ASSESSING THE GENETIC DIVERSITY OF *AGROBACTERIUM TUMEFACIENS* IN CA 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 ten 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; 190 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 27 virulent *A. tumefaciens* biovar 1 isolates from different counties. Fifteen isolates were found to be resistant to K84 from Kings, Butte, and Stanislaus counties. Further analysis of the genetic heterogeneity and K84 sensitivity of California *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.

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.

PROCEDURES

Isolation and identification of *Agrobacterium tumefaciens* biovar 1 from soil and gall tissue.
Soil or gall tissue was plated onto 1A medium amended with 80 ppm potassium tellurite (1A80), a semi-selective medium for *Agrobacterium* biovars 1 and 2 (Mougel, C., *et al*. 1997). Plates were incubated for 72 hours at 28°C. Single bacterial isolates were selected and re-streaked onto tryptic soy broth agar (TSBA) to assure purity. Purified colonies were tested for 3-ketolactose activity (Benedict's Test) and all Benedict's positive isolates were designated as *Agrobacterium tumefaciens* biovar 1 (Bouzar, *et al*. 1995).

Cultivation of *A. tumefaciens* biovar 1 strains and DNA extraction.
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 with shaking. One to 1.5 ml of bacterial 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 from frozen bacterial pellets using the MasterPure™ DNA extraction kit as per the manufacturer's instructions (Epicentre, Madison, WI). Genomic DNA extracted from *A. tumefaciens* biovar 1 strains was diluted with water to a concentration of 50 to 150 ng/µl prior to using as template for polymerase chain reaction (PCR) -based experiments.

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

Ti plasmid presence was detected using Ti plasmid specific Tip4 primers (Sudarshana, P., McClean, A., and Kluepfel, D.A., 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 followed by staining with the DNA dye ethidium bromide. *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 followed by staining with ethidium bromide (EtBr). 1.5% (w/v) agarose + 1X Tris-Acetate-EDTA Buffer (TAE) + EtBr gels were run for 16 hours at 60 V in 1X TAE buffer. Gels were photographed using a gel documentation system and imported into the computer-assisted pattern analysis program GelCompar II for further 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

This study was initiated to characterize 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 amended with 80 ppm potassium tellurite (Table 1). To assess virulence a PCR-based approach was used to detect the presence of the Ti plasmid, the virulence determinant of *A. tumefaciens*. One-hundred ninety isolates tested positive for the Ti-plasmid (Table 1). These isolates were then fingerprinted using repetitive PCR using the BOX primer (Figure 1).

Repetitive PCR using the BOX primer, or BOX PCR is commonly used to examine genetic diversity. Genomic fingerprint analysis of both human and agricultural bacterial pathogens has lead to the identification of specific bacterial strains responsible for disease outbreaks, epidemiological monitoring of bacterial strains, and source identification. The digitized BOX PCR profiles of all 190 virulent *A. tumefaciens* biovar 1 isolates were analyzed using the software GelCompar II, which uses UPGMA analysis. Distinct genetic profiles were clustered according to similarity. Figure 1 shows a subset of ninety-three virulent *A. tumefaciens* biovar 1 isolates in our current collection. We found several distinct genotypes that clustered as a function of geography. Interestingly one genotype was found in at least four counties (Figure 1).

Due to the level of genotypic diversity observed among *A. tumefaciens* biovar 1 strains, we hypothesized that the efficacy traditional biocontrol using *A. rhizogenes* K84 (K84) would be variable. Evidence of the variability of crown gall control using K84 has been documented in previous Walnut Reports (Olson, et al. 1990; Schroth, M. N. et al. 1984; Schroth, M.N. & McCain, A.H. 1985; Schroth, M.N. & Pierce, L. 1996). Also, the hypothesis that genetic variation among *A. tumefaciens* biovar 1 isolates contributes to K84 variability has been suggested (Schroth, M.N. & Pierce, L. 1996). We tested this hypothesis by subjecting 27 *A. tumefaciens* isolates from different counties and displaying different genotypes to an *A. rhizogenes* K84 bioassay (Figure 1, stars). As hypothesized not all virulent *A. tumefaciens* isolates examined in this study are susceptible to the antibiotic, Agrocin 84, produced by K84. Fifteen isolates were resistant to K84 from four counties: Kings, Butte, and Stanislaus counties (Table 2). This is consistent with and helps to explain the variable control observed by growers. At this time we are unable to link a specific genotype with K84 sensitivity.

Coupling PCR fingerprinting methods and physiological tests for K84 susceptibility is a powerful tool. However, we are unable at this time to correlate genotype with resistance to the biocontrol agent due to the small number of strains tested and only one replication. More strains from our collection need to be tested in order to make a more solid conclusion. In order for K84 to be effective, *A. tumefaciens* biovar 1 must take up certain opines, nopaline or agrocinopine that are made as a result of gall formation by the pathogen. In this case, a more biochemical approach, such as the identification of opines produced by California *A. tumefaciens* isolates in addition to genomic fingerprinting could be a more powerful predictor of K84 effectiveness in the state.

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	13	4
Fresno	5	0
Kern	0	2
Kings	118	85
San Joaquin	2	4
Solano	9	3
Stanislaus	24	26
Sutter	0	2
Unknown	14	7
Total	**190**	**150**

[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.

Table 2: Summary *A. tumefaciens* biovar 1 isolates tested for resistance to *A. rhizogenes* K84.

Sample ID	County of Isolation	Source	K84 sensitivity[‡]
CHI 115	Butte	Soil	-
CHI 117	Butte	Soil	-
JH6-1	Contra Costa	Soil	+
JH9-1	Contra Costa	Gall	+
JHS8-2	Contra Costa	Soil	+
JH13-2	Fresno	Gall	+
KIN 7-1	Kings	Soil	+
CR49T23-I4	Kings	Soil	-
CR49T23-I7	Kings	Soil	-
DR53T10BT14	Kings	Soil	-
DR55T7-2	Kings	Gall	-
DR57T18N-3	Kings	Gall	-
DR59T7SGI1	Kings	Soil	-
DR59T14SG3	Kings	Gall	-
KIN1-1	Kings	Soil	-
KIN4-1	Kings	Soil	-
KIN3-1	Kings	Soil	-
DIX R5	Solano	Soil	+
DIX R8	Solano	Soil	+
175-1-1	Stanislaus	Gall	+
186-7-1	Stanislaus	Tree Disk	-
T1HDE1	Stanislaus	Gall	+
T1HGR1	Stanislaus	Rts. from Gall	+
T3HGE1	Stanislaus	Gall	+
T3HGR2	Stanislaus	Rts. from Gall	+
T6BHRG3B	Stanislaus	Gall	-
WS9	Stanislaus	Soil	-

[‡]K84 Sensitivity was measured as halo formation on Stonier's medium. + sign indicates sensitivity to K84 and – indicates resistance to K84.

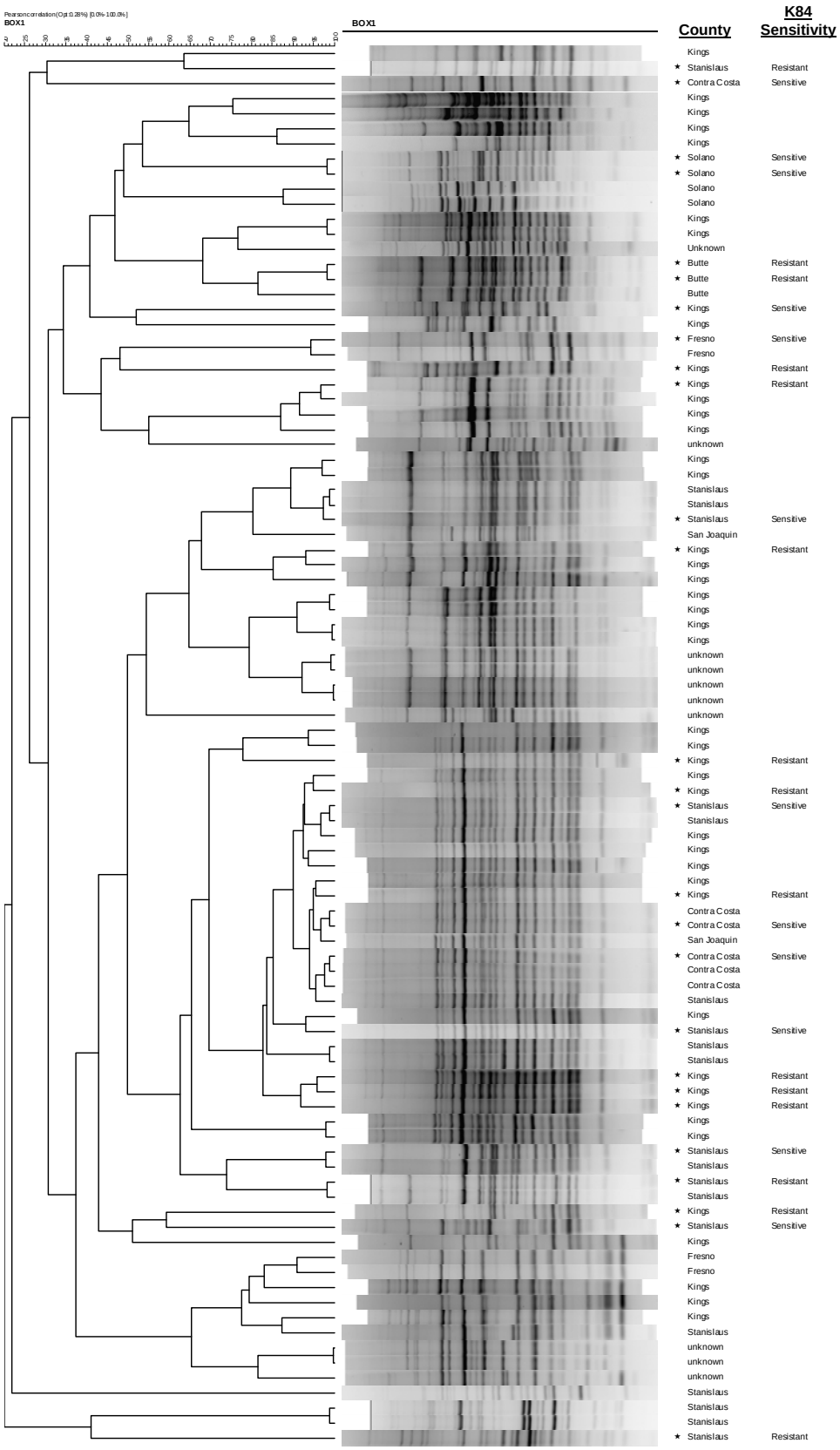

Figure 1: Cluster analysis of BOX fingerprint profile analysis of virulent *Agrobacterium tumefaciens* biovar 1 from California walnut growing regions. Ninety-three representative isolates were clustered UPGMA analysis in GelCompar II. Stars indicate strains that have been tested for sensitivity to K84.

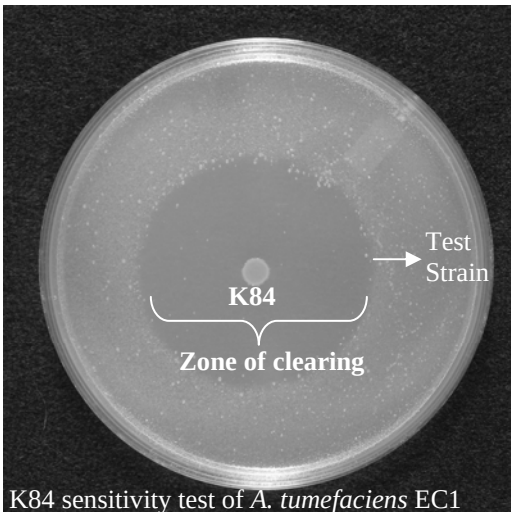

Figure 2: Photograph of K84 Sensitivity Test. A subset of virulent *A. tumefaciens* biovar 1 isolates was tested for resistance to K84. Briefly, each strain was overlaid on Stonier's plates seeded with *A. rhizogenes* K84. Sensitivity to K84 was measured as the formation of a zone of clearing or halo around the K84 colony.

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