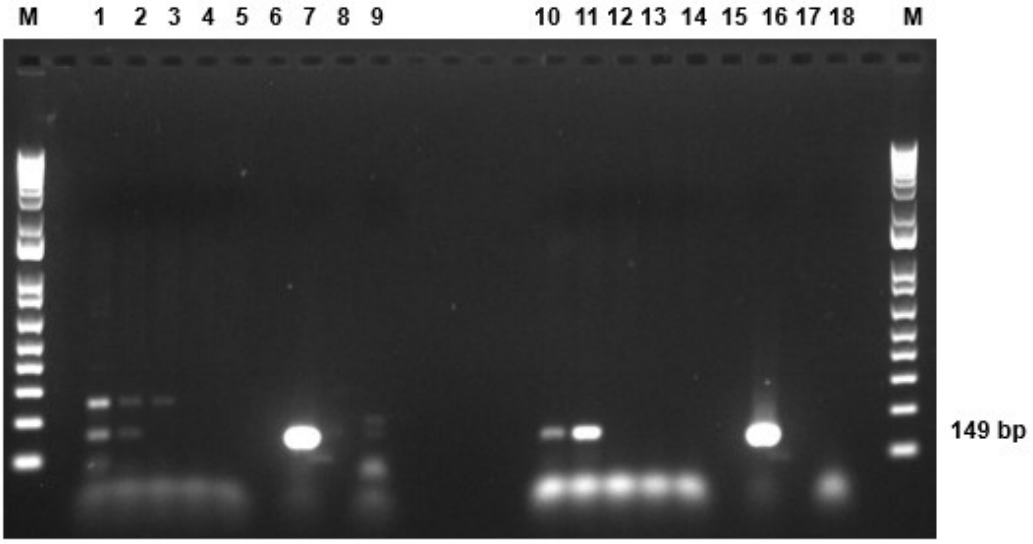

Figure 1. A comparison between the standard 'virD3cons' primer set described in the literature (left) and the elongated 35 nt 'virD3cons' primer set (right) on the same five non-vineyard soil samples. The positive control is total DNA from grapevine gall tissue.

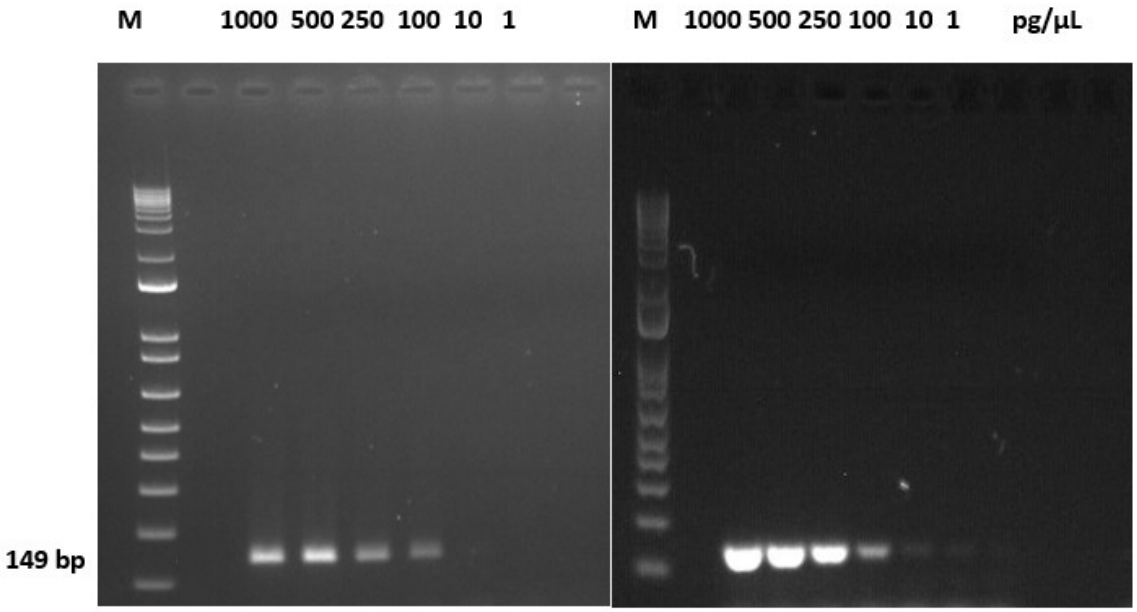

Figure 2. A comparison of the detection limit of standard (18nt) 'virD3cons' primer set (left) and elongated (35nt) 'virD3cons' primer set (right), tested on total DNA from grapevine gall tissue, in a serial dilution from 1000 pg/µL to 1 pg/µL.

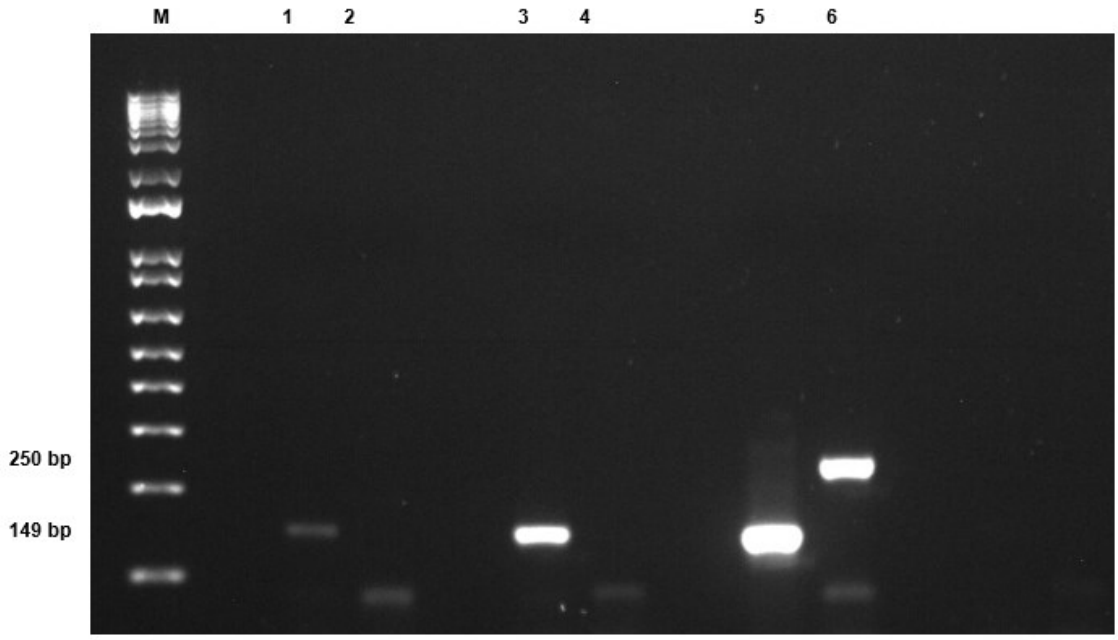

Figure 3. Two Oak tree gall tissues (left) and one grapevine gall tissue (right) were tested for the presence of 'virD3cons' amplicon (149 bp) and 'repA' amplicon (250 bp).

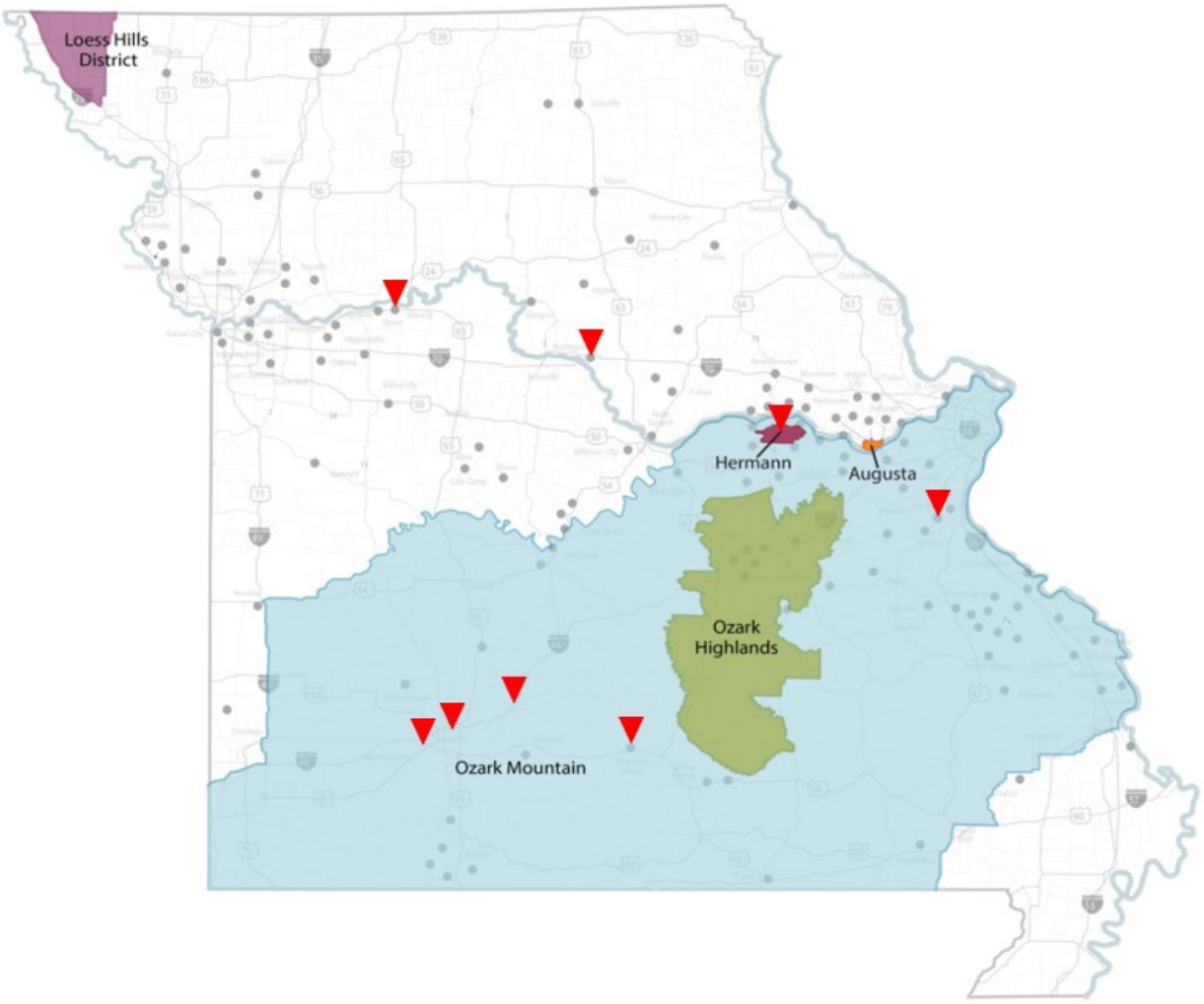

Figure 4. Map of sites surveyed, overlain on the Missouri AVAs (American Viticulture Area). Six vineyards and five non-vineyard sites were sampled for Ti plasmids harbored in *Allorhizobium vitis*.

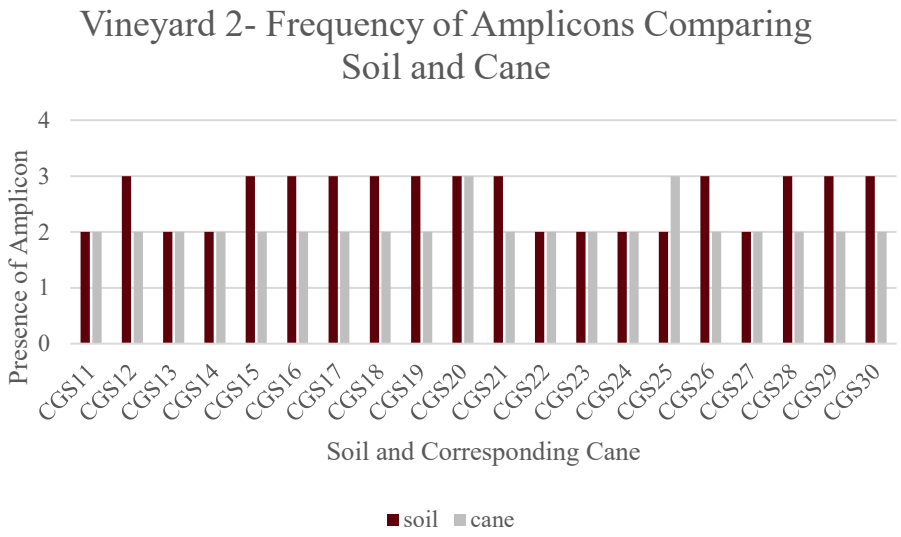

Figure 5. The incidence of *A. vitis* comparing soil to the corresponding cane tissue within a vineyard block. 0 is negative, 1 is 'repA' only, 2 is 'virD3cons' only, and 3 is both DNA fragments present.

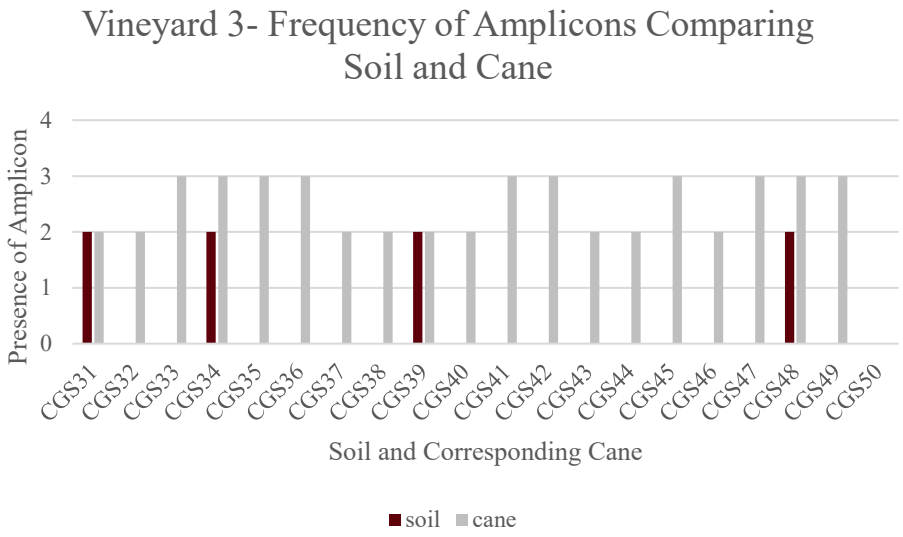

Figure 6. The incidence of *A. vitis* comparing soil to the corresponding cane tissue within a vineyard block. 0 is negative, 1 is 'repA' only, 2 is 'virDcons3' only, and 3 is both DNA fragments present.

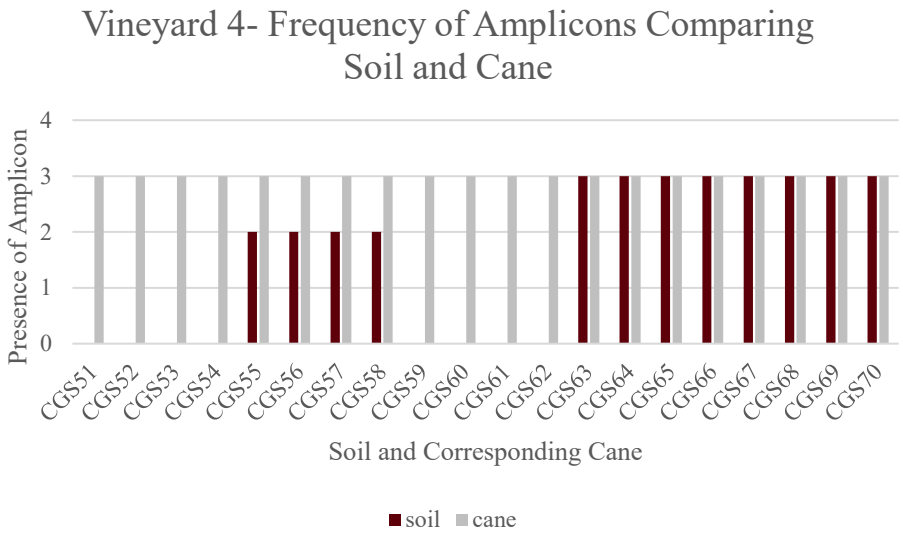

Figure 7. The incidence of *A. vitis* comparing soil to the corresponding cane tissue within a vineyard block. 0 is negative, 1 is 'repA' only, 2 is 'virD3cons' only, and 3 is both DNA fragments present.

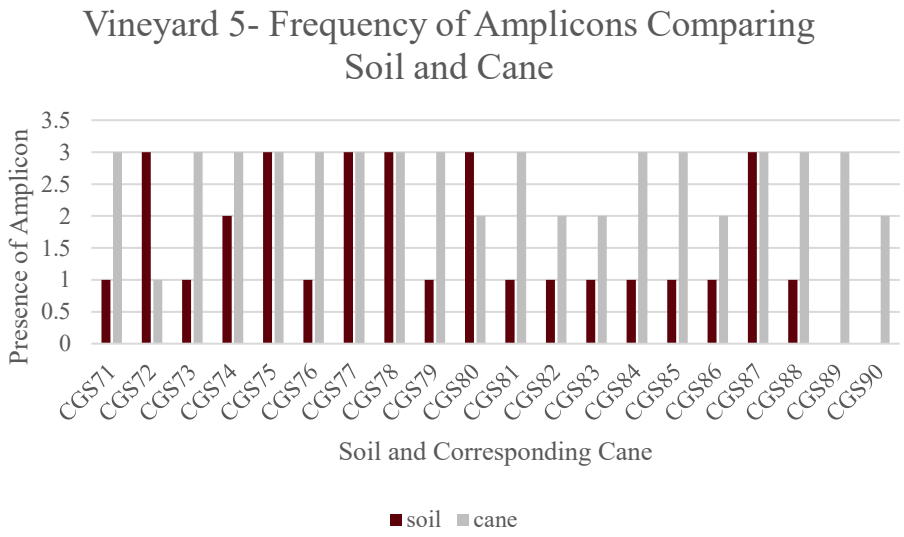

Figure 8. The incidence of *A. vitis* comparing soil to the corresponding cane tissue within a vineyard block. 0 is negative, 1 is 'repA' only, 2 is 'virDcons3' only, and 3 is both DNA fragments present.

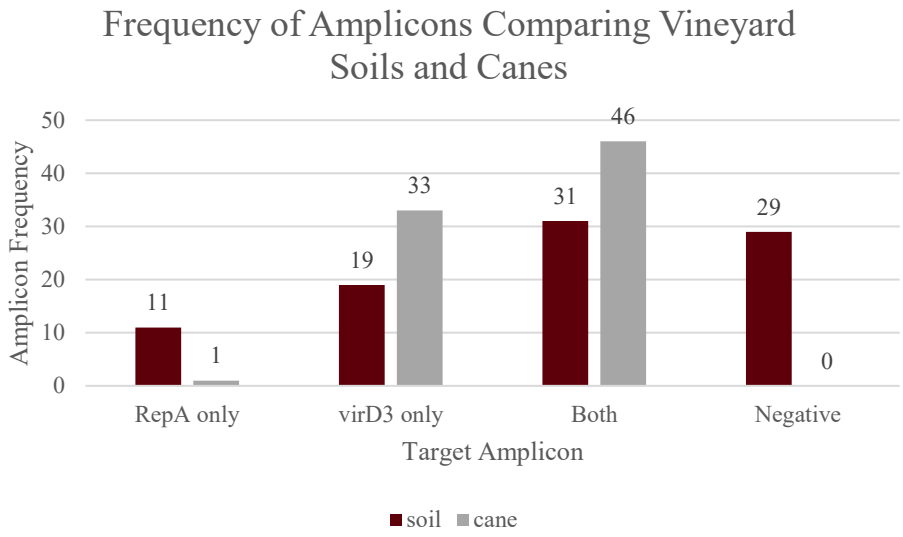

Figure 9. The incidence of *A. vitis* comparing vineyard soils to cane tissues, as indicated by the presence of 'repA,' 'virD3cons', or both DNA fragments. Results out of 90 total vineyard soil samples and 80 total vineyard cane samples.

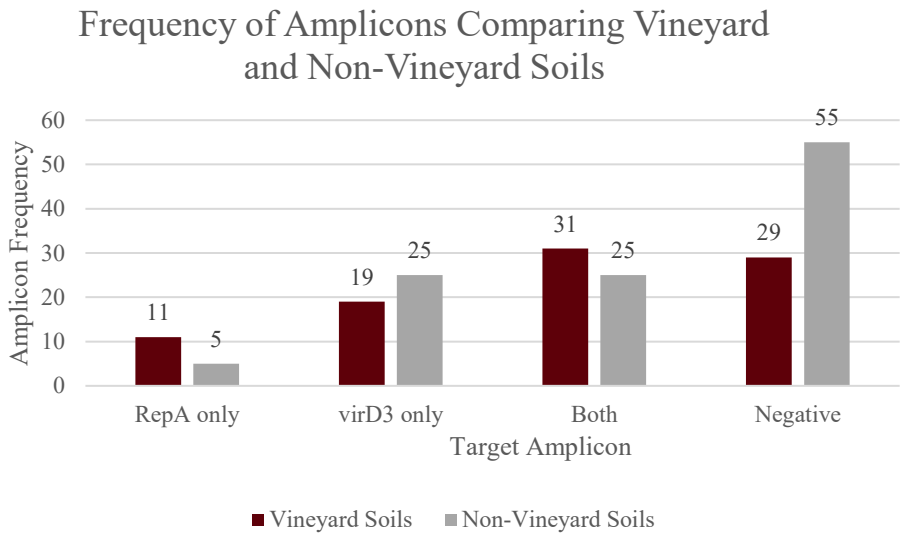

Figure 10. The incidence of *A. vitis* comparing vineyard to non-vineyard soil samples, as indicated by the presence of 'repA,' 'virD3cons', or both DNA fragments. Results out of 90 total vineyard samples and 110 non-vineyard samples.

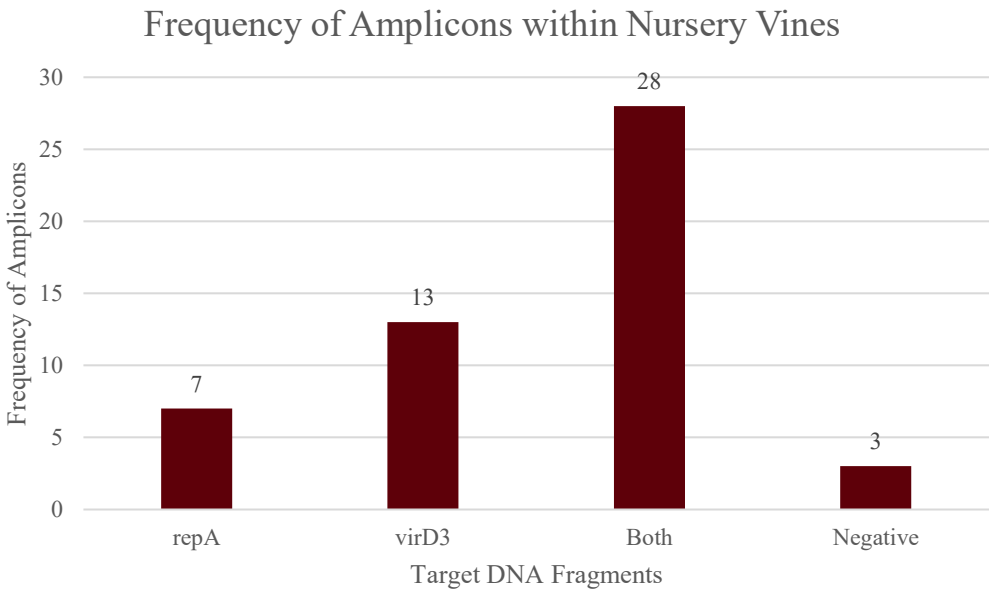

Figure 11. The incidence of *A. vitis* in cane tissues taken from nursery vines, as indicated by the presence of 'repA,' 'virD3cons', or both DNA fragments. Results out of 52 total nursery vine samples.

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