Biological Control Agent *Rhizobium vitis*, ARK-1 Reduces Incidence and Severity of Grapevine Crown Gall in Virginia

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ABSTRACT

Crown gall of grapevine (*Vitis* spp.) is a serious and economically important disease caused by the bacterial pathogen *Rhizobium vitis*, which transforms healthy plant cell genomes leading to hypertrophic and hyperplastic growth of affected plant cells. Recent studies have documented a strong inhibitory effect against Japanese tumorigenic *R. vitis* isolates by a newly identified non-tumorigenic strain of *R. vitis,* ARK-1. We conducted co-inoculation assays in tomato (*Solanum lycopersicum*) and wine grape cultivars (*Vitis vinifera)* with four tumorigenic isolates of *R. vitis* from Virginia. These tumorigenic isolates were co-inoculated with ARK-1 in various ratios and resulting gall incidence and gall size were measured. Analysis was conducted with the generalized linear mixed model (GLIMMIX) in SAS (ver. 9.4). ARK-1 significantly reduced both the mean probability of gall formation and the mean gall size ($P \leq 0.05$). ARK-1 efficacy against combinations of two or four tumorigenic isolates and up to twice as many cells of tumorigenic isolates was also significant. However, there was an indication of a loss of efficacy when ARK-1 was challenged with four isolates at four times the cell number of ARK-1. Also, the efficacy of ARK-1 was influenced by both the specific isolate and host plant used in the study. Our results suggest that ARK-1 has promising potential as an effective biological control agent for grapevine crown gall in the United States.

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GENERAL AUDIENCE ABSTRACT

Crown gall of grapevine (*Vitis* species) is a serious and economically important disease to the grape production industry caused by the bacterium *Rhizobium vitis*. This pathogen alters the genetic material of plant cells leading to cancer-like growth of affected plant cells (tumors); which may lead to the death of a grapevine. Recently, a non-tumor inducing strain of *R. vitis* (ARK-1) was documented to inhibit gall induction by tumor-inducing *R. vitis* strains in Japan. To see if ARK-1 would be a good candidate for biological control of the tumor-causing strains of *R. vitis* in the US, we tested it against four tumor-inducing strains in both tomato (*Solanum lycopersicum*) and grapevine (*Vitis vinifera)*. The tumor-inducing strains were mixed with ARK-1, injected into plant stems in various ratios, and resulting gall incidence and gall size were measured 42 to 90 days later. ARK-1 significantly reduced both the probability of gall formation and the gall size in both tomatoes and grapevines. ARK-1 was an effective agent against mixtures of one, two, or four tumor-inducing strains and provided control even when outnumbered two to one by cells of tumor-inducing strains. The efficacy of ARK-1 was influenced by the specific strain, relative cell number to tumor-inducing strains, and host plant used in the study. Our results suggest that ARK-1 has promising potential as an effective biological control agent for grapevine crown gall in the United States.

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Chapter I

Background Information and Justification of Study

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Introduction

Crown gall of grapevine (*Vitis* species), caused by *Rhizobium* (also known as *Agrobacterium*) *vitis* (Young et al. 2001), is one of the most important bacterial diseases of grapevine around the world (Burr & Otten 1999). Crown galls are hypertrophic growths (Figure 1-1) that typically form at the base or "crown" of the trunk of a vine. *R. vitis* has the ability to transform and reprogram plant host cells as part of the disease cycle and symptom development. As galls continue to grow, the gall cells act as a water and nutrient sink and reduce available nutrients for the rest of the vine (Gohlke & Deeken 2014). Large galls disrupt the vascular system of the vine, reduce vigor and yield, and can ultimately lead to vine mortality.

Currently, cultural management strategies are the predominant methods used for control of crown gall, however many of these are not effective, feasible or sustainable (Burr et al. 1998). Chemical options are very limited and often treat only the symptoms and thus cannot be justified from an economic perspective. Biological control options have been a recent focus. There are biological control products that are currently in use for the management of crown gall but further research is needed for control of crown gall of different *Rhizobium* species and plant hosts. Various bacterial isolates such as *Pseudomonas* and *Bacillus* species as well as some fungal species have recently been reported as potential biological control candidates (Ferrigo et al. 2017). The most successful crown gall biological control agents, *R. rhizogenes* (previously *R. radiobacter*) (Velázquez et al. 2010) K84 or *R. vitis* F2/5, have been investigated for their ability to prevent crown gall for various causal species and plant host species (Burr & Reid 1994; Bazzi et al. 1999; Kerr 1980; Khmel et al. 1998).

Figure 1-1. Crown gall symptoms: severe trunk gall (left) and late season, foliar symptoms due to crown gall (right). Photos by A. Wong

R. vitis, the causal agent of crown gall

Nomenclature

All *Agrobacterium* species have been re-classified as members of the genus *Rhizobium* after sequence analysis and comparison of the 16S ribosomal RNA gene revealed that *Rhizobium* and *Agrobacterium* are too closely related to each other to merit classification as different genera (Young et al. 2001). This proposal has been met with the challenge that reclassification is unnecessary and that *Agrobacterium* is a unique genus (Farrand et al. 2003). However, researchers in favor of the *Rhizobium* genus argue that this is necessary according to formal nomenclature rules (Young et al. 2003) because Agrobacterium species nest at several different locations within the Rhizobium genus. In this work we will use "Rhizobium" as the genus of the causal agent of crown gall. *Rhizobium* isolates that infect grapevines and carry the plasmid that causes crown gall disease will be referred to as "tumorigenic". "Non-tumorigenic" isolates refer to those that do not carry the plasmid and cannot cause disease.

Biology and plant host genome transformation

R. vitis is an aerobic, gram negative bacterium that infects many plant species and is commonly found in soil or as a symptomless, systemic endophyte (Burr et al. 1998). Surviving under these conditions allows the pathogen to be easily spread by infested soil or infected cuttings distributed by nurseries (Burr & Otten 1999). If clean vines are planted in infested soil, the soilborne bacterium can access the plant host via wounds in the roots or trunk. Wounds are most commonly due to winter damage, but can be caused by mechanical damage (e.g. grafting, weed whacker injury) as well. With winter injury, extreme cold temperatures can cause splitting of the vine trunk (Burr & Otten 1999). Regions with mild winters such as California have less winter injury and, therefore, less crown gall.

R. vitis has long been known to cause crown gall by insertion and expression of foreign bacterial DNA into the plant host genome (Chilton et al. 1977). This process begins with the wounding of the plant cells. Wounding is a crucial step in the infection process that allows entry of the pathogen and also stimulates the host genome transformation process for cells already established in the plant. Wounded plant cells release chemical signals, such as acetosyringone, which, when sensed by *R. vitis,* induce chemotactic movement by peritrichous flagella toward the source of the signal (Pitzschke & Hirt 2010). These plant wounding signals also stimulate the replication and expression of a plasmid that is involved in transforming plant cells. The "tumor inducing" or Ti-plasmid is a large (>200kb) plasmid that is responsible for the transformation of the plant host genome. This plasmid carries virulence genes necessary to process, shuttle and insert a region of the plasmid called the transfer DNA (T-DNA), into the host genome via a type IV secretion system (T4SS) (Pitzschke & Hirt 2010). Plant exudates from wounds will bind to an embedded membrane protein VirA that, in turn, phosphorylates the cytoplasmic VirG protein. Phosphorylated VirG acts as a transcription factor that binds to the promoter region of the *vir* gene region and induces expression of the virulence genes (Brencic & Winans 2005). Activation of the VirA and VirG system also induces replication of the entire Ti-plasmid, increasing the copy number of the plasmid (Cho & Winans 2005). To successfully transform its host, the pathogen must first attach to host plant cells by polysaccharide networks and a transfer pilus (Matthysse 2014). Once attached, transformation is induced by a T4SS pilus composed of VirB1-11 protein units to move both protein (e.g., VirE) and nucleic acid (T-DNA) from the bacterial cell to the host cell (Christie 2004). VirD2 locates the border sequences of the T-DNA, nicks the ends of the borders and covalently binds to the 5' end of the now single stranded T-DNA molecule (Herrera-Estrella et al. 1988). The single stranded T-DNA and VirD2 complex is

moved through the T4SS pilus. Once inside the host cell, the T-DNA complex is bound by VirE2 proteins that were transferred from the bacterium to the plant though the T4SS separate from the T-DNA (Christie et al. 1988). The VirD2 and VirE2 proteins localize the T-DNA complex from the cytoplasm to the host nucleus using the plant cell cytoskeleton (Herrera-Estrella et al. 1990; Citovsky et al. 1992). Once localized to the nuclear membrane, the VirE2 protein facilitates movement of the T-DNA complex into the nucleus (Dumas et al. 2001). T-DNA integration is still not fully understood, but T-DNA is incorporated into the genome of the plant host by non-homologous recombination via double stranded breaks that are repaired by host machinery (Tzfira et al. 2003; Mayerhofer et al. 1991; Dafny-Yelin et al. 2015; Gheysen et al. 1991). T-DNA integration is a semi-random event throughout the plant chromosomes that tends to integrate at sites with A-T rich motifs with low methylation and low nucleosome presence (Shilo et al. 2017).

Plant responses to infection and T-DNA expression

Most studies to elucidate the response of plants during the infection and subsequent transformation process have been done in *Arabidopsis thaliana* and *Nicotiana tabacum* because they are well understood model plant systems. During the infection and transformation process, plant defense responses are down regulated while genes that promote stable integration of the T-DNA are upregulated in the host (Veena et al. 2003). *R. vitis* cells produce and exude plant growth hormones that increase the efficiency of transformation by reprogramming the plant cells into a biological state more appropriate for DNA integration (Krens et al. 1996; Gohlke & Deeken 2014). Stimulation by these growth hormones favors transformation over defense because dividing cells express more of the genes for DNA synthesis and repair. Plant hypersensitive response is suppressed by plant growth hormones synthesized by the pathogen

(Robinette & Matthysse 1990; Lee et al. 2009). This suppression of the plant hypersensitive response is important to the pathogen's success in host transformation and failure to suppress this response can prevent gall formation, as seen in some biological control agents such as *R. vitis* F2/5 (Zheng et al. 2003). This will be addressed in more detail in later sections.

Successful and stable integration of the T-DNA will initiate the expression of synthesis genes for plant growth hormones and opines (Morris 1986). Expression of genes for the synthesis of plant growth hormones auxin and cytokinin leads to the uncontrolled proliferation and increased size of transformed cells that give rise to the gall. The rapid growth of cells changes the way that carbon and nitrogen are synthesized and how cells make ATP (Gohlke & Deeken 2014). Gall cells do not create their own carbon resources by photosynthesis so they become sinks for organic carbon made in the rest of the plant. ATP is created less from oxidative phosphorylation and more by anaerobic alcoholic fermentation (Deeken et al. 2006). The growth of the galls leads to loss of water due to the expansion of the gall and the fact that it is no longer protected by guard cells and cuticle. This triggers drought stress response genes that create a corky outer layer of periderm to reduce water loss (Efetova et al. 2007). The continued water loss also forces the water flow from the rest of the plant to be funneled to the gall tissue (Wächter et al. 2003).

The insertion and expression of the T-DNA initiates the synthesis of opines. Opines are small metabolites created by the gall cell and then metabolized by the pathogen (Burr et al. 1998). Opines found in gall tissues are synthesized by the condensation reaction of an amino acid and pyruvate, α-ketoglutarate, or a monosaccharide facilitated by the T-DNA encoded enzymes in the plant cell (Tempé & Goldmann 1982). Ti-plasmids maintained by *R. vitis* typically encode for the synthesis and metabolism of vitopine and ridéopine (Chilton et al. 2001; Szegedi et al.

1988). Opines synthesized in the gall cells are exported to the extracellular space where *R. vitis* recognizes and imports them (Lang et al. 2014).

Economic impact of crown gall

Crown gall can cause large economic loss if a large percentage of diseased vines must be removed. Overall economic losses from crown gall are difficult to estimate due to how widespread the pathogen can exist with variable symptom severity. According to the USDA National Agricultural Statistics Service (https://www.nass.usda.gov/index.php) and a report commissioned by the Virginia Wine Board in 2017: Virginia grape production in 2017 was worth close to twenty million dollars with the Virginia wine value in 2015 close to 130 million dollars (Frank & Rimerman 2017). Since effective crown gall management has not yet been established, even small (~3%) yearly losses translate to millions of dollars due to crown gall. These annual economic losses will continue to increase as the Virginia wine industry continues to grow without a feasible crown gall management program. In 1976, crown gall cost the entire United States 23 million dollars, with most of the loss from grapevine (Kennedy & Alcorn 1980). We can project that the economic cost to the US agriculture would be considerably higher today. Finally, considering global monetary losses to all crops (e.g. nuts and other fruits) due to crown gall (Pulawska 2010), we can conclude that crown gall is one of the most economically important bacterial diseases of plants. The significant loss due to crown gall disease could be greatly mitigated with the use of effective biocontrol in combination with cultural practices for crown gall prevention.

Grapevine crown gall management

Cultural practices

Crown gall management is primarily achieved through cultural practices due to a lack of effective chemical and biological options. Prevention is the best method to control the establishment and the spread of the pathogen through infested material. Purchasing vines that are not carriers of the pathogen will prevent the entry and establishment of *R. vitis* into the vineyard. However, if nursery stocks are already harboring the pathogen in vines as endophytes, the pathogen can easily be spread to vineyards (Burr et al. 1998; Burr & Otten 1999). Prevention and quarantine are not realistic due to the fact that the pathogen is so widespread. Detection of *R. vitis* in a vineyard is a crucial part of slowing the spread of the established pathogen. *R. vitis* can be isolated from vines, tissues or soil (Burr 1983). However, if *R. vitis* exists as an endophyte at a low titer, and is undetectable by isolation methods, relatively more expensive techniques, such as the serological technique, ELISA, are available (Bazzi et al. 1987; Bishop et al. 1989). Molecular techniques, such as PCR, may be sensitive enough to detect the pathogen in soil as low as 1000 cells per 100 mg soil (Picard et al. 1992). There are many primers that detect many chromosomal and plasmid genes of *Rhizobium* species and can aid in the characterization of specific isolates (Haas et al. 1995; Kumagai & Fabritius 2008; Kawaguchi et al. 2005; Szegedi et al. 2005). Molecular methods may be able to detect these very low levels of the pathogen, but would not be economically feasible for a large-scale survey.

To remove *R. vitis* from suspected plant material, propagation material may be hot-water treated. However, this is not always successful in killing every *R. vitis* cell and can damage vine buds (Burr et al. 1998). Propagating plants from shoot tips can grow a mother vine that will be pathogen-free but this is much more expensive and ineffective if propagated vines are

established in infested soil (Burr & Otten 1999). Once established in the vineyard soil, *R. vitis* can survive in the soil for years, potentially infecting healthy plants that are planted in the infested soil (Burr et al. 1995). To mitigate crown gall in vineyards that have infested soil, many growers commonly remove the infected vine once it has declined by the disease so that it is no longer productive. This practice is unsustainable and ineffective due to the fact that the soil remains infested and replacement vines may become readily infected. Alternatively, trunk renewal to replace a diseased trunk with a new, healthy trunk. However, the new trunk is at high risk of developing symptoms in the future.

Mitigating winter injury in the infested vineyards can reduce wounding and, thus, crown gall infection. Reducing winter injury can be achieved by either selecting sites with cold air drainage and hilling soil at the trunk of the vine in fall can help insulate the vine from extreme winter temperatures. Hilling soil has high labor costs due to that hilling soil has to be undone in the spring. Avoiding cultivars that are less cold weather hardy, such as *Vitis vinifera* cultivars (Pratt 1981) may also reduce the incidence of crown gall. *Vitis vinifera* cultivars such as “Chardonnay” or “Merlot” are the most susceptible to winter damage and crown gall. These cultivars are also among the premium wine cultivars. For this reason, the market drives growers to continually plant susceptible varieties. Grape species native to North America (e.g. *Vitis labrusca*) and hybrid varieties are more cold hardy and have relatively lower susceptibility to crown gall, but can still develop crown gall (Stover et al. 1997). These varieties are not as popular as wine cultivars so they are not planted as often for wine making.

Historically, development of transgenic plants for disease resistance has been successful for other crops and diseases. For the development of crown gall resistant grapevines, studies have shown the insertion and expression of antimicrobial molecules in plant cells can

significantly reduce crown gall severity (Vidal et al. 2006). Currently there are no transgenic crown gall resistant cultivars of grapevine in agricultural use. More recently, transgenic plum (*Prunus* spp.) trees that express interfering RNA specific to T-DNA genes encoding plant hormone synthesis have shown less disease symptoms (Alburquerque et al. 2017). However, transgenic apricots from the same study showed no difference in disease symptoms. While this promising approach is still under exploration, research in other options for the control of crown gall is warranted.

Chemical management options

When cultural practices fail and a vine becomes infected with the crown gall pathogen, there are very limited chemical options that can be used. The problem with attempting to manage crown gall is that once plant host cells are transformed and symptoms begin to show, it is extremely difficult to remove all of the diseased gall and pathogen cells without removing the entire vine and surrounding soil. Historically, soil fumigation has been used commonly in agriculture to kill many soilborne plant pathogens such as nematodes. Soil fumigation with methyl isothiocyanate has been shown to significantly reduce the amount of *Rhizobium* inoculum in the soil (Pu & Goodman 1993). Fumigation used in conjunction with a biocontrol agent HLB-2 (mentioned in greater detail in later section) applied as a root dip prior to planting, has been shown to significantly reduce the amount of disease. Methyl isothiocyanate registration for use has since been cancelled by the Environmental Protection Agency because it is considered too dangerous to the environment and is highly toxic. Also, fumigant applications in a vineyard setting would not be economically feasible. Vineyard vines and trellis systems would have to be removed prior to fumigation and then reinstalled.

For the treatment of galls, there are chemical products containing 2, 4-xylenol and meta-cresol used to treat the superficial symptoms of crown gall but not the disease itself. These chemical products are advertised to target and kill the gall cells without killing any of the healthy cells. The chemicals are applied by cutting as much of the gall tissue off and then painting the product directly onto the remaining gall tissue. However, this treatment does not kill all the gall cells and must be reapplied when galls reform. This product also does not have activity against the pathogen so plants are not protected against further infection.

Biological control options

Biological control for the prevention of crown gall has been studied for decades. One of the first successful biocontrol agents was *R. rhizogenes,* strain K84, found on a peach (*Prunus persica*) root gall in Australia (New & Kerr 1972). When K84 was inoculated into roots and seeds of peach trees grown in soil infested with *R. tumefaciens*, it significantly reduced crown gall compared to untreated peach trees (Htay & Kerr 1974). K84 has been used as a commercial product for the control of crown gall since the early 1970s, and derivatives of K84 continue to be used as a preventative biocontrol today for apples (*Malus* spp.), as well as other pome and stone fruits (Kerr 1980). *In vitro* plate growth assays suggested that K84 produces a chemical bacteriocin (agrocin 84) that prevents the growth of some, but not all, isolates of *R. tumefaciens* and was thought to inhibit the growth of some *R. tumefaciens* isolates by interfering with DNA synthesis (Roberts et al. 1977; Murphy et al. 1981; Kerr & Htay 1974). More recent research has shown that the agrocin 84 mode of action is interference with tRNA synthetase (Reader et al. 2005). Production of agrocin 84 is not effective against all isolates of *R. tumefaciens* and resistance to K84 can be transferred by conjugation into sensitive isolates to confer resistance. K84 production and resistance genes are expressed on the same plasmid that can be transferred

between compatible species (Stockwell et al. 1996). The transfer ability of the K84 plasmid that carries the agrocin and agrocin resistance genes was deleted to prevent transfer of resistance to sensitive isolates (Jones & Kerr 1989). These K84 strains that carry a transfer-deficient agrocin 84 plasmid are capable of preventing crown gall without conferring resistance to sensitive isolates (Penyalver et al. 2000). However, K84 is ineffective against *R. vitis*, which commonly infects grapes (Burr et al. 1998; Kawaguchi & Inoue 2012). Other strains similar to K84 have been shown to be effective against *R. vitis. R. radiobacter,* strain HLB-2 was found on hops (*Humulus lupulus*) in China and shown to be antagonistic to *R. vitis* isolates by production of an agrocin. It can reduce grapevine crown gall, especially when used in conjunction with a fumigant as mentioned earlier (Pu & Goodman 1993; Chen & Xiang 1986).

Another effective biocontrol agent that continues to be studied is *R. vitis* strain F2/5. Non-tumorigenic strains of *R. vitis* have been investigated for their ability to reduce crown gall and, with variable success, strain F2/5 stood out in its superior ability to reduce crown gall (Bazzi et al. 1999). F2/5 was originally isolated from grapevine in South Africa and was shown to be effective at reducing galls for some tumorigenic *R. vitis* isolates without causing galls itself (Burr & Reid 1994). The mechanism of F2/5 is not dependent on the production of an agrocin or by occupying sites that would otherwise be used by tumorigenic isolates to initiate infection and transformation. This study also revealed that F2/5 was ineffective at reducing gall formation in plant species other than grapevine (Burr et al. 1997). F2/5 also caused necrosis of grapevine roots and often induced high vine mortality rates (Burr et al. 1998; Bazzi et al. 1999). Creasap et al. (2005) hypothesized that the mechanism for F2/5 gall inhibition was based on the necrotizing activity of the biocontrol agent, which disrupts the natural healing process of the plant host and prevents the infection by tumorigenic isolates (Creasap et al. 2005). Continued research showed

that *R. vitis* quorum sensing genes that could induce the plant hypersensitive response (HR) were part of the inhibition mechanism of F2/5. F2/5 prevents host transformation but does not prevent growth or survival of tumorigenic isolates in grapevine tissues (Kaewnum et al. 2013; Zheng et al. 2003). The specific genes that induce HR in grapevine tissues and prevent gall formation of the host were identified using knockouts of two non-ribosomal peptide synthetases and a polyketide synthase (Zheng & Burr 2016). Transformed F2/5 strains have been developed and patented that are not necrotizing to root tissues but are still able to reduce crown gall and are the focus of research (Burr & Zheng 2012). Studies of F2/5 have had promising results, but many of the studies since 1994 have tested the efficacy of F2/5 against strains of *R. vitis* that are known to be sensitive to F2/5. More testing of F2/5 is needed to understand if F2/5 has a broader spectrum efficacy against more than a select few tumorigenic strains.

Other *Rhizobium* biological control strains have been examined in recent years. In Japan, non-tumorigenic *R. vitis* VAR03-1 was isolated and tested against tumorigenic isolates of *R. vitis* in both grapevine and rose. VAR03-1 was able to significantly suppress gall formation for most tumorigenic isolates when compared to inoculations with just the pathogen alone (Kawaguchi et al. 2007; Kawaguchi et al. 2008). In these studies, the ratio of VAR03-1 to tumorigenic isolate varied from 99:1 to 1:9 to investigate how the ratio of biological control agent to tumorigenic isolate affected efficacy. Another Japanese *R. vitis,* strain ARK-1, was found to be even more effective than VAR03-1 at reducing crown gall when co-inoculated with tumorigenic isolates of *R. vitis* (Kawaguchi & Inoue 2012). (ARK-1 will be described in more detail in the next section.) Finally, a non-tumorigenic isolate of *R. tumefaciens* UHFBA-218 was found in India on cherry (*Prunus avium*) rootstocks. This isolate was found to produce a halo of inhibition in *in vitro* assays by the production of an agrocin, similarly to K84 and HLB-2. In peach tree field trials,

UHFBA-218 root dip-treated seedlings showed reduced gall formation and size, similar to seedlings treated with K84, which is commonly used in the prevention of peach crown gall (Sharma et al. 2017). This strain has not yet been tested against other *Rhizobium* species or plant host species.

Investigations of biological control of crown gall have not been limited to non-tumorigenic *Rhizobium* species. *Pseudomonas* species, *Pseudomonas fluorescens* strains B4117 Q8r1-96 have been shown to be antagonistic to *R. tumefaciens and vitis*. These Pseudomonads were able to colonize plant hosts and reduce gall formation on grapevine, tomato and raspberry (*Rubus idaeus*) (Khmel et al. 1998; Dandurishvili et al. 2011). In addition to *Pseudomonas*, some *Bacillus* and *Curtobacterium* species have also been shown to possess activity against *Rhizobium* and reduce gall formation (Eastwell et al. 2006; Ferrigo et al. 2017). Another *Bacillus* strain has potential as a biological agent for the control of crown gall. *B. subtilis* 14B produces a molecule that has antimicrobial activity (Hammami et al. 2012; Hammami et al. 2009). Biological control candidate strains from the *Bacillus* genus tend to share the antimicrobial *bmyB* gene and researchers suggested that this gene is an excellent gene to use in order to screen for additional potential *Bacillus* biological crown gall control agents (Frikha-Gargouri et al. 2017). Fungal species from the *Acremonium* and *Trichoderma* genera have also been shown to have the ability to suppress gall formation in grapevine (Ferrigo et al. 2017). *Rahnella aquatilis* HX2 significantly reduces crown gall in sunflower and grapevine. HX2 also exudes an antimicrobial molecule, gluconic acid, which is antagonistic to *Rhizobium* spp., thus preventing gall formation, but does not disrupt the microbiome of the plant host (Chen et al. 2007; Li et al. 2014).

Biological control candidate ARK-1

Recently in Japan, Dr. Akria Kawaguchi isolated a non-tumorigenic strain of *R. vitis* from Pione (*Vinifera* hybrid 'Kyoho' x 'Muscat of Alexandria') (Yamada & Sato 2016) grapevine tissue. This isolate demonstrated significant gall suppression activity when co-inoculated with tumorigenic isolates of *R. vitis* in grapevine seedlings. Interestingly, heat killed or cell filtered ARK-1 did not suppress gall formation (Kawaguchi & Inoue 2012). From this it was concluded that ARK-1 was not exuding any molecule that was responsible for gall inhibition and ARK-1 had to be alive to be effective (Kawaguchi et al. 2017). ARK-1 was shown to be able to colonize and survive in the roots of grapevine when treated as a root dip without causing any type of necrosis as seen with F2/5. The colonized roots showed a reduced rate of gall formation compared to roots not treated with ARK-1 (Kawaguchi 2013). These results suggest that ARK-1 could persist for years and protect vines for at least two years after planting, the period when vines are most vulnerable to crown gall. To begin investigation of the mechanism of ARK-1, the populations of both ARK-1 and tumorigenic isolates *in planta* and *in vitro* were examined. ARK-1 was shown to cause a halo of inhibition in *in vitro* antibiosis assays, but this varied from 20 to 0 mm of inhibition, depending on the growth media and indicator strain used. *In planta*, ARK-1 was shown to reduce the population of the tumorigenic isolate when symptoms were absent, but populations were not affected in galls (Kawaguchi 2014). The main mechanism of ARK-1 reducing gall formation is suspected to be due to a decrease of gene expression of *virD* and *virE* genes on the Ti-plasmid. Tumorigenic isolates co-inoculated with ARK-1 had significantly lower expression levels of the virulence genes. However, this decrease in expression was not observed in co-inoculations of cell-free ARK-1 culture supernatant (Kawaguchi 2015). This result confirms that cells of the bacterial isolate, ARK-1, must be alive and present to inhibit gall

formation and ARK-1 is not producing a small molecule exudate (e.g. agrocin) that interferes with host transformation.

These promising results have led to ARK-1 testing in plant hosts beyond grapevine. ARK-1 efficacy as a biological control agent has been tested in tomato, rose, pear and apple inoculated with *R. vitis, tumefaciens* or *rhizogenes*. ARK-1 root dip-treatments were able to effectively prevent gall formation compared to untreated plants (Kawaguchi et al. 2015). Unlike F2/5, which is only effective in grapevines, ARK-1 has the potential to be effective against more than one species of *Rhizobium* and more than one plant host species. In these studies, ARK-1 will be examined as a biological control agent in preventing grapevine crown gall.

Justification of study

In this study, we examined biological control candidate ARK-1. Discovered in Japan, ARK-1 was found on the surface of gall tissue of grapevine, but it does not carry the pathogenic plasmid required for plant host transformation (Kawaguchi & Inoue 2012), so it is not tumorigenic. Studies of ARK-1 revealed that crown gall inhibition is governed by slowing the growth of tumorigenic *R. vitis* isolates that cause crown gall and reducing expression of genes that are required for plant cell transformation (Kawaguchi et al. 2017). Our work evaluated ARK-1 as a biological control agent in the United States. Japanese studies evaluated the efficacy of ARK-1 as a biological control agent when challenged with tumorigenic isolates of *R. vitis* that were isolated in Japan. Studies in Japan have also only investigated ARK-1 with co-inoculations in grapevine seedlings of table grapes, which are genetically distinct from wine grapes (Zhou et al. 2017). Finally, only the soft, green tissue of seedlings was co-inoculated to test efficacy in Japan. We sought to expand on these methods to further investigate the potential of ARK-1 as an effective biological control agent for crown gall. We tested tumorigenic isolates of *R. vitis*

recovered from galls on grapevines in Virginia vineyards against ARK-1 to evaluate the efficacy of ARK-1 to prevent crown gall. Since the predominant use of grape production in Virginia is to produce wine, we tested how ARK-1 can be used as a biological control agent in *Vitis vinifera* wine grape varieties. We inoculated woody tissue and green seedling stems with ARK-1 to determine if ARK-1 could be effective in woody tissue, as well as green tissue.

This work is significant because there are no highly effective control options for the management of crown gall. This is especially important in the mid-Atlantic where crown gall incidence is relatively high compared to regions with moderate climates, such as California, Quarantine of *R. vitis* is impossible due to the widespread nature of the pathogen in soil and nursery stocks. A biological control product that could be used to protect vines for years from *R. vitis* infection and disease development would reduce the monetary loss to vineyards due to crown gall.

Chapter II

Biological Control Agent ARK-1 Reduces Single-Isolate Induced Crown Gall of Grapevine in Virginia

This chapter was written with the intent to submit to *Plant Disease* for publication

References are in the combined Literature cited section following Conclusions and future directions

Abstract

Non-tumorigenic *Rhizobium* (=*Agrobacterium*) *vitis,* strain ARK-1 has been shown to reduce crown gall in both tomato (*Solanum lycopersicum*) and grapevine (*Vitis vinifera*) seedlings when co-inoculated with Japanese tumorigenic isolates of *R. vitis*. To date, however, the efficacy of ARK-1 as a biological control agent for *R. vitis* isolates from outside Japan has not been investigated. The objective of this study was to test ARK-1 efficacy as a biological control agent for crown gall caused by *R. vitis* native to Virginia. Four tumorigenic *R. vitis* isolates from a collection of different Virginia grapevine gall samples were selected for biocontrol assays. To challenge ARK-1 with Virginia isolates, ARK-1 was co-inoculated into tomato seedling stems with one of four tumorigenic *R. vitis* isolates at a 1:1 cell ratio. Compared to inoculation with tumorigenic isolates alone, ARK-1 co-inoculation significantly decreased gall formation as well as gall diameter ($P < 0.05$). Co-inoculation of three-year-old grapevine trunks with ARK-1 and tumorigenic *R. vitis* isolates also led to a significant reduction of gall numbers and gall diameter at inoculated locations when compared to inoculation with the tumorigenic isolates alone ($P < 0.05$). These results demonstrate that efficacy of ARK-1 challenged with Virginia *R. vitis* isolates was similar to results with Japanese isolates and suggest that ARK-1 is a good candidate for biocontrol of crown gall in the mid-Atlantic region of the United States.

Introduction

Crown gall of grapevine is a devastating bacterial disease caused by *Rhizobium vitis*, previously known as *Agrobacterium vitis* (Young et al. 2001). Galls are hypertrophic growths that form at wounds on the roots or trunk of a vine. Wounds can result from a variety of sources, but primarily result from winter or mechanical injury, such as grafting (Burr & Otten 1999). When *R. vitis* senses a wound based on the release of secreted plant chemical signals, the transfer DNA (T-DNA) region from the tumor-inducing bacterial plasmid (Ti-plasmid) is transferred, and subsequently integrated, into the plant host genome (Gelvin 2012; Chilton et al. 1977; Pitzschke & Hirt 2010). The inserted T-DNA codes for plant growth hormones and opine synthesis genes (Burr et al. 1998; Morris 1986). The high levels of plant growth hormones induce hyperplastic and hypertrophic cell growth that results in galls. Galls are water and nutrient sinks that weaken plants and can eventually lead to vine death (Gohlke & Deeken 2014; Wächter et al. 2003). Opines are small metabolites synthesized in the gall cells that are not utilized by the plant (Tempé & Goldmann 1982), but serve as a nitrogen and energy source for *R. vitis* (Lang et al. 2014; Szegedi et al. 1988; Chilton et al. 2001). In this paper, *R. vitis* isolates that carry the Ti-plasmid and, thus, are capable of causing crown gall will be referred to as "tumorigenic".

The history of investigation for viable biological control agents for crown gall goes back to the early 1970s (New & Kerr 1972). There have been a number of studies investigating various bacteria other than *Rhizobium* spp., as potential biological controls for crown gall. *Serratia plymuthica* IC1270, *Pseudomonas fluorescens* Q8r1-96, *P. fluorescens* B-4117 (Dandurishvili et al. 2011) all caused a significant reduction in the mass of galls formed on tomatoes compared to pathogen-alone controls. However, none of the strains of tumorigenic *Rhizobium* used were from the United States. *Rahnella aquatilis* HX2 exudate, gluconic acid,

suppressed gall formation in both sunflower and grapevine when inoculated with *Rhizobium vitis, tumefaciens,* or, *rhizogenes* (Chen et al. 2007; Li et al. 2014). These studies only examined one strain of tumorigenic *R. vitis.* The *V. vinifera* endophyte *Curtobacterium* spp. (Ferrigo et al. 2017) had a significant decrease in gall incidence and size when co-inoculated with *R. vitis.* The three *R. vitis* strains used were originally isolated in Italy. *B. subtilis* 14B (Hammami et al. 2009) produces an antimicrobial protein (Hammami et al. 2012) that can significantly reduce the formation of galls and the gall mass. This study only shows the results of the biological control assay using tumorigenic *R. tumefaciens* in tomato co-inoculations. Fungal species have also been examined for potential efficacy as a biological control agent. *Trichoderma asperellum* T1 (Ferrigo et al. 2017) has shown significant reduction of grapevine crown gall caused by the Italian *R. vitis* strains mentioned above.

Among these potential agents, two biological control agents have been extensively studied for the prevention of crown gall. *Rhizobium rhizogenes* (previously *R. radiobacter*) (Velázquez et al. 2010) strain K84 has been studied and used since the early 1970's. *R. rhizogenes* K84 does inhibit the growth of some *Rhizobium* species and did reduce crown gall (New & Kerr 1972; Htay & Kerr 1974; Kerr & Htay 1974). K84 produces an antimicrobial agrocin 84 that is antagonistic to sensitive strains of *Rhizobium* (Kerr 1980; Reader et al. 2005). K84 is used today for the prevention of crown gall predominantly in stone fruit (*Prunus* spp.*)* and apples (*Malus* spp.*)* but also other fruit and nut trees. However, K84 is ineffective at reducing crown gall in grapevine caused by *R. vitis* (Burr et al. 1998; Kawaguchi & Inoue 2012).

Another effective biological control agent is a non-tumorigenic *R. vitis* strain F2/5 (Burr & Reid 1994; Kaewnum et al. 2013). F2/5 reduces crown gall by stimulating the hypersensitive defense response in grapevine (Zheng et al. 2003; Kaewnum et al. 2013; Zheng & Burr 2016).

However, this hypersensitive response also induces necrosis of grapevine tissue, which can lead to vine mortality (Bazzi et al. 1999). In addition, F2/5 is host specific, and only able to prevent crown gall in grapevines and is only effective against some isolates of *R. vitis* (Burr et al. 1997; Burr & Reid 1994). Since first testing F2/5 against many *R. vitis*, F2/5 has only been tested against *R. vitis* isolates that have high sensitivity to F2/5. Derivatives of F2/5 that do not induce necrosis, but are still capable of preventing crown gall have been developed and patented (Burr & Zheng 2012).

Recently in Japan, a new strain of *R. vitis*, designated ARK-1 by Dr. Akira Kawaguchi, was recovered from 'Pione' grapevine (*V.* hybrid 'Kyoho' x 'Muscat of Alexandria') (Yamada & Sato 2016). ARK-1 neither carries the Ti-plasmid nor causes disease (Kawaguchi & Inoue 2012). When ARK-1 was inoculated onto grapevine seedlings together with isolates that carry the Ti-plasmid (tumorigenic isolates), gall incidence decreased by approximately 90% without causing necrosis to grapevines (Kawaguchi & Inoue 2012). Interestingly, if ARK-1 cultures were filtered to remove cells or heat-killed before co-inoculation, there was no reduction in gall formation when co-inoculated with tumorigenic isolates (Kawaguchi & Inoue 2012). ARK-1 slows the population growth of tumorigenic *R. vitis* isolates at inoculation locations (Kawaguchi 2014) and suppresses Ti-plasmid gene expression (*virD2* and *virE2*) critical to plant cell transformation by *R. vitis* (Kawaguchi 2015). This suggests that ARK-1 must be alive to be effective and suppresses host transformation without the use of a secreted factor (Kawaguchi et al. 2017). ARK-1 has been shown to be effective at controlling crown gall in other plant species such as tomato, pear, peach, and rose (Kawaguchi et al. 2015). Although ARK-1 seems to show great potential as a biological agent against crown gall, these studies have investigated the efficacy of ARK-1 only against Japanese isolates of *R. vitis*. Therefore, in this study, the effect of ARK-1 in

reducing gall formation was tested when co-inoculated in tomato stems and grapevine trunks at a 1:1 cell ratio using individual tumorigenic *R. vitis* isolates from Virginia (VA), United States vineyards.

Materials and Methods

Origin and preparation of ARK-1

ARK-1 was obtained by the Virginia Tech Alson H. Smith Jr. Agricultural Research and Extension Center through Kumiai Chemical Industry Co, LTD in 2015. The freeze-dried ARK-1 cells were reconstituted in sterile distilled water at a rate of 0.2 g freeze-dried cells per liter of sterile distilled water (colony forming units (CFU) of 5 x 10^7 CFU/ml). One loopful of the ARK-1 suspension was streaked on semi-selective Roy and Sasser medium (Roy & Sasser 1983) and incubated at 28°C. All growth on RS medium from the reconstituted freeze-dried ARK-1 cells was expected to be ARK-1.

Isolation of *R. vitis* from Virginia vineyards

Virginia isolates of *R. vitis* were obtained by collecting gall tissue from grapevines in Virginia vineyards. Gall tissue was cut into pieces, surface sterilized with 70% ethanol for 2 minutes and rinsed twice with sterile distilled water. The tissue was then soaked in 5 ml of sterile distilled water for 30 minutes in a sterile 15 ml tube to allow the bacteria to move out of the tissue and into the water. A 100 µl aliquot of the suspension was then streaked onto Roy and Sasser plates (Roy & Sasser 1983) and incubated at 28°C. After 5-7 days, single colonies with the expected phenotype for *Rhizobium* species (i.e., mucoid and purple color at the center of a colony) were re-streaked onto potato dextrose agar (Difco, Sparks, MD) and yeast extract mannitol (YEM) plates (Morton & Fuqua 2012) for storage at 4°C.

Identification of tumorigenic *R. vitis* species

To extract DNA from *R. vitis* isolates, a small amount of a *R. vitis* colony was transferred with a sterile dissecting needle to 50 µl of sterile distilled water to 0.2 ml PCR tubes; this was vortexed for 10 seconds and incubated for 10 minutes at 95°C. The tubes were centrifuged at 20817 x g RCF (14,000 rpm) in an Eppendorf 5424 (Eppendorf, Hamburg, Germany) for 5 minutes. Forty µl of supernatant were transferred into a 1.5 ml microfuge tube and stored at -20°C.

Multiplex PCR for *R. vitis* characterization was performed using the primers and method described by Kawaguchi et al. (2005). A Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany). The primer pair to identify *R. vitis* was Ab3-F3 (5'-ATGACGGTAGTCGGAGAAGAAGCC-3') / Ab3- R4 (5'-CTGTCTCTGTGTCCCCGAAAGG-3') (Integrated DNA Technologies, Coralville, IA). This primer set produces a 568 base pair amplicon of the 16S ribosomal RNA gene region (accession no. D14502). The 414 base pair amplicon from the VCF3/R3 primer set overlaps the ends of the *virC1* and *virC2* genes (accession no. M16397). The primer pair used to identify the Ti-plasmid was VCF3 (5'GGCGGGCGYGCYGAAAGRAARACYT -3') / VCR3 (5'-AAGAACGYGGNATGTTGCATCTYAC-3') (Y = C or T; R = A or G; N = A, C; T, or G) (Integrated DNA Technologies, Coralville, IA). Twenty five µl reaction mixture volumes were as follows: 12.5 µl 2x Qiagen multiplex PCR master mix, 0.5 µl VCF3 (20 pmol/µl), 0.5 µl VCR3 (20 pmol/µl), 0.5 µl Ab3-F3 (5 pmol/µl), 0.5 µl Ab3-R4 (5 pmol/µl), 1 µl DNA template (~15 ng/µl), and 9.5 µl nuclease free water. Thermal cycling for the C1000 thermocycler (Bio Rad Laboratories, Hercules, CA) was programed as follows: 95°C for 15 min, followed by 34 cycles of 95°C for 30 s, 56°C for 90 s, 72°C for 90 s, and a final extension at 72°C for 10 min. PCR products were resolved on a 2% agarose gel. PCR products were stained with GelRed™

(Biotium, Fremont, CA) nucleic acid stain, and visualized with ultraviolet Trans illuminator (Fotodyne, Hartland, WI).

PCR products from the Ab3-F3/R4 primer set (16S rRNA gene) were purified with QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany), to verify correct amplification. Sequencing was performed bi-directionally by the Genomics Sequencing Center at the Virginia Tech Biocomplexity Institute. The sequences of the tumorigenic Virginia isolates were aligned and compared using Geneious® alignment software (Geneious, Version 11.1.2, Auckland, New Zealand). Consensus sequences generated from the partial 16S rRNA amplicon of the tumorigenic Virginia isolates were submitted to NCBI GenBank (MH330681, MH333801, MH333802, and MH333803) and compared to other *Rhizobium* spp. nucleotide sequences using NCBI BLASTN program version 2.8.0 (Zhang et al. 2000) (blast.ncbi.nlm.nih.gov).

***In planta* ARK-1 biocontrol efficacy assays in tomato**

Tomato seedlings, *Solanum lycopersicum* “Beefsteak” (Ferry-Morse, Fulton, KY), were grown from seed for one month in a greenhouse under natural light in summer and 24 hour fluorescent light in early spring. Bacterial inocula were prepared by inoculating 5 ml of YEM broth in a 15 ml culture tube with a *R. vitis* colony with a sterile loop. Cultures were incubated at a 45° angle on a shaker that was set at 135 rpm for 48 hours at 28°C. After 48 hours, optical densities of cell cultures were measured at 600 nm (OD_{600}) and adjusted to OD_{600} of 0.1. Inoculum was mixed with equal volumes of distilled water or ARK-1. Five stab wounds (~1-2 cm apart) were made on the tomato stem with a sterilized metal dissection needle. Five µl of the bacterial suspension was transferred into each wound location using a micropipette. Control plants received 5 µl of water or ARK-1 inoculum.

Gall formation was recorded and galls were measured at their widest diameter using a digital caliper (Electron Microscopy Sciences, Hatfield, PA) at 42 days post-inoculation (Fig. 2-1). Locations that did not form galls were assigned a diameter value of 0.00 mm. This experiment had three internal replications with four experimental trials. Each treatment was tested on 12 tomato plants, each with five inoculation wounds for a total of 60 inoculation locations for each treatment.

In planta ARK-1 biocontrol efficacy assays in grapevine

Vitis vinifera 'Cabernet Sauvignon' vines (3 years old and self-rooted) were grown in root bags with a mushroom compost and sand mixture in a raised bed in a high tunnel structure. The same bacterial isolates from the tomato assay were used in the grapevine assay. Cell suspensions as inocula were prepared as described above. Wounds were made into the trunks of grapevines 3-4 inches above the soil line by making 3.2 mm diameter drill wounds to the core of the vine, with approximately 2.5 cm spacing between drill wounds. Wounds were inoculated with 100 µl of bacterial inoculum for each drill location and then wrapped in Parafilm. Control plants were inoculated with water or ARK-1 alone. Gall formation was recorded and gall diameter (mm) at the widest point was measured with a digital caliper at 90 days post-inoculation. At inoculation locations where a gall had formed internally but was not measurable externally, galls were recorded as a formed gall and assigned a gall diameter value equal to the size of the inoculation wound (3.2 mm). Inoculation locations that did not form galls were given a gall diameter value of 0.00 mm. This experiment had three internal replications and three experimental trials. For each treatment, there were a total of nine plants, each with five inoculation locations, thus, a total of 45 inoculation locations were measured per treatment.

Figure 2-1.Measurement of tomato stem galls 42 days after inoculation with a digital caliper. Each gall diameter was measured at the widest point.

Statistical analyses

The probability of gall formation and the average gall diameter per treatment were analyzed using the generalized linear mixed model (GLIMMIX) in SAS (ver. 9.4, SAS, Cary, NC). The logit and the identity link function were used for the mean probability of gall formation, and gall diameter data, respectively. Data are presented in figures as the proportion of inoculation locations that formed a gall, but the analysis was performed to test the probability of successful inoculation (gall formation) for each treatment effect. Treatment and isolate factors were considered as fixed effects and experimental run factor was considered as a random effect. The effects of treatment, isolate, and their interaction on the probability of gall formation and the mean gall diameter per treatment were examined. Once the effect of a fixed factor(s) or their interaction was found to be significant ($P < 0.05$), Fisher's Least Significant Difference (LSD, $\alpha = 0.05$) was used as a mean separation method.

Results

Selection of Virginia *R. vitis* isolates

A total of 33 *R. vitis* isolates were obtained and characterized in 2015 from galls on several cultivars of *Vitis vinifera* from different geographical regions of Virginia (*data not shown*). Based on the geographic locations (Northern, Central, and Shenandoah Valley, Virginia) and the relative growth rate of isolates on the RS media (mucoid colonies with red centers), four tumorigenic isolates were selected for further investigation (Table 2-1). The presence of Ti-plasmid for each isolate was confirmed by the multiplex PCR described above. Sequence alignment confirmed that the 16S rRNA partial sequences of each Virginia tumorigenic isolate were 100% identical to each other (Appendix A). A BLASTN search showed that nine *R. vitis* sequences (NR_115517, AB680781, FR828339, CP000633, KC196483, NR_036780,

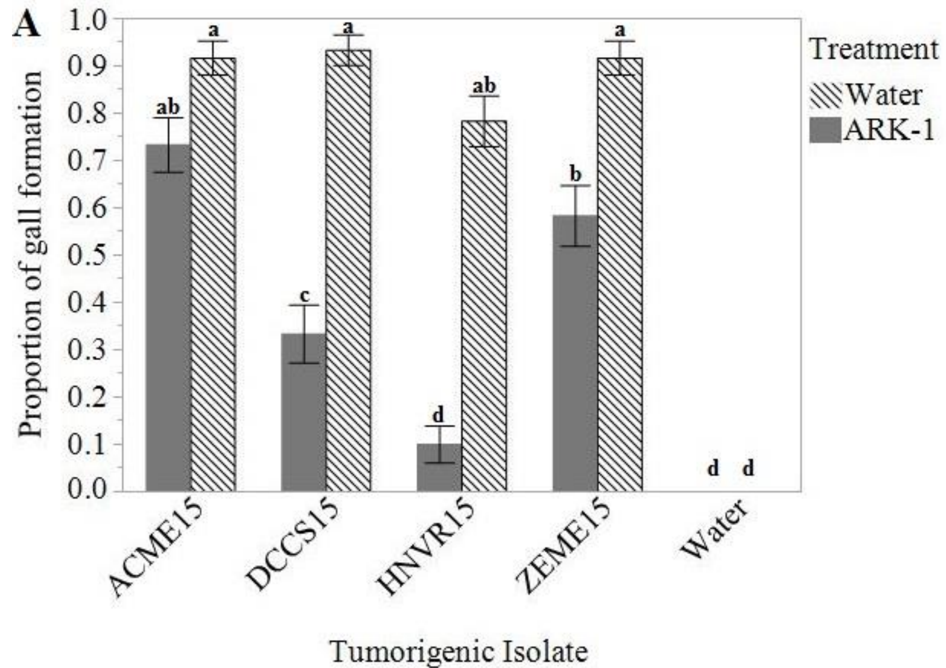
JN185718, NR_18989, and NR1137535) were 99-100% identical to sequences of the Virginia tumorigenic isolates. *R. tumefaciens* sequences (LT899962, MG062741, MG819299, and MF967403) and R. rhizogenes sequences (AB289616, LN845892, NR_104207, KC196497, NR_113607, and NR_043398) were all less than 98% identical.

Table 2-1. *Rhizobium vitis* isolates used in inoculation studies.

Isolate ID (GenBank Accession no.)	Location (Region)	Grapevine host cultivar	Ti-Plasmid present?
ACME15 (MH330681)	Winchester (Shenandoah Valley Virginia)	'Merlot' (*V. vinifera*)	Yes
DCCS15 (MH333801)	Etlan (Northern Virginia)	'Cabernet Sauvignon' (*V. vinifera*)	Yes
HNVR15 (MH333802)	Gordonsville (Central Virginia)	'Viognier' (*V. vinifera*)	Yes
ZEME15 (MH333803)	Hamilton (Northern Virginia)	'Merlot' (*V. vinifera*)	Yes
ARK-1	Okayama, Japan	'Pione' (*V.* hybrid)	No

Biocontrol gall inhibition assays in tomato seedlings

Tomato stem galls were assessed 42 days after inoculation. The effect of the interaction between treatment and isolate on the mean probability of gall formation was significant ($F = 7.1$, $P < 0.001$). The mean probability of gall formation per treatment ranged from 0.00 to 0.93 (Fig. 2-2A). When ARK-1 was co-inoculated with isolates DCCS15, HNVR15, and ZEME15, the mean probability of gall formation was significantly reduced ($P \leq 0.05$). The average gall diameter varied from 0.00 to 9.75 mm (Fig. 2-2B). The interaction between the fixed effects of treatment and isolate significantly affected the mean gall diameter ($F = 8.2$, $P = 0.001$). Inoculation with ACME15 and ZEME15 resulted in significantly larger mean gall diameters than the other two tumorigenic isolates (Fig. 2-2B). ARK-1 treatment significantly reduced ($P \leq 0.05$) the mean diameter of galls for every isolate compared to inoculation with the isolate alone. As expected, treatments that received ARK-1 or water alone did not result in any gall formation.



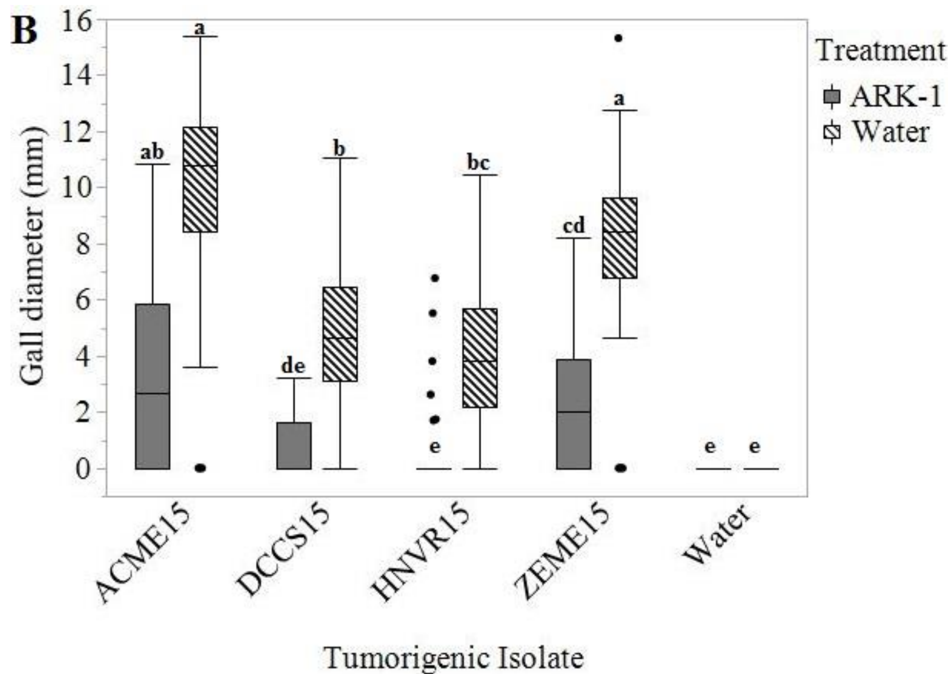


Figure 2-2. Effect of ARK-1 co-inoculation on the A) mean probability of gall formation and B) mean gall diameter 42 days after 1:1 cell ratio co-inoculation of tumorigenic isolates with ARK-1 in tomato stems. Solid grey columns represent ARK-1 co-inoculation and striped columns represent water co-inoculations. Error bars represent the standard error of the mean proportion of gall formation (n = 60). Different letters represent significant differences according to Fisher's LSD (P < 0.05). The points outside the box and whisker plot are outliers.

Biocontrol gall inhibition assays in grapevine

Assessments of gall formation and gall diameter were made 90 days after inoculation with *R. vitis* isolates (Fig. 2-3). The mean probability of gall formation per treatment varied from 0.00 to 0.84 (Fig. 2-4A). As with the tomato trial, the effect of the interaction between treatment and isolate on the mean probability of gall formation per treatment was significant ($F = 3.0$, $P = 0.046$). With solo inoculation, both HNVR15 and ZEME15 resulted in significantly higher mean probability of gall formation ($P \leq 0.05$) than ACME15 and DCCS15. With HNVR15 and ZEME15, ARK-1 treatment significantly reduced ($P \leq 0.05$) the mean probability of gall formation per treatment.

The mean gall diameter per treatment ranged from 0.00 to 4.74 mm (Fig. 2-4B), and a significant interaction of treatment and isolate effect on the mean gall diameter was observed ($F = 30.2$ and $P < 0.001$). Similar to the mean probability of gall formation, the mean gall diameters of isolate HNVR15 and ZEME15, when inoculated alone, were significantly larger than galls resulting from inoculation with the other two isolates alone ($P \leq 0.05$). When ARK-1 was co-inoculated, the mean gall diameter was reduced significantly ($P \leq 0.05$) for isolates DCCS15, HNVR15 and ZEME15, but not for ACME15 (Fig. 2-4B). However, ACME15 had a very small mean gall diameter when inoculated alone. As expected, inoculation with water or ARK-1 alone did not result in gall formation.

Figure 2-3. *R. vitis* inoculated “Cabernet Sauvignon” grapevine trunk, 3 months after inoculation with A) ARK-1 + HNVR15 and B) tumorigenic *R. vitis* isolate HNVR15 alone.

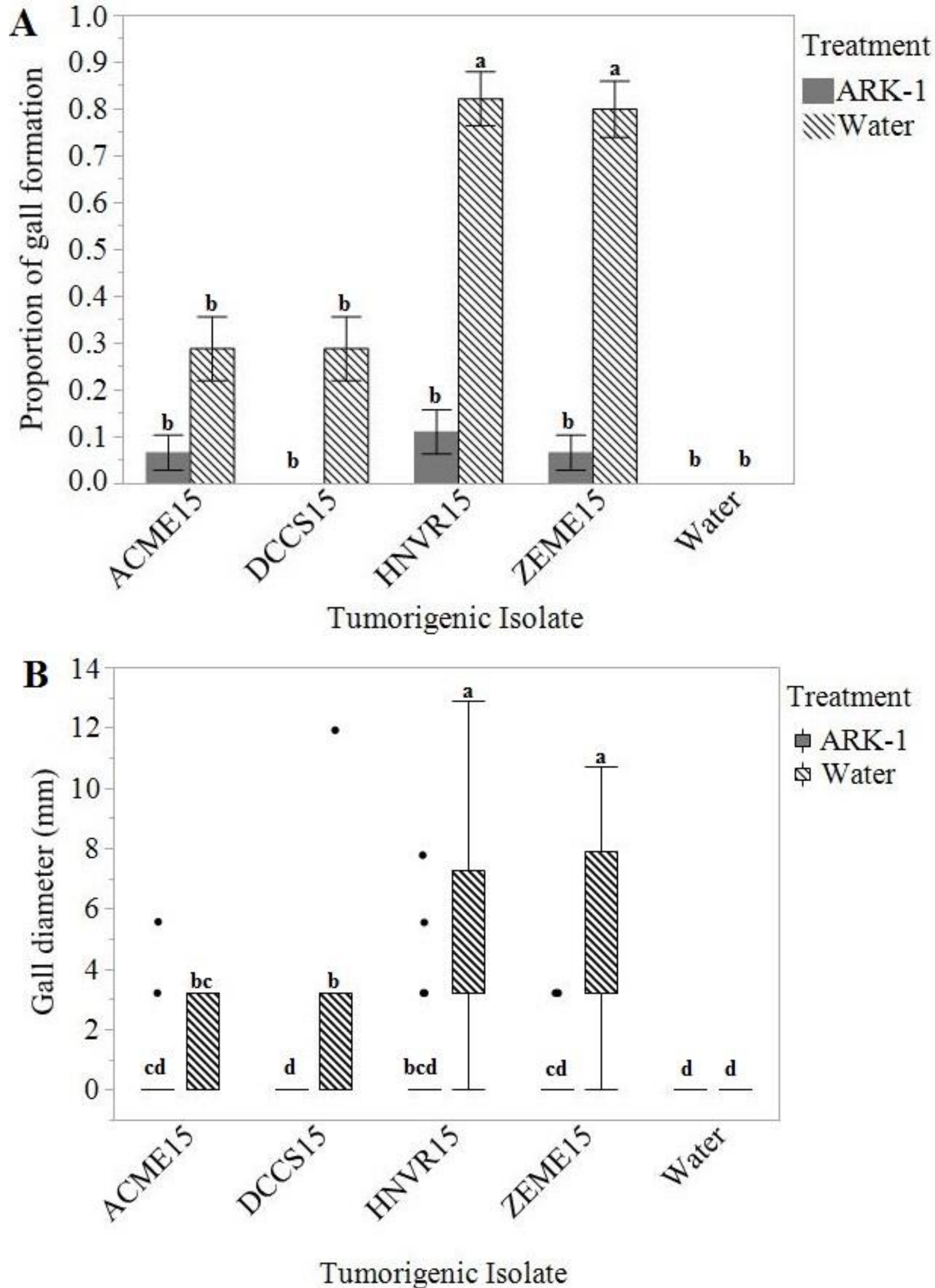


Figure 2-4. Effect of ARK-1 inoculation of the A) mean probability of gall formation and B) mean gall diameter 90 days after 1:1 cell ratio co-inoculation with ARK-1 in grapevine trunks. Solid grey columns represent ARK-1 co-inoculation and striped columns represent water co-inoculations. Error bars represent the standard error of the mean proportion of gall formation ($n = 45$). Different letters represent significant differences according to Fisher's LSD ($P < 0.05$). Points outside the box and whisker plot are outliers.

Discussion

Our results indicate that ARK-1 has significant potential for grapevine crown gall management. A simultaneous 1:1 cell ratio co-inoculation of grapevine with ARK-1 and a tumorigenic *R. vitis* isolate in grapevines resulted in an average of 89% reduction for both the mean probability of gall formation and the mean gall diameter. Previous studies have shown that simultaneous 1:1 cell ratio co-inoculation of ARK-1 with a mix of seven tumorigenic Japanese *R. vitis* isolates reduced gall formation in grapevine by approximately 90% (Kawaguchi & Inoue 2012). Our study confirms that the levels of reduction provided by the ARK-1 treatment against four Virginia isolates are similar to the previous findings with Japanese table grapes. In a previous study of another potential biocontrol agent for grapevine crown gall, a 1:1 cell ratio co-inoculation of *R. vitis* F2/5 was 100% effective against some but not all US native tumorigenic *R. vitis* strains (Burr and Reid 1994). ARK-1 and F2/5 have both demonstrated efficacy to significantly reduce crown gall incidence and severity in grapevine.

Interestingly, the reduction in mean probability of gall formation by ARK-1 was not as significant with tomato seedling inoculation as was seen in grapevine trunk inoculations. In this study, the percent reduction of mean probability ranged from 20% to 87%. In a study conducted in Japan, ARK-1-treated tomato plants showed a similar reduction in gall formation that ranged from 69% to 80% of plants with galls (Kawaguchi et al. 2015). In the same study, the efficacy of ARK-1 did vary among crops and experimental repetitions. The tested crops and the mean percent reductions of the number of galled plants were: apple (69%), Japanese pear (78%), peach (86%), rose (71%), and tomato (80%). The overall range varied from 45% to 100%, indicating that the efficacy of ARK-1 treatment can be influenced by host species and other conditions.

There was significant variation in the mean probability of gall formation among isolates that were inoculated with ARK-1 in tomatoes ($P < 0.05$). In a study conducted using *R. vitis* F2/5 co-inoculated with individual *Rhizobium* isolates in grapevines, Burr and Reid (1994) also reported varying efficacy of F2/5 among tested isolates. Among eleven *R. vitis* isolates tested in their study, five isolates were completely inhibited, three were inhibited by 80-90%, one by about 60%, one by approximately 10%, and one had a 0% reduction of gall incidence at the inoculation locations.

In some cases, opine production and metabolism, which are encoded by the Ti-plasmid, influenced the function of a biological agent against crown gall. For example, *R. rhizogenes* strain K84 is ineffective against *R. vitis* strains that typically carry vitopine, octopine and/or nopaline type plasmids (Perry & Kado 1982; Szegedi et al. 1988). Nopaline can induce bacterial conjugation and transfer of the agrocin 84 plasmid that would confer resistance to previously sensitive strains (Kerr 1980; Kerr & Roberts 1976). However, sensitive *R. tumefaciens* strains can also carry octopine or nopaline type plasmids (Knauf et al. 1983; Szegedi et al. 2005), so the resistance to agrocin 84 may simply be correlated to opine utilization and be caused by other unique characteristics of *R. vitis*. When a mixture of seven tumorigenic *R. vitis* strains were used in previous Japanese experiments, ARK-1 was shown to be effective against different opine producing tumorigenic isolates [vitopine, nopaline, and octopine strains] (Kawaguchi & Inoue 2012). However, the study did not test individual strains separately from each other. Further investigation is needed to understand tumorigenic *R. vitis* populations from Virginia vineyards. The efficacy of ARK-1 could vary from isolate to isolate due to the specific Ti-plasmid types. Previous studies showed that isolates that utilize different opines can be correlated with gall virulence in tomatoes (Dandurishvili et al. 2011).

Differences among isolates also differed between tomato and grapevine trials. In particular, both isolates ACME15 and DCCS15 resulted in high mean probability of gall formation and large mean gall diameters with tomato inoculation, but with grapevine inoculation, these isolates resulted in significantly lower probabilities and smaller gall diameters ($P \leq 0.05$) than inoculation with the other two isolates. As noted earlier, a wide range of variability in the percent reduction of gall formation with ARK-1 co-inoculation has been shown in other crops. We suspect that the isolates ACME15 and DCCS15 may be more virulent when inoculated in tomato seedlings than grapevine trunks. We also speculate that the lignified tissue of grapevine trunks may be less susceptible to infection than the young, green tissue of tomato or grapevine seedling stems.

Unlike *R. rhizogenes* K84, which produces the antibacterial compound, agrocin 84, ARK-1 does not seem to produce an effective amount of an antibacterial compound (Kawaguchi & Inoue 2012). One potential mode of action for ARK-1 is interference with expression of virulence genes, such as *virD2* and *virE2* in tumorigenic *R. vitis* (Kawaguchi 2015). These genes are critical in the transfer of T-DNA from the pathogen to the host plant cell (Gelvin 2012). Once the transfer is successful, ARK-1 may not be able to control tumorigenic strains. Therefore, a possible explanation for the lower efficacy of ARK-1 in tomato is that the tested *R. vitis* isolates grow faster than ARK-1 in tomato, colonize the cells and insert T-DNA into the host cell before the intervention by ARK-1. Similar to ARK-1, *R. vitis* F2/5 was found to be dependent on the timing of application (Kaewnum et al. 2013). F2/5 has to be applied either prior to or at the time of inoculation to be effective (Burr & Reid 1994; Kaewnum et al. 2013).

The other possible cause of the variability is the difference in environmental conditions during the incubation period. Although not statistically significant, there were higher numbers of

gall formation and gall diameters observed with the tomato trial when experimental trials were conducted during the summer than when trials were conducted in the spring. The tomato plants also tended to grow faster in the summer than the spring. The rapid growth of the host cells may favor some isolates over others. Another possibility is that ambient temperatures influenced the pathogen's level of activity. For instance, on agar media plates, the rate of colony expansion of all tumorigenic isolates tended to be faster at 30°C than 25°C (*data not shown*). Thus, the higher temperatures observed during the summer might have affected the results.

To our knowledge this is the first study of *R. vitis* ARK-1 biocontrol efficacy using the woody tissue of a wine grape cultivar (*Vitis vinifera* cv. 'Cabernet Sauvignon') and individual US (Virginia) *R. vitis* isolates. Previous studies of ARK-1 used *V. vinifera* 'Neo Muscat' seedlings, a table grape cultivar (Kawaguchi & Inoue 2012; Yamada & Sato 2016; Zhou et al. 2017). Those studies also used a mixture of Japanese tumorigenic *R. vitis* isolates without testing each isolate individually when challenging ARK-1 (Kawaguchi & Inoue 2012). In this study, the lignified woody portion of the grapevine was co-inoculated with ARK-1 and Virginia tumorigenic isolates. In the previous studies (Kawaguchi & Inoue 2012; Kawaguchi 2013), green stem stab inoculations and roots of the grapevines grown in infested soil were used for assessment of gall formation. However, crown galls often form on the main trunk above the ground in the field. In this study, the ability of ARK-1 to prevent gall formation was demonstrated under conditions that more closely resembled those in the vineyard, in terms of tissue type.

Based on these results, we conclude that ARK-1 is a promising candidate for biocontrol of grapevine crown gall in the United States. Reduction in gall incidence and severity (gall diameter) would provide protection to grapevine from the disease and limit the losses for grape

growers. Because ARK-1 is able to establish in roots and act as an effective antagonist to crown gall-causing isolates (Kawaguchi 2013), ARK-1 is currently being tested by Kumiai Chemical Industry Co., LTD as a before planting-root dip treatment for grapevine. Future studies will investigate whether use of ARK-1 as a soil drench root treatment is a feasible option in the United States. Future studies will also examine how well ARK-1 inhibits gall formation when challenged with a mixture of two or more isolates of tumorigenic *R. vitis* and whether inhibition is increased with an increase in the ratios of ARK-1 cells relative to the tumorigenic isolates.

Chapter III

A Biological Control Agent *Rhizobium vitis* ARK-1 Reduces Crown Gall Incidence and Size when Challenged by Multiple Tumorigenic *R. vitis* Isolates

This chapter was written with the intent to submit to *Plant Disease* for publication

References are in the combined Literature cited section following Conclusions and future directions

Abstract

Biological control agent *Rhizobium vitis* ARK-1 has been shown to reduce gall incidence and gall size of grapevine crown galls caused by tumorigenic *Rhizobium vitis.* Studies have been conducted in both Japan and the United States using native tumorigenic *R. vitis* isolates. The objective of this study was to investigate the efficacy of ARK-1 as a biological control agent. In vineyard conditions, more than one individual isolate of *R. vitis* may be present as a potential source of infection and at different cell concentrations. To simulate dynamic populations of *R. vitis*, ARK-1 was co-inoculated with four individual tumorigenic *R. vitis* isolates and with different relative ratios and mixtures of the tumorigenic isolate(s). Decreasing the relative ratio of ARK-1 to the tumorigenic isolate by two-fold resulted in a significant reduction in both gall formation and size ($P \leq 0.05$). ARK-1 also significantly reduced gall formation and gall size ($P \leq 0.05$) when co-inoculated with mixtures of either two or four tumorigenic isolates. We conclude that ARK-1 is an effective biological control agent for the control of crown gall in grapevine when co-inoculated with more than one isolate of *R. vitis* and at ratios that would translate to practical application rates in an agricultural setting.

Introduction

Crown gall of grapevine (*Vitis* spp.) is an economically important disease caused by the bacterial pathogen *Rhizobium vitis* (Burr et al. 1998). This disease negatively impacts wine grape production in the United States by millions of dollars every year (Burr & Otten 1999; Kennedy & Alcorn 1980). *R. vitis* uses a tumor-inducing plasmid (Ti-plasmid) to transfer DNA (T-DNA) into the host plant cell genome (Chilton et al. 1977; Pitzschke & Hirt 2010; Gelvin 2012). Host transformation by the Ti-plasmid of the pathogen reprograms the cell to synthesize growth hormones that induce rapid cell growth and division (Morris 1986). Gall cells transformed by T-DNA also synthesize opine metabolites that serve the pathogen as a carbon and nitrogen source (Chilton et al. 2001; Tempé & Goldmann 1982; Szegedi et al. 1988). These rapidly proliferating cells form galls on the roots and trunk of grapevines, which negatively affect vine production by diverting water and nutrient resources to support bacterial growth (Wächter et al. 2003; Deeken et al. 2006). Severe galling that girdles the trunk can disrupt xylem and phloem flow, leading to vine collapse. This pathogen is commonly found in vineyards and nurseries in the United States and has significant economic consequences, especially in the mid-Atlantic US where crown gall symptoms are more prevalent than in areas with milder winters due to frequent winter injury (Burr et al. 1998).

Due to the lack of chemical control options, crown gall management in the field is limited to cultural and biological options, most of which are not feasible for routine or large scale use. Cultural options focus on prevention of injury because the bacterium requires a wound to infect grape tissues (Burr et al. 1998). One example is the hilling of soil at the crown of the grapevine to reduce the risk of winter injury (Burr & Otten 1999). This method requires high labor cost, since hilling of soil is labor-intensive and must be undone in spring. A biological agent for

control of crown gall for pome and stone fruits, *Rhizobium rhizogenes,* (previously *R. radiobacter*) (Velázquez et al. 2010) strain K84, produces an antimicrobial molecule, agrocin 84, which is inhibitory to some *Rhizobium* species. (New & Kerr 1972; Htay & Kerr 1974; Kerr & Htay 1974; Kerr 1980). K84 has been used for decades in fruit and nut trees as a biological control agent against crown gall, but is not an effective biological control agent for the prevention of crown gall of grapevine caused by *R. vitis* (Kawaguchi & Inoue 2012; Burr et al. 1998).

Rhizobium vitis F2/5, which stimulates the plant hypersensitive response, has shown very promising results in preventing grapevine crown gall only (Burr & Reid 1994; Zheng & Burr 2016; Kaewnum et al. 2013; Creasap et al. 2005; Burr et al. 1997). The stimulation of the hypersensitive response by F2/5 causes grapevine tissue necrosis (Bazzi et al. 1999). To address this issue, researchers have developed non-necrotic derivatives of F2/5 that retain the ability to suppress crown gall (Burr & Zheng 2012). F2/5 is effective against some but not all tumorigenic isolates (Burr & Reid 1994).

Non-pathogenic *Rhizobium vitis* strain ARK-1 (hereafter referred as ARK-1) is an effective biological control agent in Japan against endemic isolates of *R. vitis* that cause disease in *V. vinifera* table grape varieties and other plant species (Kawaguchi & Inoue 2012; Kawaguchi et al. 2015; Zhou et al. 2017). ARK-1 suppresses expression of Ti-plasmid virulence genes and can slow the growth of the tumorigenic *R. vitis* populations (Kawaguchi 2014; Kawaguchi 2015). In a previous study, ARK-1 was demonstrated to be an effective biological control agent challenged with four different tumorigenic isolates of *R.* vitis from Virginia vineyards. ARK-1 suppressed crown gall incidence and severity (gall size) when ARK-1 was co-inoculated with a tumorigenic *R. vitis* at a 1:1 cell ratio. In a study in Japan, ARK-1 was challenged with a co-

inoculation mixture of up to seven tumorigenic *R. vitis* isolates endemic to Japan (Kawaguchi & Inoue 2012).

In a study with another biological control, *R. vitis* strain, VAR03-1, changing the ratios of VAR03-1 to tumorigenic isolates has shown that as the relative amount of VAR03-1 decreases, the efficacy decreases as well (Kawaguchi et al. 2007). VAR03-1 is 100% effective at a ratio of 3:1 and greater of VAR03-1 to the tumorigenic isolate tested and was also significantly effective at a 1:1 ratio. The efficacy of VAR03-1 was no longer significant at a 1:3 ratio of VAR03-1 to tumorigenic isolate. With *R. vitis* F2/5, a ten or one-hundred-fold increase of the tumorigenic isolate to F2/5 greatly reduced the efficacy of F2/5 (Burr & Reid 1994). In this study the effect on gall incidence and size from inoculation with a higher ratio of a tumorigenic isolate to ARK-1 will be investigated.

In order to determine the effect of ratio of ARK-1 to tumorigenic isolate(s), we challenged ARK-1 with a two-fold higher and lower cell number of a tumorigenic isolate. We also challenged ARK-1 with both two and four isolates mixed together in tomato and grapevine seedling co-inoculations. These studies will help to understand whether ARK-1 can be effective under more dynamic populations that may better mimic field populations. Understanding how well ARK-1 suppresses crown gall when challenged by multiple tumorigenic *R. vitis* isolates is an important consideration for potential use of ARK-1 as an effective biological control agent in the United States.

Materials and methods

Isolation of ARK-1 and tumorigenic *R. vitis*

ARK-1 and tumorigenic isolates of *R. vitis* were prepared and characterized using the same method described in Chapter 2. The ARK-1 biological control agent was obtained from Kumiai Chemical Industry and was prepared by suspending 0.2 grams of the ARK-1 formulation per liter of sterile water (5 x 10^7 colony forming units (CFU)/ml). The suspension was streaked onto Roy and Sasser medium and incubated at 28 °C for five to seven days (Roy & Sasser 1983). Tumorigenic isolates from previous studies used to test the efficacy of ARK-1 were used in this work (Chapter 2). They are designated as ACME15, DCCS15, HNVR15, and ZEME15 (GenBank accession nos. are MH330681, MH333801, MH333802, and MH333803). The presence of the Ti-plasmid in these isolates was determined by multiplex PCR using the VCF3/VCR3 primer set (Kawaguchi et al. 2005). The 16S rRNA gene (Ab3-F3/Ab3-R4 primer set) was used to confirm the identity of the tumorigenic isolates of *R. vitis* (Kawaguchi et al. 2005).

Preparation of bacterial inocula for *in planta* biocontrol efficacy assays

Bacterial inocula were prepared by transferring a single *R. vitis* colony into 5 ml of yeast extract mannitol (YEM) broth (Morton & Fuqua 2012) in a sterile 15 ml culture tube, which was placed in a 45 degree slant rack and incubated with shaking at 135 rpm for 48 hours at 28°C. After 48 hours, optical densities of cell suspensions were measured at 600 nm (OD_{600}) and adjusted to OD_{600} = 0.1, which correlates to approximately10^8 CFU/ml (Kawaguchi & Inoue 2012).

The effect of relative cell ratios of ARK-1 to a tumorigenic isolate was tested in one-month old tomato seedlings and two-month old grapevine seedlings. For tomato inoculations, 250 µl cell suspension of ACME15 or DCCS15 was mixed with either 125, 250, or 500 µl of

ARK-1 suspension or sterile distilled water for controls. The total number of cells inoculated in each plant remained constant (~1 x 10^5 cells μl^{-1}), but the ratio of ARK-1 to tumorigenic isolate(s) varied. For grapevine experiments, the isolate HNVR15 was substituted for DCCS15, since DCCS15 did not produce as many galls in grapevine (Chapter 2). In the grapevine seedling inoculations, cell suspensions of ARK-1 and a tumorigenic isolate were both adjusted to OD_{600} = 0.1 or 0.2, and then mixed. For example, at a 2:1 ratio of ARK-1 to tumorigenic isolate, 100 µl of OD_{600} = 0.2 ARK-1 suspension was mixed with 100 µl of OD_{600} = 0.1 tumorigenic cell suspension, and at a 1:1 ratio, 100 µl of OD_{600} = 0.1 of both ARK-1 and the tumorigenic isolate cell suspensions were mixed. Thus, the total number of cells for each ratio differed (~ 1.5 x 10^5 cells μl^{-1} for the 0.5:1 and 1:2 ratios and ~ 1 x 10^5 cells μl^{-1} for the 1:1 ratio) [Tables 3-1 and 3-2]).

Table 3-1. Estimation of the cell number (x 10^5) inoculated in a 5 µl volume to tomato for ARK-1 to tumorigenic isolate(s) ratio experiments

Inoculum	ARK-1 +ACME15			ARK-1 + DCCS15			ARK-1 + four isolate mixture	
Ratio	**0.5:1**	**1:1**	**2:1**	**0.5:1**	**1:1**	**2:1**	**0.25:1**	**1:1**
***R. vitis* strain**								
ARK-1	1.66	2.50	3.33	1.66	2.50	3.33	1.00	2.50
ACME15	3.33	2.50	1.66	-	-	-	1.00	0.63
DCCS15	-	-	-	3.33	2.50	1.66	1.00	0.63
HNVR15	-	-	-	-	-	-	1.00	0.63
ZEME15	-	-	-	-	-	-	1.00	0.63
Total	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00

Table 3-2. Estimation of the cell number (x 10^5) inoculated in a 5 µl volume to grapevine for ARK-1 to tumorigenic isolate(s) ratio experiments

Inoculum	ARK-1 + ACME15			ARK-1 + HNVR15			ARK-1 + four isolate mixture
Ratio	**0.5:1**	**1:1**	**2:1**	**0.5:1**	**1:1**	**2:1**	**1:1**
***R. vitis* strain**							
ARK-1	2.50	2.50	5.00	2.50	2.50	5.00	4.00
ACME15	5.00	2.50	2.50	-	-	-	1.00
DCCS15	-	-	-	-	-	-	1.00
HNVR15	-	-	-	5.00	2.50	2.50	1.00
ZEME15	-	-	-	-	-	-	1.00
Total	7.50	5.00	7.50	7.50	5.00	7.50	8.00

When four tumorigenic isolates were used to challenge ARK-1, the four-isolate mixture was prepared by combining 100 µl of each OD_{600} adjusted tumorigenic isolate suspension with water or ARK-1 suspension. In experiments using tomato plants ARK-1 cell suspensions at an adjusted $OD_{600} = 0.1$. One hundred µl of ARK-1 ($OD_{600} = 0.1$) were mixed with 100 µl of each tumorigenic isolate for a 0.25:1 ratio of ARK-1 to tumorigenic isolate (~ 2×10^4 cells μl^{-1} for each isolate). Preparing a 1:1 inoculum mixture for tomato inoculations was performed by mixing 400 µl of ARK-1 suspension (~ 5×10^4 cells μl^{-1}) with 100 µl of each tumorigenic isolate suspension (~ 1.25×10^4 cells μl^{-1}) (Table 3-1). Preparing a 1:1, four-isolate inoculum for grapevine seedling inoculations was prepared by adjusting OD_{600} of ARK-1 to 0.4 and the tumorigenic isolates to 0.1. For ARK-1 and all tumorigenic isolates, 100 µl of ARK-1 (~ 8×10^4 cells μl^{-1}) and each tumorigenic isolate (~ 2×10^4 cells μl^{-1}) was mixed (Table 3-2). In all studies, co-inoculations were performed immediately after preparation of inoculum mixtures.

In planta ARK-1 biocontrol efficacy assay in tomatoes

Tomato seedlings, *Solanum lycopersicum* 'Beefsteak' (Ferry-Morse, Fulton, KY) were grown for one month in a greenhouse under natural light (~ 12 hours sunlight). Using a sterile dissecting needle, five stab wounds were made on the seedlings' stems starting at the base with approximately 2 cm spacing between wounds and wounds were immediately inoculated with 5 µl inoculum or water. Inoculations with ARK-1 or water alone served as controls. Galls were measured at 42 days after inoculation at their widest diameter using a digital caliper (Electron Microscopy Sciences, Hatfield, PA). Wound locations that did not form galls were assigned a diameter value of 0 mm. Three replications for each treatment and three independent experimental trials were performed. Each treatment/isolate combination was tested on nine plants with five inoculation locations/per plants for a total of 45 inoculations for each treatment. The assay with four isolates challenging ARK-1 in tomatoes had four internal replications for a total of 60 inoculation locations per treatment and isolate assessed.

In planta ARK-1 biocontrol efficacy assay in grapevine seedlings

Wine grape (*Vitis vinifera* 'Chardonnay') seeds were harvested and grown in a greenhouse for ten weeks. Five stab wounds spaced approximately 2 cm apart were made on the seedling stem using a sterile dissection needle (Electron Microscopy Sciences, Hatfield, PA). Five microliters of prepared inoculum or water was pipetted into each wound location. Control seedlings received water or ARK-1 inoculum. Gall formation was measured at 42 days after inoculation (Fig. 3-1) at their widest diameter using a digital caliper. Inoculation locations that did not form galls were assigned a diameter value of 0 mm. Experiments included three internal replications and three independent experimental trials. Each treatment was tested on nine plants with five inoculation locations per plant for a total of 45 inoculations for each treatment.

Figure 3-1. Grapevine seedling inoculation locations 42 days after inoculation with ARK-1 and tumorigenic isolates mixture (left) or tumorigenic isolates (right).

Statistical analyses

Effect of treatment on the probability of gall formation was analyzed using the generalized linear mixed model (GLIMMIX) in SAS (ver. 9.4, SAS, Cary, NC), using the logit link function. Although data are presented as a mean proportion of gall formation per treatment, the model was run to test the probability of successful inoculation (gall formation) for each treatment effect. For gall diameter, the normal distribution was assumed and the identity link function in GLIMMIX was used. For the analysis of relative ratio, each relative ratio was analyzed separately since the total number of cells for a tumorigenic isolate and/or ARK-1 differ. The effect of ARK-1 treatment and tumorigenic isolate(s) were considered as fixed factors and experimental trial was considered as a random factor. For the analysis of two or four tumorigenic isolates challenging ARK-1, ARK-1 treatment and isolate combination were considered as fixed effects and experimental trial was considered as a random effect. When the effect of the fixed effects and/or their interaction were found to be significant ($P \leq 0.05$), Fisher's Least Significant Difference (LSD, $\alpha = 0.05$) was used for mean separation.

Results

Tomato, ARK-1 biocontrol efficacy assays

Relative Concentration: To study whether ARK-1 efficacy is dependent on the ratios of ARK-1 to a tumorigenic isolate, inoculations were performed using either twice, equal or half the number of ARK-1 cells relative to a tumorigenic isolate. At the 0.5:1 ratio, the main effects of isolate ($F = 10.8$, $P = 0.02$) and treatment ($F = 10.8$, $P = 0.02$) at a 0.5:1 ratio were significant, but the interaction ($F = 0.5$, $P = 0.53$) was not significant. There was a 29% average reduction in gall formation from ARK-1 co-inoculation at a 0.5:1 ratio (Fig. 3-2A). The main effects of tumorigenic isolate ($F = 26.2$, $P < 0.01$) and treatment ($F = 83.7$, $P < 0.01$) on mean gall diameter was significant but the interaction ($F = 0.5$, $P = 0.47$) was not. ARK-1 co-inoculation at a 0.5:1 ratio reduced the mean gall diameter 62% on average (Fig. 3-2D).

For a 1:1 ratio of ARK-1 to the tumorigenic isolate, the main effects of isolate ($F = 18.8$, $P < 0.01$) and treatment ($F = 33.1$, $P < 0.01$) on the probability of gall formation were significant, but the interaction ($F = 0.1$, $P = 0.79$) was not significant. The co-inoculation of ARK-1 at a 1:1 ratio resulted in a 56% reduction in gall formation on average (Fig. 3-2B). The interaction effect of ARK-1 treatment and the tumorigenic isolate on the mean gall diameter was significant ($F = 11.9$, $P \leq 0.001$) at the 1:1 ratio. The mean gall diameter was reduced by an average of 84% by ARK-1 co-inoculation at a 1:1 ratio (Fig. 3-2E).

For a 2:1 ratio of ARK-1 to the tumorigenic isolate, the main effects of tumorigenic isolate ($F = 15.2$, $P < 0.01$) and treatment ($F = 19.4$, $P < 0.01$) were significant on the probability of gall formation, but the interaction ($F = 0.2$, $P = 0.64$) was not. A 2:1 ratio co-inoculation of ARK-1 to tumorigenic isolate resulted in a 29% reduction in gall formation on average (Fig. 3-2C). The interaction of ARK-1 treatment and isolate was significant ($F = 40.5$, $P < 0.01$) with a

2:1 ratio. A 2:1 ratio of ARK-1 to tumorigenic isolate resulted in a 55% reduction in mean gall diameter (Fig. 3-2F).

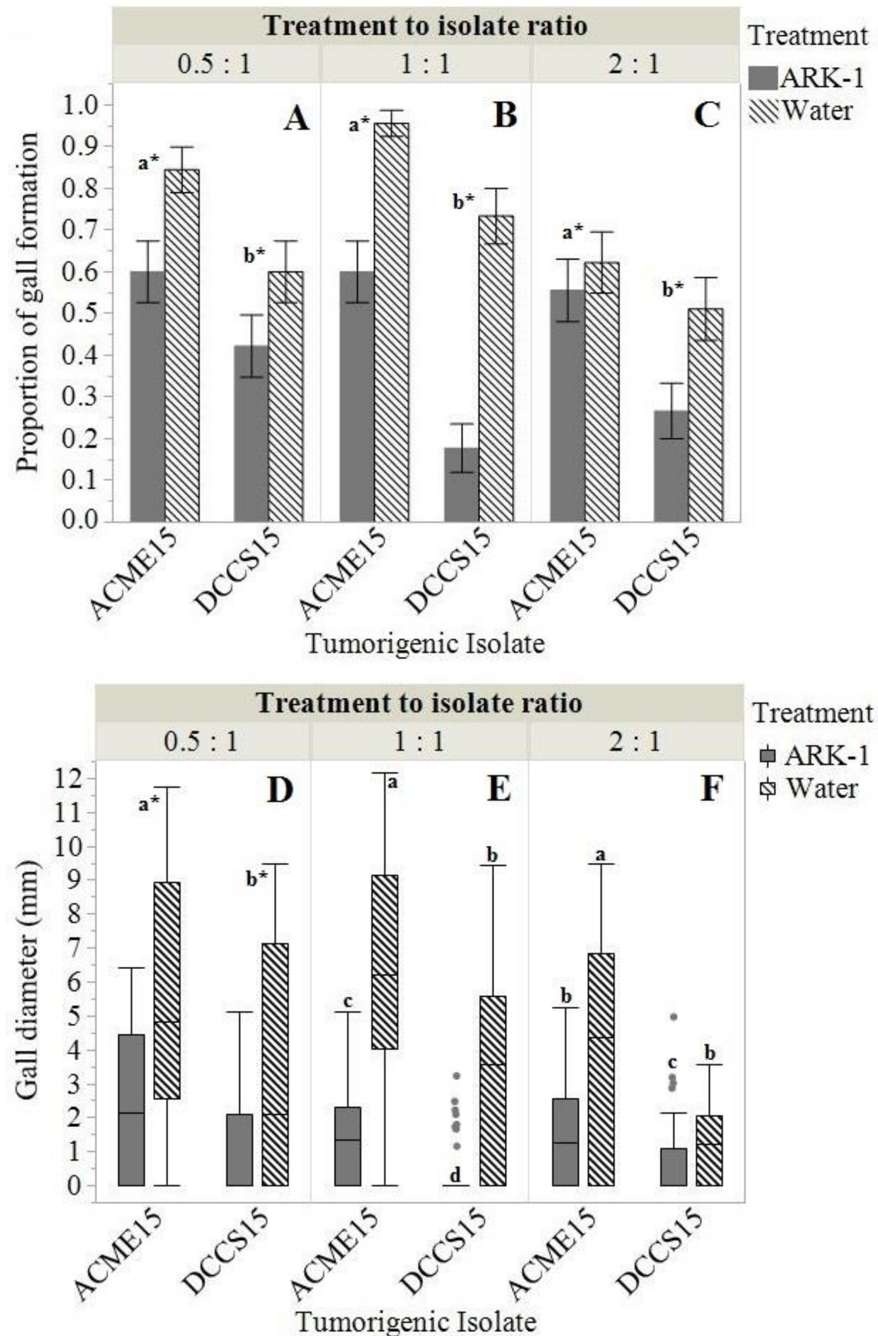


Figure 3-2. Effect of ARK-1 at a 0.5:1, 1:1, and 2:1 ratio of treatment to tumorigenic isolate in tomato stems. A-C) Bar plot of the mean proportion of inoculation locations that formed galls and D-F) Box plot of the mean gall diameter (mm). Solid grey columns represent co-inoculations with ARK-1 and striped columns represent co-inoculation with water. Error bars represent standard error of the mean (n = 45). Points outside the box plot are outliers. The statistical analysis was performed separately for each relative ratio. In the panels A-D, different letters indicate significant difference between tumorigenic isolates and an asterisk indicate significant difference between ARK-1 and water treatment ($P \leq 0.05$). In the panels E and F, different letters indicate significant difference ($P \leq 0.05$) between tumorigenic isolates. Fisher's LSD was used as a post-hoc mean separation method.

Multiple tumorigenic isolates: The effect of the interaction of the two-isolate mixture and treatment with ARK-1 on mean probability of gall formation was significant ($F = 2.9$, $P = 0.03$). Co-inoculation of ARK-1 with two tumorigenic isolates significantly reduced the mean probability of tomato gall formation and gall diameter as opposed to inoculations with the two tumorigenic isolates alone. The mean probability of gall formation for each combination of isolates and ARK-1 treatment ranged from 0.00 to 0.98, and with co-inoculation of ARK-1 was reduced 57% on average (Fig. 3-3A). In all cases, the mean probability of gall formation with ARK-1 treatment resulted in a significant reduction ($P \leq 0.05$). There was no significant difference among the mean probability of gall formation without ARK-1 treatment with an exception of DCCS15 and ZEME15 combination, which resulted in significantly lower mean probability of gall formation ($P \leq 0.05$) than the other isolate mixtures. When the two-isolate combination contained the isolate ACME15, the mean probability of gall formation tended to be higher than the other two-isolate combinations.

The interaction effect of isolate mixture and ARK-1 treatment for gall diameter was also significant ($F = 7.4$, $P < 0.01$). The mean gall diameter for each isolate and ARK-1 combination ranged from 0.00 to 7.61 mm (Fig. 3-3B). On average, ARK-1 co-inoculation treatment reduced gall diameter by 83% across all two-isolate combinations. For every two-isolate combination, the mean gall diameter was significantly lower with ARK-1 co-inoculation compared to co-inoculations without ARK-1 ($P \leq 0.05$). Similar to the results of the mean probability of gall formation, two-isolate combinations that included ACME15 tended to result in larger mean gall size than the combinations without it, and in some cases, the difference was significant ($P \leq 0.05$). As expected, the control plants inoculated with ARK-1 or water alone did not form any galls.

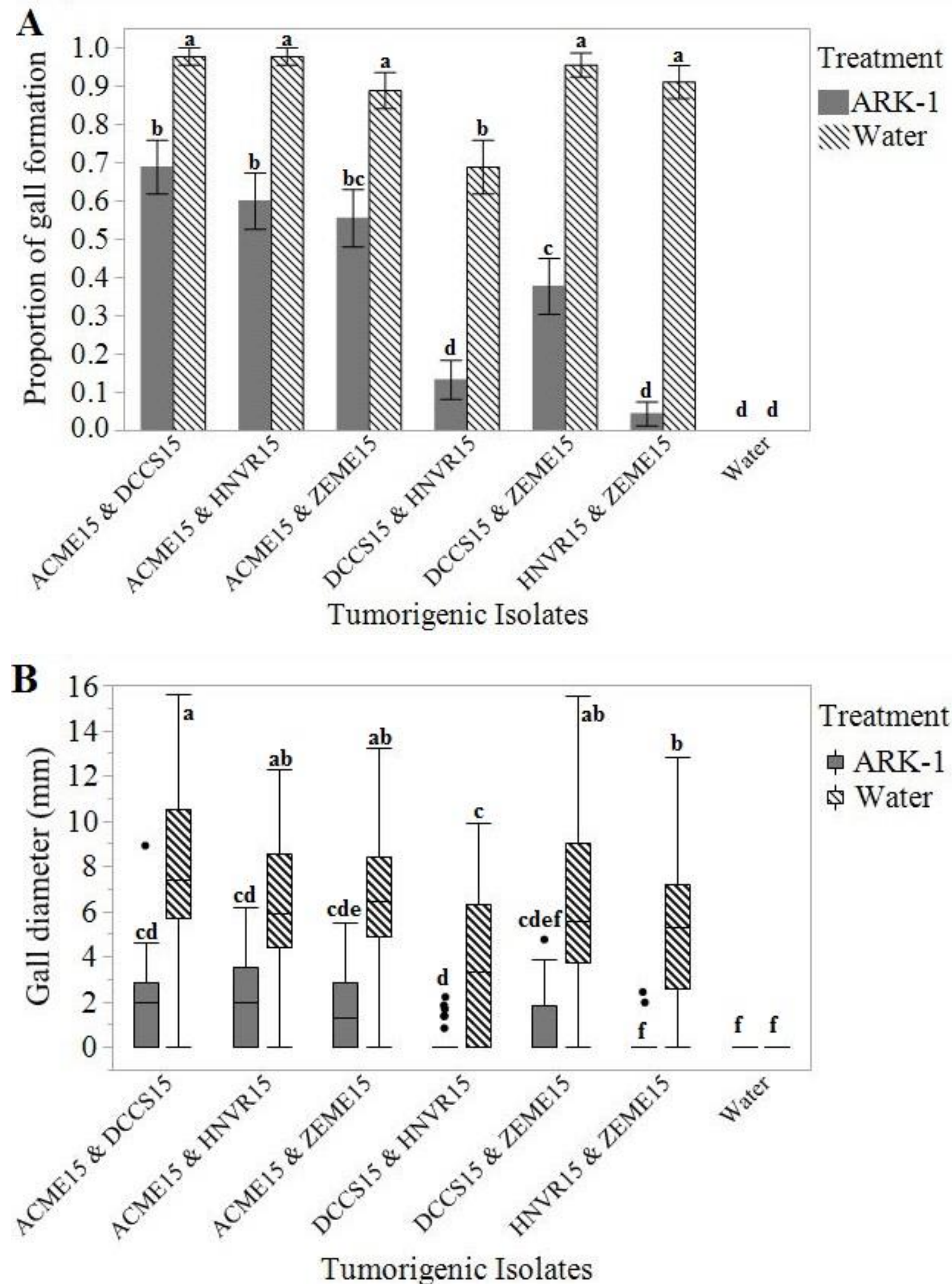


Figure 3-3. Effect of ARK-1 co-inoculation using a two-isolate mixture on the A) mean probability of tomato gall formation and B) mean gall diameter. Solid grey columns represent ARK-1 co-inoculation and striped columns are a water co-inoculations. Error bars represent standard error of the mean of the proportion of gall formation (n = 45). Different letters represent significant differences according to Fisher's LSD ($P \leq 0.05$). Points outside the box and whisker plot are outliers. If there was no significant interaction, different letters indicate significant difference between tumorigenic isolates based on Fisher's LSD ($P \leq 0.05$) and an asterisk indicate significant difference between ARK-1 and water treatment according to Fisher's LSD ($P \leq 0.05$).

Co-inoculation at a 1:4 ratio of ARK-1 to the tumorigenic isolates mixture (ACME15, DCCS15, HNVR15, ZEME15) reduced the probability of gall formation by 23% but this reduction was not significant ($F = 6.3$, $P = 0.13$) (Fig. 3-4A). On the other hand, the ARK-1 treatment effect was significant on the mean gall size ($F = 40.4$, $P < 0.01$), with a 58% reduction in mean gall diameter from an ARK-1 co-inoculation treatment (Fig. 3-4C). Co-inoculation of ARK-1 with a mix of four tumorigenic isolates at a 1:1 ratio resulted in a significant reduction in the probability of gall formation ($F = 25.0$, $P = 0.04$) (Fig. 3-4B) and the mean gall diameter ($F = 40.4$, $P < 0.01$) (Fig. 3-4D). A 1:1 ARK-1 to tumorigenic isolate ratio resulted in a 53% and 62% reduction in mean probability of gall formation and gall diameter, respectively.

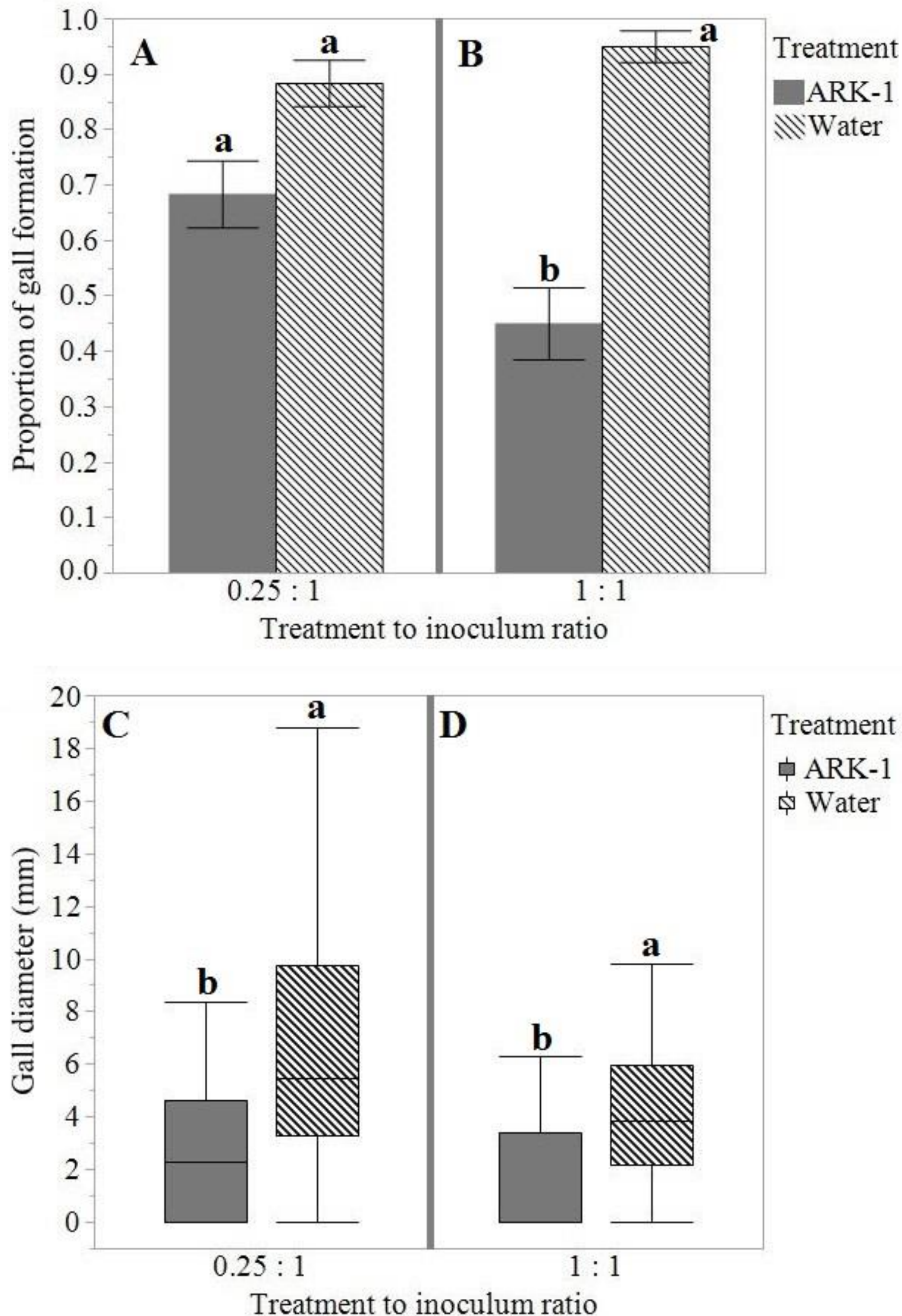


Figure 3-4. Effect of ARK-1 at a 0.25:1 and 1:1 ratio of treatment to tumorigenic isolates mixture in tomato stems. A-B) Bar plot of the mean proportion of inoculation locations that formed galls and C-D) Box plot of the mean gall diameter (mm). Solid grey columns represent co-inoculations with ARK-1 and striped columns represent co-inoculation of tumorigenic isolates with water only. Error bars represent standard error of the mean (n = 60). Points outside the box plot are outliers. The statistical analysis was performed separately for each relative ratio. Different letters indicate significant difference between tumorigenic isolates ($P \leq 0.05$). Fisher's LSD was used as a post-hoc mean separation method.

Grapevine seedling, ARK-1 biocontrol efficacy assay

Relative Concentration: Inoculations of ARK-1 at differing relative ratios to a tumorigenic isolate were undertaken in grapevine seedlings to determine the effect of the relative ratio on the mean probability of gall formation and the mean gall diameter. Two tumorigenic isolates (ACME15 and HNVR15) were used in this experiment. The effect of treatment ($F = 9.3$, $P = 0.02$) at a 0.5:1 ratio of ARK-1 to tumorigenic isolate was significant; isolate ($F = 0.8$, $P = 0.40$) and the interaction ($F = 3.6$, $P = 0.11$) was not significant (Fig. 3-5A). The interaction ($F = 4.1$, $P = 0.045$) of isolate and treatment effect on the mean gall diameter was significant (Fig. 3-5D). A 0.5:1 co-inoculation ratio of ARK-1 resulted in a 26% and 50% reduction in the mean probability of gall formation and the mean gall size, respectively.

When the ratio of ARK-1 to tumorigenic isolate was increased to 1:1, the main effect of tumorigenic isolate ($F = 6.9$, $P = 0.04$) and treatment ($F = 31.8$, $P < 0.01$) were significant, while the interaction ($F = 3.7$, $P = 0.10$) was not significant (Fig. 3-5B). ARK-1 co-inoculation resulted in a 50% reduction in the probability of gall formation. The isolate HNVR15 resulted in higher mean probability of gall formation than the isolate ACME15. The interaction ($F = 10.9$, $P < 0.01$) of isolate and treatment on mean gall diameter was significant. On average the mean gall diameter with ARK-1 treatment was reduced by 65%; however, the mean gall diameter of HNVR15 Co-inoculated with ARK-1 was significantly larger than that of ACME15 co-inoculated with ARK-1 (Fig. 3-5E).

The 2:1 ratio of ARK-1 to the tumorigenic isolate main effects of isolate ($F = 12.0$, $P = 0.01$) and treatment ($F = 9.6$, $P = 0.02$) were significant, but the interaction ($F = 2.0$, $P = 0.21$) was not significant. The 2:1 treatment ratio resulted in a 70% reduction in the probability of gall formation (Fig. 3-5C), and similar to the 1:1 ratio, HNVR15 resulted in higher mean probability

of gall formation. The interaction of isolate and treatment significantly affected the mean gall size ($F = 8.6$, $P < 0.01$) with an average of 78% reduction in mean gall diameter with ARK-1 treatment (Fig. 3-5F). HNVR15 co-inoculated with ARK-1 resulted in a larger mean gall size than ACME15 with ARK-1.

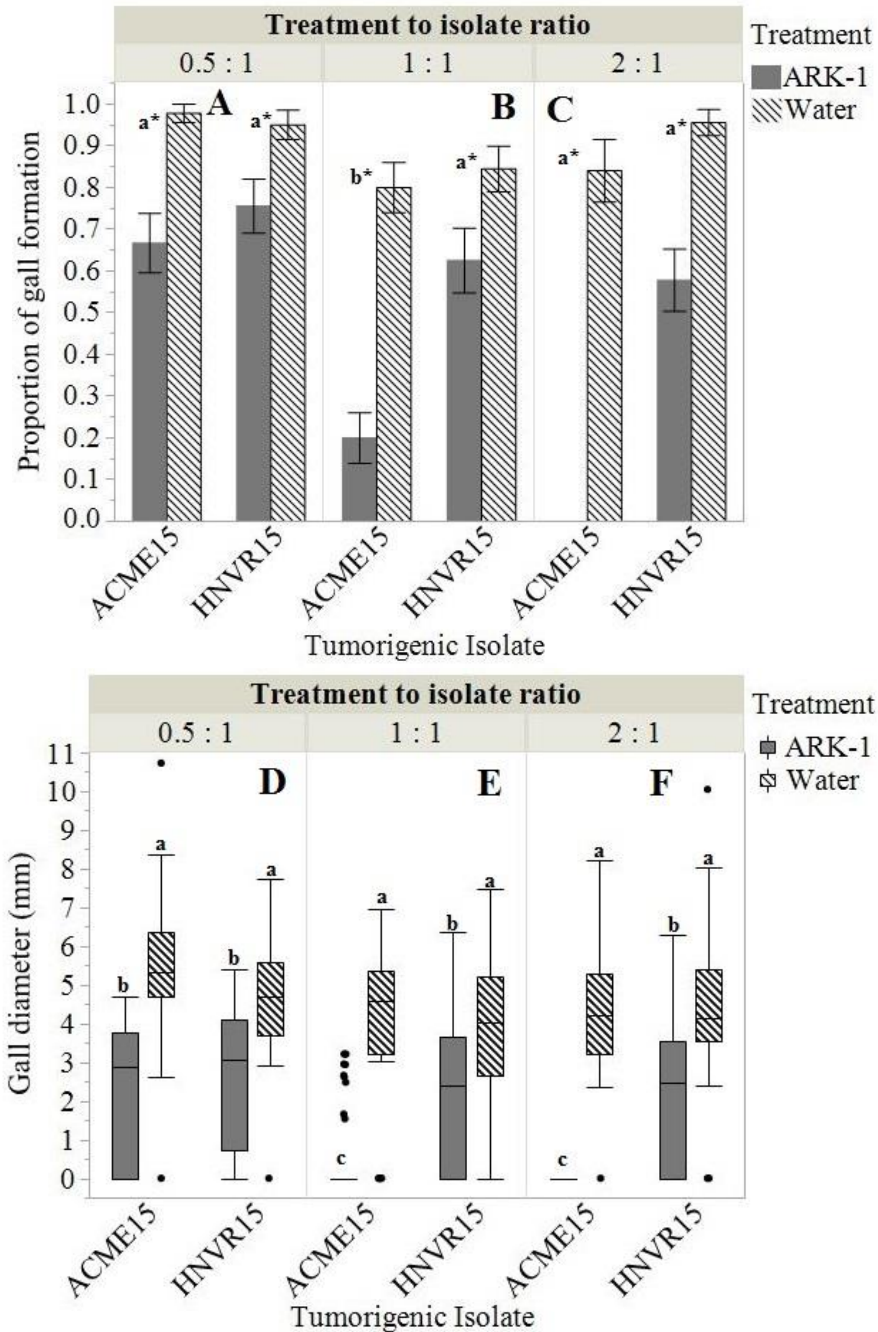


Figure 3-5. Effect of ARK-1 at a 0.5:1, 1:1, and 2:1 ratio of treatment to tumorigenic isolate in grapevine seedling stems. A-C) Bar plot of the mean proportion of inoculation locations that formed galls and D-F) Box plot of the mean gall diameter (mm). Solid grey columns represent co-inoculations with ARK-1 and striped columns represent co-inoculation of tumorigenic isolates with water only. Error bars represent standard error of the mean (n = 45). Points outside the box plot are outliers. The statistical analysis was performed separately for each relative ratio. In the panels A-C, different letters indicate significant difference between tumorigenic isolates and an asterisk indicate significant difference between ARK-1 and water treatment ($P \leq 0.05$). In the panels D-F, different letters indicate significant difference ($P \leq 0.05$) between two combinations of a tumorigenic isolate and treatment. Fisher's LSD was used as a post-hoc mean separation method.

Multiple tumorigenic isolates: Co-inoculation of grapevine seedling stems using ARK-1 and a mixture of two tumorigenic isolates significantly reduced the mean probability of gall formation. The interaction effect of ARK-1 treatment and the two-isolate mixture on the mean probability of gall formation was significant ($F = 2.7$, $P = 0.04$). Gall formation mean probability ranged from 0.00 to 0.98. The mean probabilities of gall formation among six two-isolate combinations without ARK-1 treatment were not significantly different from each other, with the exception of ACME15 co-inoculated with DCCS15 which resulted in a higher mean probability of gall formation than ACME15 co-inoculated with ZEME15 (Fig. 3-6A). ARK-1 co-inoculation treatment reduced the mean probability of gall formation by 78% for all two-isolate mixtures (Fig. 3-6A). With co-inoculation of ARK-1, the mean probability of gall formation of ACME15 co-inoculated with HNVR15 was significantly higher than that resulting from the three mixtures of isolates that did not contain the isolate HNVR15.

The interaction effect of ARK-1 co-inoculation and two-isolate mixture on the diameter of galls was significant ($F = 8.1$, $P < 0.01$). The mean gall diameters ranged from 0.00 to 7.61 mm. Although there were slight numerical differences among different two-isolate combinations without ARK-1, the mean gall diameter was not significantly different among combinations (Fig. 3-6B). Co-inoculation of ARK-1 with the two-isolate mixture led to a significant reduction of the mean gall diameter for all two-isolate combinations ($P \leq 0.05$), with an average reduction of 84% (Fig. 3-6B). Similar to the mean probability of gall formation, the mean gall diameter with a two-isolate combination that contained HNVR15 resulted in numerically higher means, and the mean gall diameter of ACME15 co-inoculated with HNVR15 was significantly larger than two of three combinations of isolates that did not contain the isolate HNVR15. Inoculations of ARK-1 or water alone resulted in no gall formation.

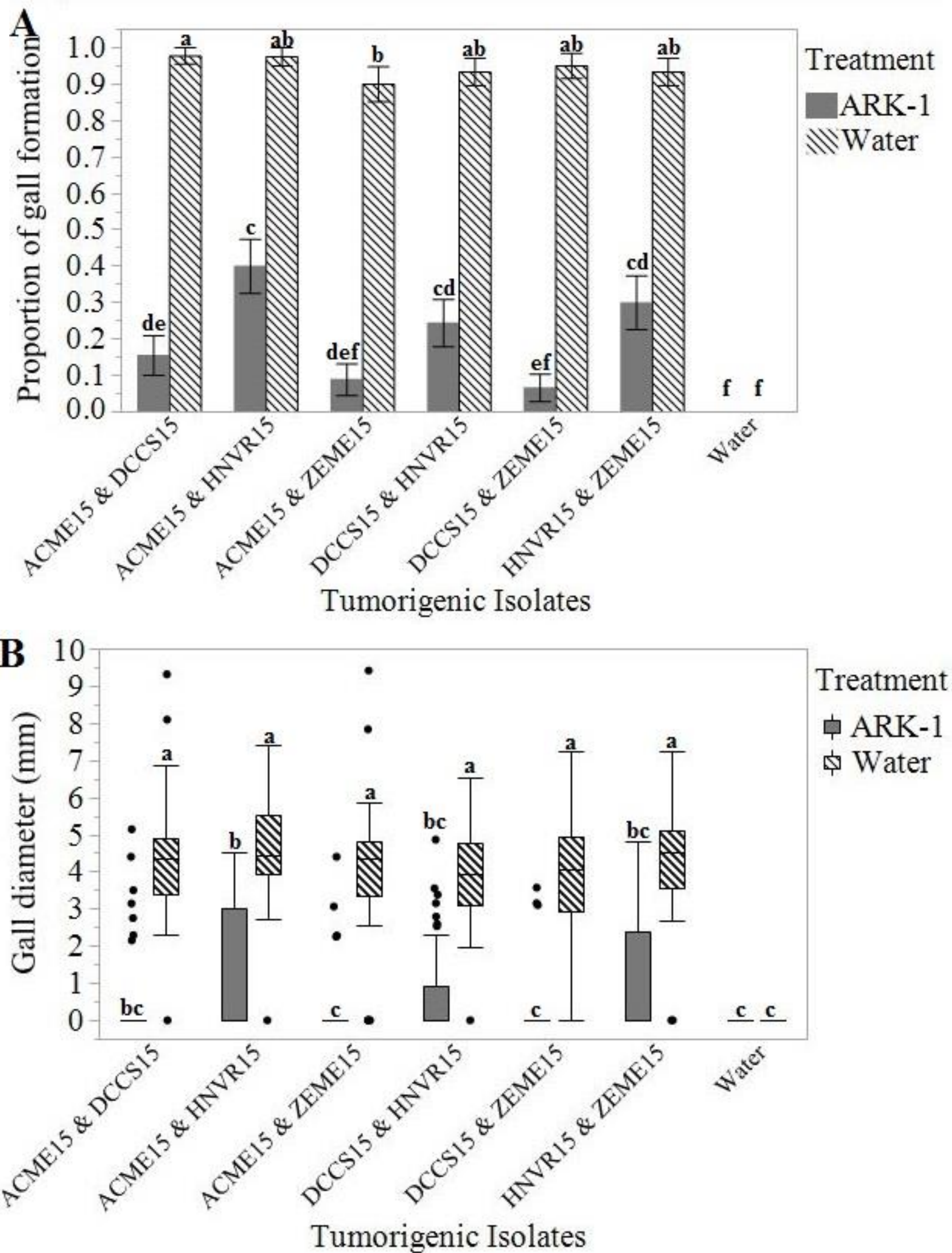


Figure 3-6. The effect of ARK-1 co-inoculation on grapevine seedlings A) gall formation and B) mean gall diameter when co-inoculated with two tumorigenic isolates. Solid grey columns are co-inoculations with ARK-1 and striped columns are co-inoculations with water. Error bars represent one standard error of the mean proportion of gall formation (n = 45). Different letters represent significant differences according to Fisher's LSD ($P \leq 0.05$). Points outside the box and whisker plot are outliers.

When ARK-1 was challenged with a mixture of four tumorigenic isolates, the effect of ARK-1 treatment on both the mean probability of gall formation ($F = 10.2$, $P = 0.02$), and the mean gall diameter ($F = 76.3$, $P < 0.01$) were significant (Fig. 3-7A, B). The co-inoculation of ARK-1 with the mixture of four tumorigenic isolates significantly reduced both the mean gall diameter and the mean probability of gall formation. The mean probability of gall formation was reduced from 0.93 to 0.09, or a 90% reduction (Fig. 3-7A). Average gall diameter was reduced from 3.65 to 0.19 mm or a 95% reduction when co-inoculated with ARK-1 (Fig. 3-7B). Control treatments of ARK-1 or water alone did not result in gall formation (*data not shown*).

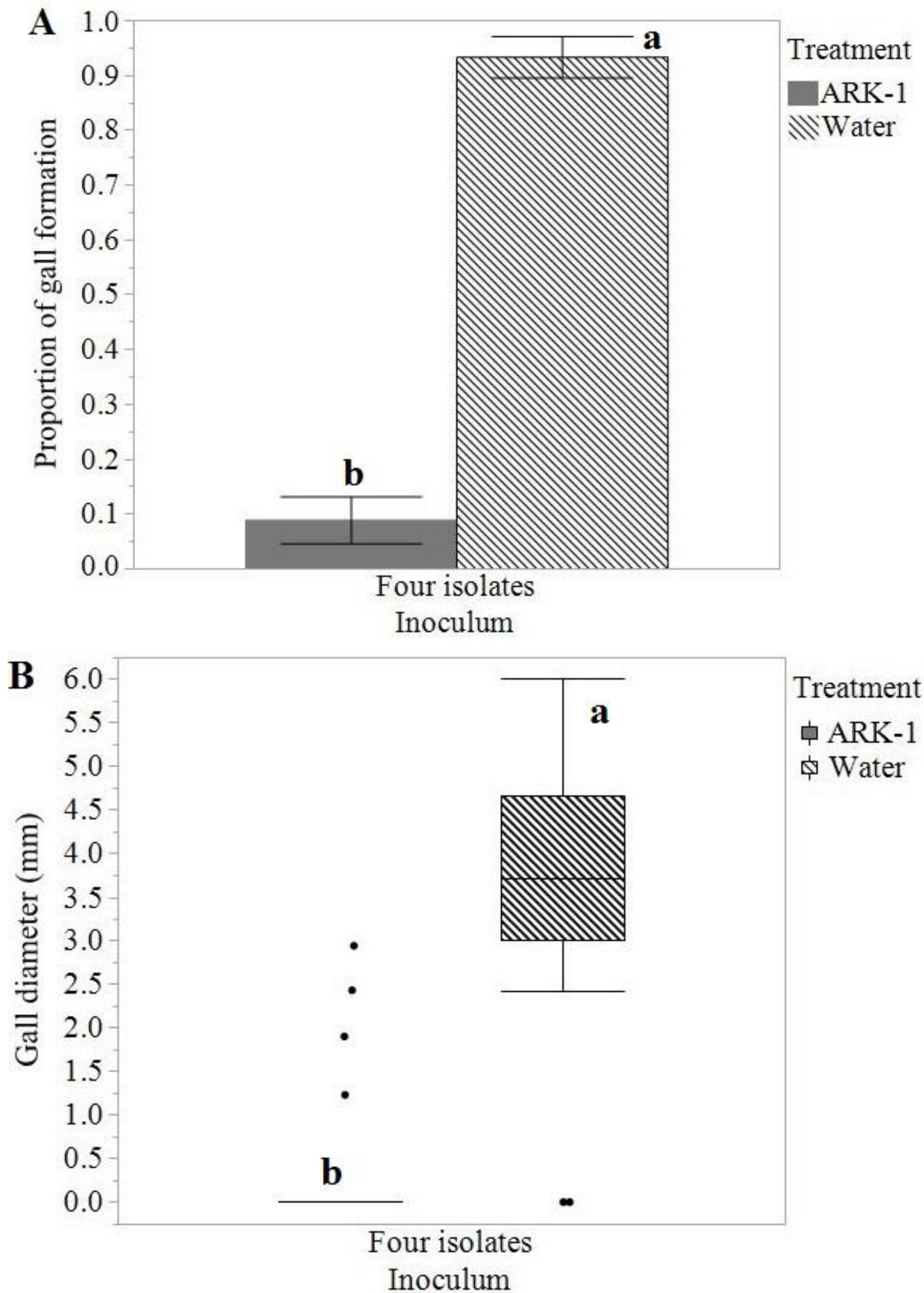


Figure 3-7. Effect of four tumorigenic isolates co-inoculated with ARK-1 in grapevine seedlings. A) Mean probability of gall formation when a mix of four isolates co-inoculated with ARK-1 or water. B) Mean gall diameter of inoculation locations co-inoculated with the tumorigenic isolates and ARK-1 or water. Solid grey columns represent co-inoculation with ARK-1 and striped columns represent water co-inoculation. Error bars represent the standard error (n = 45). Different letters indicate significant differences according to Fisher's LSD ($P \leq 0.05$). Points outside box and whisker plot are outliers.

Discussion

Chapter 2 described the efficacy of ARK-1 when challenged against individual tumorigenic isolates of *Rhizobium vitis* in both tomato and grapevine. In Japan, ARK-1 has been shown to be an effective biological control agent against a mixture of tumorigenic isolates with unique *R. vitis* genotypes (Kawaguchi & Inoue 2012). Our previous results in grapevine woody trunk inoculations revealed that ARK-1 is an effective biological control agent, but there is variation in ARK-1 efficacy against the tumorigenic isolates causing grapevine crown gall. Results in this chapter demonstrate that ARK-1 is an effective biological control agent when challenged with combinations of more than one tumorigenic Virginia isolate in both tomato and grapevine seedlings. For some isolates, the results also demonstrate that a lower relative ratio of ARK-1 to the tumorigenic isolate(s) can negatively affect the efficacy of ARK-1.

Previous studies have shown that the efficacy of *R. vitis* biological control agents VAR03-1 (Kawaguchi et al. 2007) and F2/5 (Burr & Reid 1994) are dependent on the relative ratio of the biological control agent and the tumorigenic isolate. Biological control agent F2/5 was able to reduce gall incidence and severity when the concentration of the tumorigenic isolate was ten times that of F2/5, but not nearly as effectively at a 1:1 ratio (Burr & Reid 1994). Application of F2/5 90 minutes prior to inoculation with the tumorigenic isolate at a concentration one hundred times that of the applied F2/5 did not reduce gall formation.

We hypothesized a similar trend for ARK-1 efficacy as a function of the ratio of ARK-1 to tumorigenic isolate. Since the proposed mechanism of control by ARK-1 is via interaction with the pathogen and not the host (Kawaguchi 2014; Kawaguchi 2015; Kawaguchi et al. 2017), ARK-1 must be at a ratio high enough to inhibit all of the tumorigenic cells. VAR03-1 was an effective biological agent until the tumorigenic isolate concentration was greater than a 1:1 ratio.

We tested one tumorigenic isolate with ARK-1 at 0.5:1, 1:1, and 1:2; two tumorigenic isolates with ARK-1 at a 0.5:1 ratio; and a four tumorigenic isolate mixture with ARK-1 at 0.25:1 and 1:1. ARK-1 was consistently effective at a 0.5:1 ratio of ARK-1 to the tumorigenic isolate. In tomato when the relative ratio of ARK-1 to the four tumorigenic isolate mixture was 0.25:1, the probability of gall formation was not significantly reduced with co-inoculation of ARK-1. Our studies did not explore a wide range of ratios, but these results provide a guide for future studies that should examine ratios beyond those investigated here.

Some of the first investigations of ARK-1 tested this biological control agent against a mixture of seven Japanese tumorigenic isolates (Kawaguchi & Inoue 2012), but as we reported in Chapter 2, there were variations in the sensitivity among the Virginia tumorigenic isolates to ARK-1. In the current studies, when the two-isolate mixture was used, there was a trend that a tumorigenic isolate mixture that contained the isolate ACME15 in tomato inoculations, and HNVR15 in grapevine inoculations, resulted in numerically, and sometimes statistically, higher mean probabilities of gall formation and/or gall diameters than the other isolate mixtures. The results from grapevine trunk inoculations from our previous study (Chapter 2) showed that the isolate HNVR15 had significantly higher mean probability of gall formation and gall diameter than the isolate DCCS15. However, in tomato inoculations DCCS15 resulted in significantly larger galls and numerically greater mean probabilities of gall formation than HNVR15. Isolate DCCS15 had numerically lower proportion of gall formation and significantly lower gall size compared to ACME15 when inoculated in tomatoes at a 1:1 ratio with ARK-1 (Chapter 2). Similarly, in the study of *R. vitis* F2/5, Burr and Reid reported that the efficacy of the strain F2/5 was not equal among their tested isolates (Burr & Reid 1994). However, to our knowledge, F2/5 has not been challenged with a simultaneous inoculation of more than one tumorigenic isolate.

When four tumorigenic isolates were mixed and co-inoculated with ARK-1 the treatment was more efficacious compared to co-inoculation with a two-isolate mixture. The average reduction provided by ARK-1 treatment with the two-isolate co-inoculation with grapevine was 78% (mean probability of gall formation) and 84% (mean gall size), while that of the four-isolate co-inoculation with grapevine was 90% (mean probability of gall formation) and 95% (mean gall size). The difference in efficacy of ARK-1 between these two experiments might be due to the number of each tumorigenic isolate cells inoculated differed. In the two-isolate experiment, the total number of cells of the two-isolate mixture was twice as high as that of ARK-1, but in the four-isolate experiment, the total cell ratio was 1:1. Thus, if there was a particular isolate that was less sensitive to the ARK-1 treatment, than the other isolates, the lower ratio of the less sensitive isolate to ARK-1 might have been critical. The difference could also be due to competition among tumorigenic isolates. Since tumorigenic cells are competing for limited resources within the artificial wound inoculation, the higher number of tumorigenic isolate cells may have negatively affected their ability to overcome ARK-1 and/or induce galls.

Similar to the results from Chapter 2, the percentage reduction in gall diameter was similar between the tomato and grapevine studies, but there was a greater reduction of gall formation probability in grapevine seedlings than in tomato. This could be due to the fact that ARK-1 may be more effective in suppressing grapevine galls than tomato galls or that certain Virginia isolates are more effective at inducing tomato galls. In the previous study by Dr. Kawaguchi (Kawaguchi et al. 2015), they reported varying efficacy of ARK-1 among different hosts they examined. Their study only used Japanese *R. tumefaciens* and *R. rhizogenes* as the tumorigenic isolates, thus, the differences in the *R. vitis* virulence were not studied, but as we observed in our studies, some isolates of *R. vitis* may favor a certain host over the others. Based

on our results, we suspect that isolate ACME15 is a more virulent pathogen of tomatoes and that HNVR15 is a more virulent pathogen of grapevine. In any case, ARK-1 was able to significantly reduce the probability of gall formation and gall diameter whether a tumorigenic isolate was applied alone (Chapter 2) or as a mixture of multiple isolates (this study).

Results from this study further confirm the findings from Japan that ARK-1 is an effective biological control agent against crown gall of grapevine. We challenged ARK-1 with higher concentrations and/or multiple tumorigenic *R. vitis* isolates from Virginia. There was an indication of rate response among tumorigenic isolates and also variable sensitivity to ARK-1. Co-inoculation with ARK-1 resulted in statistically significant reduction in both mean probability of gall formation and the mean gall size when challenged at a 0.5:1 ratio of ARK-1 to tumorigenic isolate cells. The same result was observed when ARK-1 was challenged with up to four isolates simultaneously. Understanding how ARK-1 efficacy is influenced by the ratio of ARK-1 to tumorigenic *R. vitis* isolate cells and also how ARK-1 efficacy is affected by the presence multiple tumorigenic isolates helps prepare for the future introduction of ARK-1 into agricultural use. Since ARK-1 is effective at reducing crown gall when co-inoculated with more than one isolate and maintains efficacy even when outnumbered by tumorigenic cells, we conclude that ARK-1 has great potential as an effective biological option for the control of crown gall of grapevine in the United States.

Conclusions and future directions

Based on the work described in the previous chapters, *R. vitis* ARK-1 has shown the potential to be an effective biological control agent for the management of grapevine crown gall in the United States. Challenging ARK-1 with individual tumorigenic isolates of *R. vitis* from Virginia at a 1:1 cell ratio in co-inoculations of tomato and grape significantly reduced both the probability of gall formation and the gall size. ARK-1 reduced the probability of gall formation for all four isolates tested, and the reduction was statistically significant for three and two of the four isolates used in tomatoes and grapevine co-inoculations, respectively. The effect of ARK-1 on different isolates varied somewhat. A possible source of variation may arise from tumorigenic isolate and host interactions. Certain isolates tested appeared to be better pathogens of tomatoes than grapes and vice versa. Overall, this study had comparable results from previous studies in Japan that documented the efficacy of ARK-1 to suppress gall formation and severity.

When the cell ratio of ARK-1 to the tumorigenic isolate was 0.5:1, 1:1 or 2:1, ARK-1 significantly reduced both the probability of gall formation and gall size, regardless of the ratio. In addition, ARK-1 was able to significantly reduce the probability of gall formation and gall size when challenged with either two- or four-isolate mixture. However, when ARK-1 was challenged with four-isolate mixture at a cell number that was four times greater than that of ARK-1, no significant reduction in the probability of gall formation was observed in tomatoes. This result indicates that the efficacy of ARK-1 will likely decrease as the number of ARK-1 cells relative to tumorigenic *R. vitis* cells decrease.

In conclusion, the studies presented in this thesis demonstrated the efficacy of ARK-1 against individual tumorigenic isolate, higher relative cell ratios, and the efficacy against multiple isolates. Moreover, this is the first study to document the efficacy of ARK-1 beyond

Japanese *R. vitis* isolates, and also using grapevine woody trunk tissue, where crown gall symptoms appears in the field. ARK-1 consistently provided reduction in the gall incidence and size.

Future directions examining the efficacy of ARK-1 as a feasible biological control agent for crown gall should investigate the timing and method of application of ARK-1. In the current studies shown in this thesis, ARK-1 was simultaneously co-inoculated with the tumorigenic isolate(s). Future studies should include inoculation of ARK-1 at different time points relative to the inoculation of the tumorigenic isolate(s). This would be important factor in the use of ARK-1 as an agricultural product. If ARK-1 is effective only when applied to a plant prior to infection by tumorigenic *R. vitis,* then ARK-1 application to the vine would be required prior to planting or prior to a wounding event. Once the effect of timing of ARK-1 is understood, the application method should be investigated. Currently, Kumiai Chemical Industry is testing ARK-1 as a pre-planting root dip. The future studies would focus primarily on whether there are effective methods to apply ARK-1 to grapevines after planting, assuming the grapevines are already contaminated with native *R. vitis* isolates. These methods may include a soil drench or a bark or shoot surface treatment with a suspension of ARK-1 applied as a paint or spray. Understanding how ARK-1 can be effectively applied in the field is important for maximizing the efficacy of ARK-1 to prevent crown gall. Finally, for ARK-1 to be used as a commercial biological control agent, the impact of ARK-1 on the soil microbiome would have to be minimal. Studies would be required to demonstrate that ARK-1 would not significantly disrupt the populations of potentially beneficial bacteria and fungi in the soil.

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Appendix

A. 16S genomic consensus sequence of tumorigenic Virginia R. vitis isolates and primers.

Ab3-F3

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  1 atgacggtag tcggagaaga agccccggct aacttcgtgc cagcagccgc ggtaatacga
 61 agggggctag cgttgttcgg aattactggg cgtaaagcgc acgtaggcgg ataattaagt
121 caggggtgaa atcccgcagc tcaactgcgg aactgccttt gatactggtt atcttgagta
181 tggaagaggt aagtggaatt gcgagtgtag aggtgaaatt cgtagatatt cgcaggaaca
241 ccagtggcga aggcggctta ctggtccatt actgacgctg aggtgcgaaa gcgtggggag
301 caaacaggat tagataccct ggtagtccac gccgtaaacg atgaatgtta gccgtcggca
361 agttgacttg tcggtggcgc agctaacgca ttaaacattc cgcctgggga gtacggtcgc
421 aagattaaaa ctcaaaggaa ttgacggggg cccgcacaag cggtggagca tgtggtttaa
481 ttcgaagcaa cgcgcagaac cttaccagct cttgacatcc tgtgaccgcc acggagacgt
541 ggttttcctt tcggggacac agagacag
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Ab3-R4