Workgroup/Department: Walnut/Plant Pathology

University of California
Division of Agricultural Sciences
Project Plan/Research Grant Proposal

Project Year: 2008/2009 **Anticipated Duration of Project**: 1 yr

Project Leader: D.A. Kluepfel **Location**: USDA-ARS, Dept. of Plant Pathology

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Project Title: An ecological and molecular study of the biocontrol agent *A. rhizogenes* K84 and its effectiveness in controlling crown gall of walnut.

Keywords: Biocontrol, Crown gall, walnut, *Agrobacterium tumefaciens*, *A. rhizogenes* K84

Commodity: Walnut **Relevant AES/CE Project No**._____

Biological Control of Crown Gall by *A. rhizogenes* K84

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 parts of the world, California walnut growers have had limited success using this agent to manage crown gall of walnut. There are several reasons that may explain the lack of control. First, *A. tumefaciens* strains in California may not be susceptible to the antibiotic, agrocin 84, produced by strain K84. Second, 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. Here we propose to continue the work we initiated last year to examine and document why K84 is ineffective in controlling crown gall in most of the walnut growing regions of California.

This will be done by characterizing, at the genetic level, an *A. tumefaciens* collection comprised of approximately 300 isolates from 8 counties in California for genes that are known to be required for K84 susceptibility. Identifying the locations in California where susceptible or resistant strains occur will facilitate targeted use of K84; this will reduce unnecessary expense due to using K84 in orchards where K84-resistant virulent *A. tumefaciens* are found. Orchards in locations where K84 susceptible *A. tumefaciens* are found may benefit from treatment with K84. However, control may be limited due to K84 establishing inadequate populations in the field and in the walnut rhizosphere, due to its inability to compete with other microorganisms or survive in

this environment during the growing season. A twelve-month study of K84 persistence in the field and in the walnut rhizosphere will determine whether populations of K84 can be sustained over an extended period or time. Together, data gathered from these experiments will help to better understand the biology of K84 resistance in California *A. tumefaciens* populations and to fine-tune biocontrol initiatives. This information also will serve as a basis for developing new and improved biocontrol strategies for crown gall of walnut.

We have completed all of the preliminary work required to establish the in vitro and in vivo procedures to quantify agrocin 84 sensitivity. By using our work describing the genetic diversity of *A. tumefaciens*, we were able to chose 25 genetically distinct *A. tumefaciens* isolates and examine them in our invitro K84 sensitivity test. As hypothesized, we found *A. tumefaciens* isolates from specific regions that are resistant to K84 while in other areas of the state virulent *A. tumefaciens* isolates are sensitive to K84. These observations have important implications for the future biological control of *A. tumefaciens* and control of crown gall disease.

Objective I: Determine the distribution of Californian *A. tumefaciens* isolates that exhibit sensitivity to *A. rhizogenes* K84.

Procedure: Preliminary results from our *A. tumefaciens* diversity study has revealed the presence of genetically distinct *A. tumefaciens* populations in Californian soils. Although all the virulent *A. tumefaciens* isolates in our collection possess the machinery to cause disease as detected by the presence of the tumor-inducing DNA fragment, we do not know if these bacteria possess the genes for K84 susceptibility. In order for K84 to be effective, target *A. tumefaciens* isolates must be able to take up certain compounds known as opines, i.e. nopaline or agrocinopine. The agrocinopine transporter also facilitates the entry of agrocin 84, the toxic component produced by strain K84, which is a mimic of agrocinopine (1). Using DNA primers to detect the agrocinopine transporter gene in our collection of CA *A. tumefaciens* isolates will allow us to classify strains as K84 resistant or susceptible based on the presence of this gene. Biological confirmation of K84 resistance or susceptibility will be follow by *in vitro* screening.

Last year we completed the development of PCR primers and protocols required to examine the presence of the agrocinopine transporter genes. Here we propose to continue this work by examining up to 30 genetically and geographically distinct *A. tumefaciens* populations for the occurrence of agrocinopine transporters. In addition we will examine the soil collected from the walnut growing regions of the state for the occurrence of this important class of genes. This examination will be performed in a culture independent manor where DNA will be directly isolated from the soil, purified and used in the PCR detection protocols described above.

Outcome: These experiments will allow us to determine which regions of California are most likely to benefit from K84 application. Moreover, this allows us to identify regional isolates that are resistant to K84 and allow for the development of more defined management measures for these strains. This work also will facilitate the development of culture-independent detection of K84 sensitive isolates using DNA extracted directly from soil. In addition it will be very useful to determine whether culture independent direct DNA detection of opine transporters will serve as a useful and robust predictive tool for determining future success of K84 applications.

Objective II: Determine the population dynamics and persistence of *A. rhizogenes* K84 in the rhizosphere of walnut trees.

Procedure: One requirement for effective and continual biocontrol by K84 is long-term colonization of the soil and rhizosphere of the plant to be protected. In 1993, Stockwell, *et al*., reported that K84 was able to survive well in Oregon soils immediately surrounding cherry tree roots as well as persisting for weeks on plants (2). However, K84 survival in bulk soil was low with populations dropping from 10^6 colony forming units per gram dry weight soil (cfu/g d.w. soil) to 10^2 cfu/g d.w. soil in only 16 weeks (2). K84 also has been shown to be able to colonize the rhizosphere of peach tree seedlings reaching 10^8 cfu/g of fresh weight root tissue (4). However, it is unknown how well K84 colonizes the walnut rhizosphere. We propose to examine the long-term survival of K84 in the walnut tree rhizosphere and in soils immediately surrounding trees at various distances from the tree (6, 12 and 36 inches). Walnut trees (Paradox root stock) will be treated with K84 by spraying the roots with a suspension of K84 or K1026 (a genetically modified derivative of K84) of known cell density. In order to enumerate K84 populations in the soil, we will generate a spontaneous rifampacin or streptomycin resistant strain in order to trace K84 or K1026 populations. This spontaneous mutant plus the use of *A. rhizogenes* semi-selective medium amended with the appropriate antibiotics will facilitate enumeration K84 or K1026 while suppressing populations of other rhizobacteria. Sampling of tree roots, rhizosphere soil, and soil surrounding the tree will occur bi-weekly for the first 2 months and then every month for the next 10 months.
Note: This objective remains unchanged from last year. Due to personnel and time constraints last year, we were only able to generate and characterize the K84 strains that we genetically tagged with resistance to the appropriate antibiotics. This year we will complete the proposed soil population dynamics studies using these tagged K84 isolates.
Outcome: Understanding the population dynamics of K84 will allow for the design of methods to promote population sustainability in walnut orchards. It is known that K84 can survive in irrigation water and on the roots of grasses surrounding cherry trees (2). However, mature California walnut orchards are relatively weed-free around the tree trunk and therefore reservoirs of K84 do not exist due to the lack of alternate grass hosts. Improving K84 efficacy may include the re-establishment of K84 populations by applying the microbial biocontrol agent through the irrigation system. This approach would be inexpensive and require limited time to perform. The results of this work will provide a sound basis for the timed application of microbial biocontrol agents through the irrigation system.

Objective III: Incorporate the use of a high through put in vitro screen to characterize K84 sensitivity of genetically distinct virulent *A. tumefaciens* populations collected from the walnut growing regions of Ca.

Procedure: Data obtained from Objectives I and II will lead to a better understanding of K84 resistance at the molecular level and the physiology of K84 in the field, respectively. However once K84-susceptible or resistant California *A. tumefaciens* isolates are identified at the molecular level, it is imperative to biologically confirm these results. To quantitatively assess resistance or susceptibility of California *A. tumefaciens* isolates to strain K84, a modified Stonier's method will be used (3). Briefly, a seed culture of K84, will be suspended in a 500-microliter droplet of 0.7% agar-media and placed in the center of individual wells of a 16-well

plate; this will be allowed to solidify and grow overnight. Once solidified, indicator strains, or strains to be tested for their resistance or susceptibility to K84 will be overlaid as 5 ml cultures suspended in 0.9% agar into each well containing a seed culture. 16-well plates will be allowed to solidify and grow at 28 degrees Celsius. The appearance of clear zones surrounding the seed strain indicates susceptibility to K84. *In vitro* biocontrol efficacy can be calculated based on the diameter of the zone measured. Control strains such as K84 sensitive *A. tumefaciens* C58 and K84 resistant *A. tumefaciens* B49c will be used to compare relative sensitivity or resistance to K84, respectively.

Note: The procedure described above was included from last years proposal to remind readers how we will be conducting the in vitro screening. Development of this high through put in vitro screening was accomplished as stated in last year's proposal. Here we propose to put this screening method to work examining the existing *A. tumefaciens* isolates in our collection and the new ones being continuously added. This collection is especially valuable given the fact that we have established the genetic diversity/relatedness of the isolates as a function of both host and collection site. Consequently it will now be possible to interpret the observed differences in K84 sensitivity with genetic diversity and the geographic source of isolation. The results of the preliminary screen can be found in this year's annual report.

Here we will also extend the work proposed in objective 3 to include greenhouse tests of the effectiveness of K84 against isolates shown above to be sensitive to K84. Paradox seedlings will be inoculated with K84 sensitive *A. tumefaciens* strains followed by introduction of K84 at two times after inoculation of the virulent isolates (i.e. at time 0 and 2hr post inoc.). Ten groups of plants will then be wounded at; 30 min, 2hours and 4 hours post K84 inoculation and then planted in pasteurized soil. Four to six weeks after inoculation plants will be evaluated for tumor production. Since we will be using virulent *A. tumefaciens* strains that are tagged with antibiotic resistance markers we will also determine the population dynamics of both the virulent and K84 strains used in the experiments. Populations of both isolates will be determined 7days and 21 days after inoculation. These data will allow us to quantify the ability of K84 to control both populations of the virulent populations and tumor formation.

The experiment described above will be repeated using a virulent *A. tumefaciens* isolate that is insensitive to K84. By comparing both tumor formation and population dynamics for both the K84 sensitive and K84 insensitive virulent isolates we will be able to gain considerable insight into the mechanisms that control the effectiveness or failures of *Agrobacterium* biological control strains under orchard conditions.

Outcome: From the experiments above we will be able to validate results from Objective I while at the same time be able to quantitatively assess the *in vitro* efficacy of K84 toward susceptible California *A. tumefaciens* isolates. Also, K84 resistant strains may now serve as indicators for potential biocontrol agents isolated from *A. tumefaciens* suppressive sites. Targeting specific strains of *A. tumefaciens* may be lead to more effective control measures in California against crown gall.

Project Duration: 12 months. We anticipate approximately 12 months to complete the genetic and K84-sensitivity invitro screening of our *A. tumefaciens* collection and the culture independent examination of soil collected from the major walnut growing regions in CA for the presence of agrocinopine transporter genes. Finally, the population dynamics of K84 strains tagged with antibiotic resistance markers, in the soil/rhizosphere will examine concomitantly with the molecular and in vitro screening work.

Literature Cited:

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Budget Request

Budget Period: May 1, 2008 to April 30, 2009
Funding Source: Walnut Marketing Board

Item	**Amount ($)**
Post doctoral associate, salary and benefits	11,500
Supplies and Expenses:	2,000
Travel:	500
Total:	14,000

Signature of Principal Investigator: _____

Signature of Authorized Institution Rep,
USDA-ARS, Davis; Location Coordinator: _____

Signature of Authorized Institution Rep.,
University of California, Davis; Dept. Chair:_____