ASSESSING THE GENETIC DIVERSITY OF *AGROBACTERIUM TUMEFACIENS* IN CALIFORNIA WALNUT GROWING REGIONS AND RESISTANCE TO THE BIOCONTROL AGENT, *A. RHIZOGENES* K84.

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ABSTRACT

Crown gall of walnut (*Juglans* sp.), caused by the bacterium *Agrobacterium tumefaciens*, greatly impacts the CA walnut industry. To determine the genetic diversity of *A. tumefaciens* throughout the Central Valley of CA, we collected isolates from 12 walnut growing counties. A total of 340 *A. tumefaciens* biovar 1 isolates were collected and tested for the presence of the Ti plasmid by PCR; 267 isolates were Ti-plasmid positive or virulent. To assess genetic diversity, BOX PCR profiles were generated for the confirmed virulent isolates. Additionally, we tested the ability of the biocontrol agent *A. rhizogenes* K84 to inhibit the growth of virulent *A. tumefaciens* biovar 1 isolates. Further analysis of the genetic heterogeneity and K84 sensitivity of CA *A. tumefaciens* isolates will facilitate the design of effective crown gall management strategies, including more precise application of K84 depending on *A. tumefaciens* genotype present.

INTRODUCTION

Each year the walnut industry of California suffers significant yield and tree losses due to crown gall caused by the soil-borne bacterium *Agrobacterium tumefaciens*. To manage crown gall disease, growers rely on soil fumigation, culling of infected trees, or surgical removal of the tumor tissue. These methods have limited efficacy and are costly and labor intensive. The commercially available biological control agent, *A. rhizogenes* K84 is used around the world to control crown gall-causing *A. tumefaciens* on a variety of plants. However, while very effective in other parts of the world, California walnut growers have had quite variable success using this agent to manage crown gall. There are several reasons that may explain this lack of control. First, *A. tumefaciens* strains in California may not be susceptible to the antibiotic, agrocin 84, produced by strain K84. Second, resident *A. tumefaciens* strains, though initially susceptible, may rapidly develop resistance to K84 when under intense selection. Third, K84 may have a limited ability to colonize the rhizosphere of walnut trees or to sustain populations capable of suppressing crown gall formation over an extended period of time.

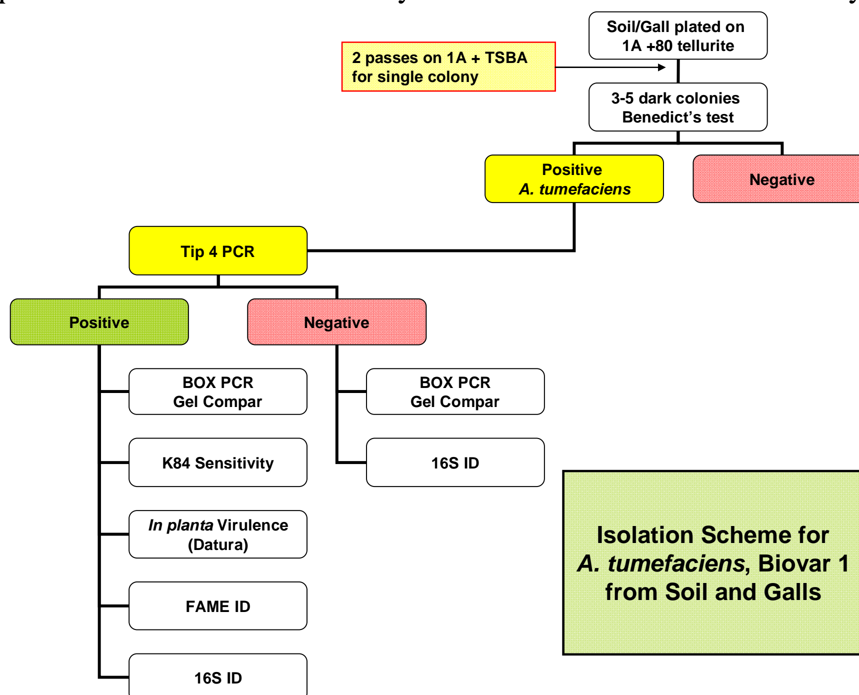
We hypothesized that the efficacy of biocontrol using *A. rhizogenes* K84 is variable due to the genotypic diversity observed among *A. tumefaciens* biovar 1 strains. To test this hypothesis we selected a subset of virulent strains representing distinct BOX PCR profiles collected across 12 CA counties and tested their resistance to the biological control agent *A. rhizogenes* K84. This work will facilitate the design of more cost effective crown gall management strategies, including targeted application of *A. rhizogenes* K84 in regions shown to contain virulent, and K84 sensitive, *A. tumefaciens* strains.

OBJECTIVES

1. Assess the diversity of *A. tumefaciens* biovar 1 isolates from walnut growing regions in California.
2. Test the susceptibility of a subset of *A. tumefaciens* biovar 1 isolates to the biocontrol agent *A. rhizogenes* K84.
3. Examine the development and selection of K84 resistance in selected *A. tumefaciens* strains.

PROCEDURES

See flow chart below for *A. tumefaciens* isolation from soil and galls. Briefly, soil or galls were plated onto 1A medium + 80 ppm potassium tellurite, a semi-selective medium for *Agrobacterium* biovars 1 and 2 (1). Single colonies were picked, re-streaked onto tryptic soy broth agar, and tested for 3-ketolactose activity (Benedict's test). All Benedict's positive isolates were designated as *A. tumefaciens* biovar 1, and genomic DNA was extracted for further PCR analyses. The presence of the Ti plasmid was detected using Ti-plasmid specific Tip4 primers. BOX PCR fingerprinting analyses of both Ti+ and Ti- *A. tumefaciens* isolates were analyzed using GelCompar® II software ver. 4.0 (Applied Maths, Inc., Austin, TX)(4). Selected Ti-plasmid $^{+}$ isolates were tested for sensitivity to *A. rhizogenes* K84 and virulence. 16s rDNA sequence and FAME analysis were used to identify the isolates tested.



<u>Cultivation of *A. tumefaciens* biovar 1 strains and DNA extraction.</u> To extract total DNA from *A. tumefaciens* biovar 1 strains, individual isolates were grown in 2-3 ml of tryptic soy broth (TSB) at 28°C for 24 hours. One to 1.5 ml of culture was aliquoted into 1.5 ml centrifuge tubes and cells were collected by centrifugation at 14,000 RPM for 8-10 minutes. The supernatant was decanted and bacterial cell pellets were stored at -20°C until DNA extraction was performed. DNA was extracted using the MasterPure™ DNA extraction kit (Epicentre, Madison, WI).

Molecular identification of virulent, Ti-plasmid containing *A. tumefaciens* biovar 1 isolates.

Ti plasmid presence was detected using Ti plasmid specific Tip4 primers (Sudarshana, McClean, and Kluepfel, unpublished). PCR mixtures were prepared as follows in a final volume of 25 µl: 1X Taq buffer, 1.5 mM $MgCl_2$, 200 mM each dNTP mix, 100 pmol Tip4F primer, 100 pmol Tip4R primer, 50-100 ng genomic DNA template, and 1 unit of Taq polymerase (Promega Corp, Madison, WI). PCR cycling parameters consisted of an initial dentaturation step (92°C for 2 minutes) followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C and a final elongation step of 72°C for 2 minutes.

PCR products were resolved by agarose gel electrophoresis. *A. tumefaciens* biovar 1 isolates that displayed a 220 bp band were designated as Ti-plasmid containing or virulent.

Analysis of virulent *A. tumefaciens* biovar 1 isolates using BOX PCR genomic fingerprinting and computer-assisted pattern analysis.

BOX-PCR analysis was used to generate genetic fingerprints of *A. tumefaciens* biovar 1 isolates from California walnut growing regions (Versalovic, J., *et a*l. 1994). PCR mixtures were prepared as follows to a final volume of 25 µl: 1X Gitschier Buffer, 10% (v/v) DMSO, 2.5 mM each dNTP, 50 pmol of BOX primer (BOX 1AR), 2 units of Taq polymerase (Promega), and 50-150 ng genomic DNA templates. PCR cycling parameters consisted of an initial denaturation step (95°C for 7 minutes) followed by 35 cycles of 94°C for 1 minute, 53°C for 1 minute, 65°C for 8 minutes, and a final extension time of 65°C for 16 minutes. PCR samples were stored at -20°C until further processing.

BOX-PCR fingerprint patterns were visualized using agarose gel electrophoresis. Gels were photographed using a gel documentation system and imported into the computer-assisted pattern analysis program GelCompar II for analysis (AppliedMaths, Inc., Austin, TX).

A. rhizogenes 84 bioassay of California *A. tumefaciens* biovar 1 isolates.

A subset of *A. tumefaciens* biovar 1 isolates were tested for their resistance to agrocin 84 produced by the biocontrol strain *A. rhizogenes* K84 using Stonier's bioassay plates and a modified Agrocin 84 bioassay (Stonier, T. 1960; Kim, H. & Farrand, S.K. 1997). Briefly, individual *A tumefaciens* biovar 1 isolates were grown overnight and suspended in a phosphate buffered soft agar and overlaid on a Stonier's plate seeded with *A. rhizogenes* K84. Susceptibility was measured as the appearance of a zone of inhibition or halo around the K84 colony (Figure 2).

RESULTS AND DISCUSSION

Here we examined the genetic diversity of *A. tumefaciens* isolated from walnut growing regions in California and determine their sensitivity to the widely used crown gall biocontrol agent *A. rhizogenes* K84. A total of 340 isolates were cultured from soil or from infected plant tissue (galls and roots) using the *Agrobacterium* semi-selective medium 1A. To assess virulence a PCR-based approach was used to detect the presence of the Ti plasmid, the virulence

determinant of *A. tumefaciens*. Two hundred and sixty seven isolates tested positive for the Ti-plasmid (Table 1). These isolates were fingerprinted using repetitive PCR using the BOX primer (Figure 3).

Repetitive PCR using the BOX primer is commonly used to examine genetic diversity. The digitized BOX PCR profiles of all 340 virulent *A. tumefaciens* biovar 1 isolates were analyzed using the software GelCompar II, which uses UPGMA analysis (data not shown). The result of this analysis revealed the presence of at least 30 distinct BOX-PCR profiles with many of them clustering as function of geography. Interestingly, at lease one genotype can be found in numerous counties through out the central the valley (Figure 3).

Figure 3 shows a subset of 73 virulent *A. tumefaciens* biovar 1 isolates in our current collection that were chosen for further analysis because they represent the genetic diversity found across California. Analysis of this group of isolates revealed no relationship, between BOX PCR profiles and K84 sensitivity. For example, the isolates that are described by the fragment profiles found in profile #18 are both K84 sensitive and K84 resistant. Consequently, these profiles will not be predictive for the effectiveness of commercially available, K84-based, crown gall control agents. In addition, these results suggest that one could expect a significant amount of variability in biocontrol efficacy of K84 agents due to the presence of both K84 sensitive and K84 resistant isolates at a single location (Figure 3). Even more importantly, in several counties we found that less than 30% (19/ 65) of the tested virulent *A. tumefaciens* biovar 1 isolates are sensitive to *A. rhizogenes* K84 (Fig. 1).

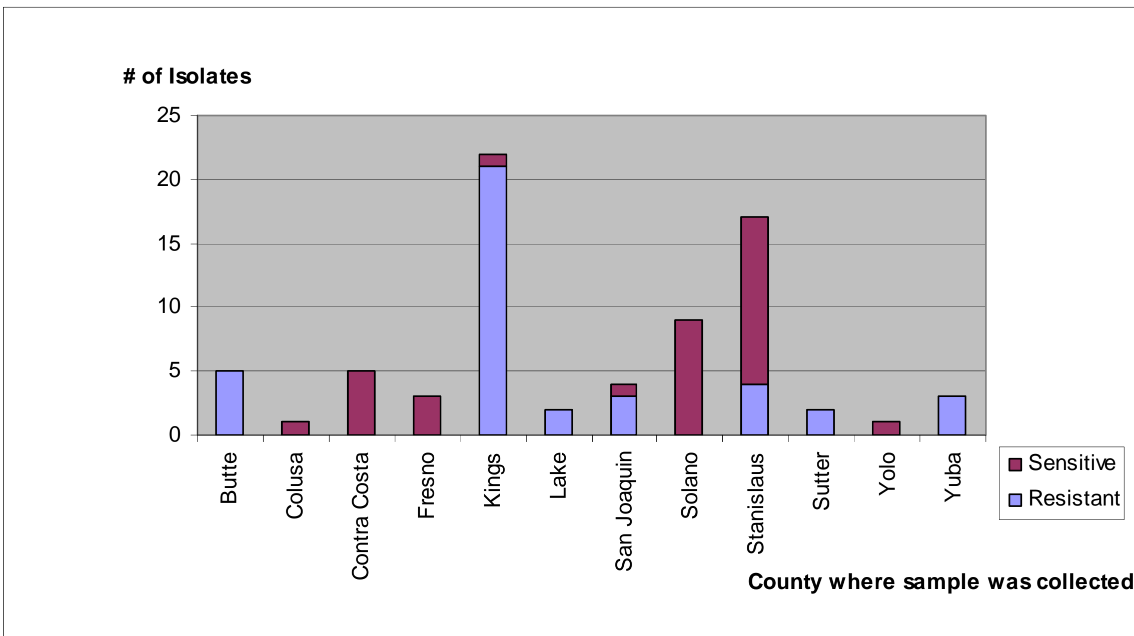
A new curious result was observed this year. When screening *A. tumefaciens* isolates for K84 sensitivity, we observed that within 2 to 3 days after plating the strain to be tested, we observed individual colonies developing within the “cleared” zone of inhibition (see Figure 2). This occurred with most *A. tumefaciens* isolates examined using this bioassay. We subsequently re-isolated, and single colony purified, all the “K84-resistant” isolates developing with in the zone of inhibition. These isolates were confirmed to be the original *A. tumefaciens* strain and upon subsequent K84-sensitivity testing, were found to be resistant to K84. The speed with which K84 resistance develops invitro may be another explanation for the inconsistent nature of the biological control of crown gall using K84 derivatives observed in the field.

Table 1: Summary data of *A. tumefaciens* biovar 1 collection from June 2005 until present.

County	*A. tumefaciens* bv. 1 virulent isolates[a]	*A. tumefaceins* bv. 1 avirulent isolates[b]
Butte	5	0
Colusa	0	17
Contra Costa	9	4
Fresno	5	0
Kern	0	16
Kings	118	85
San Joaquin	4	6
Solano	11	3
Stanislaus	24	26
Sutter	18	5
Yuba	10	2
Placer	19	3
Glenn	3	21
Napa	34	0
Lake	3	2
Total	263	190

[a]virulent isolates are defined as those that yielded at 220 bp band in Ti-plasmid specific PCR.
[b]avirulent isolates are defined as those that did not yield a positive PCR result for Ti-plasmid.

Fig 1. K84 Sensitivity of environmental *A. tumefaciens* strains isolated from 17 sites across 12 counties. K84 sensitivity was measured as halo formation on Stonier's medium.



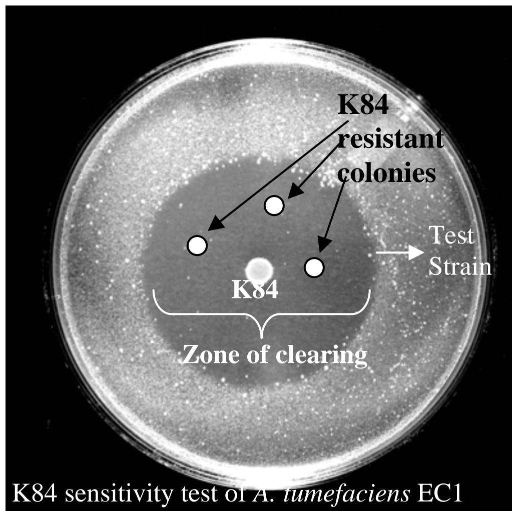
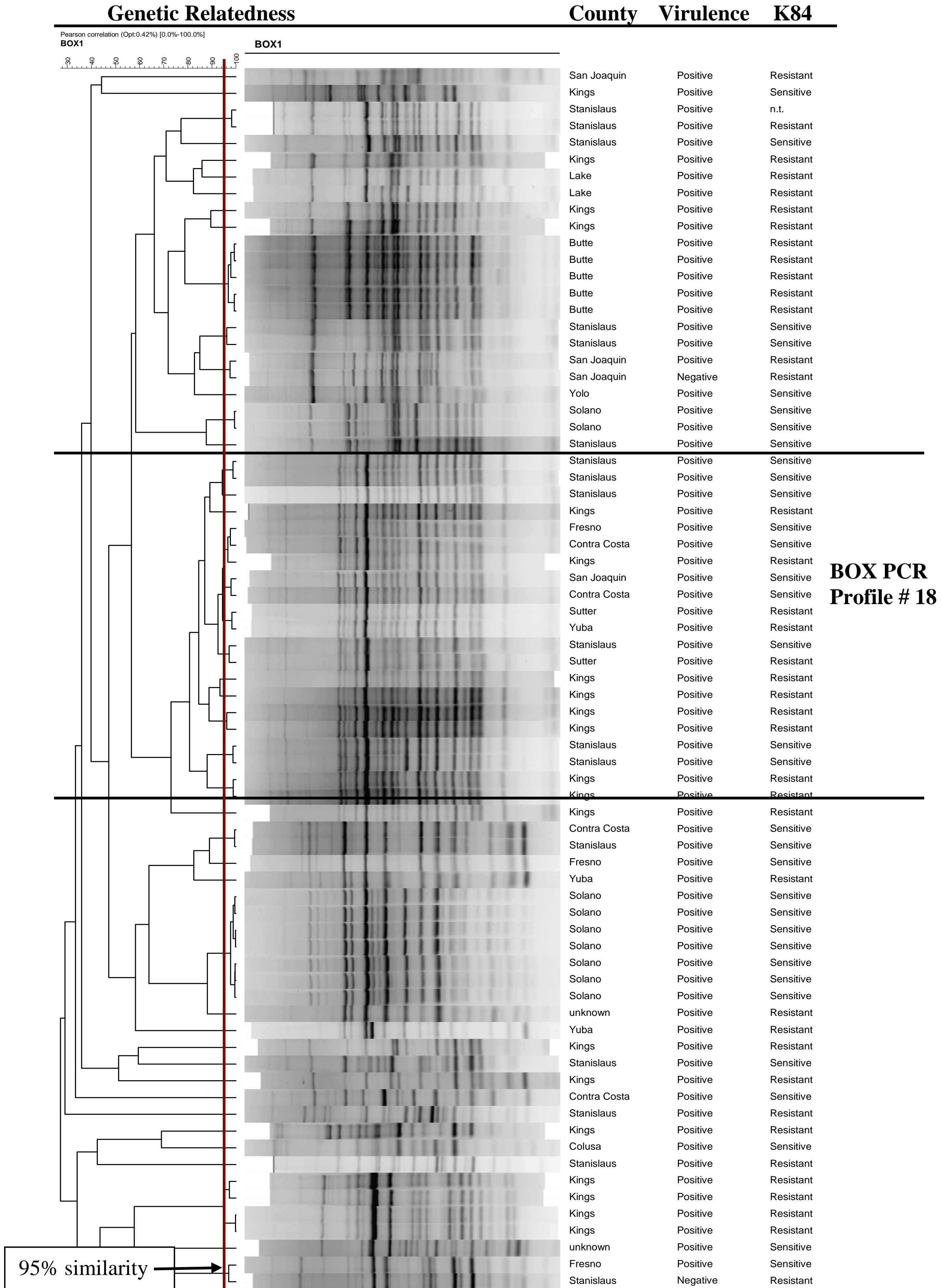


Figure 2: K84 Sensitivity Test. Virulent *A. tumefaciens* biovar 1 isolates were tested for resistance to K84 using a Stonier's plate where a zone of inhibition develops around the dot in the center of the plate which contains K84. Briefly, each strain was overlaid on Stonier's plates seeded with *A. rhizogenes* K84. Sensitivity to K84 was measured as the formation of a zone of clearing or halo around the center K84 colony.

Figure 3. BOX-PCR analysis of representative *A. tumefaciens* isolates.



CONCLUSIONS

1. There are at least 30 distinct BOX PCR profiles of A. *tumefaciens* biovar 1 isolated from California walnut growing regions. A total of 12 California counties encompassing the majority of California walnut growing regions were represented in this study. The presence of many distinct genotypes points to the diversity of gall-forming *A. tumefaciens* strains that growers and nurseries encounter in their orchards.

2. Less than 30% (19/ 65) of the tested virulent *A. tumefaciens* biovar 1 isolates are sensitive to *A. rhizogenes* K84. Stanislaus, Kings, and San Joaquin counties have both K84 resistant and sensitive *A. tumefaciens* present. As hypothesized, not all virulent *A. tumefaciens* biovar 1 isolates from commercial orchards are susceptible to K84. Additionally, we demonstrated the presence of both K84 susceptible and resistant strains in commercial orchards which, in part, explains the inconsistencies in K84 control of crown gall. This is consistent with grower observations that variable crown gall control is achieved with K84 in a walnut orchard setting.

3. At this time, no single BOX PCR fingerprint can be linked to K84 resistance or susceptibility. For example, strains belonging to fingerprint group 18 can be found in 6 counties and display variable resistance to K84 (Figure 1B). Investigations into Ti plasmid content and the identification of opines produced and utilized by CA *A. tumefaciens* isolates are ongoing and could yield better predictors for K84 effectiveness in CA walnut orchards and nurseries.

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