PREVENTION OF CROWN GALL ON WALNUT: DETECTION, EPIDEMIOLOGY AND CONTROL OF AGROBACTERIUM

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ABSTRACT

We studied the diversity of Agrobacterium strains in growers' fields and in the walnut nurseries. When farm advisers were requested to send gall samples from growers' fields, 5 farm advisers sent galls from 19 growers' fields. Surprisingly, all 19 of the 19 growers had purchased their trees from just one of the walnut nurseries. In addition to receiving galls from growers' fields, we also received galls from six walnut nurseries. Overall, we isolated a total of 518 pathogenic Agrobacterium isolates from galls on 233 walnut trees. Pathogenic isolates were characterized for biovar, agrocin-sensitivity, and rep-PCR “fingerprint.” Of all the isolates from walnuts, 65% were biovar one, 34% were biovar two, and none of the isolates were biovar three. According to the agrocin-test, 66% of the isolates were agrocin-resistant. Using the primer REP, we characterized 452 pathogenic isolates from 205 galls and obtained 241 unique REP-PCR fingerprints. Similar data were obtained using the primers ERIC and BOX. Thus, there is extremely high diversity in the population of Agrobacterium on walnuts. One ramification of the extreme diversity of strains is that we cannot provide evidence on whether or not isolates were transmitted from a nursery to a grower's field. A lux-tagged isolate that bioluminesces was used to demonstrate that Agrobacterium multiplies and spreads on the Paradox root surface within the 30-day experimental period. Thus, a transplant without any visible galls can be infested with Agrobacterium on the root surface.

OBJECTIVES

Objective I. To complete the analysis of the distribution of strains of Agrobacterium in California using REP-PCR.

Objective II. To determine whether our current model for epidemiology of the disease adequately predicts disease incidence and severity in grower’s fields.

Objective III. To determine the impact for growers of crown gall on yield and tree longevity.

PROCEDURES

Crown galls from walnut trees were obtained from five farm advisors, who sent samples from a total of 19 growers' fields, and from six nurseries. To isolate bacteria from galls, the surface bark was removed, and several pieces of healthy tissue were excised, wetted with 0.1M MgSO4 and then ground in a mortar and pestle. The cell suspension was streaked onto 1A and 2E media (Bouzar, 1995; Moore et al., 1988), and incubated for 4 days. Then, large mucoid colonies were picked and streaked onto King’s B (KB) agar. To store cultures, cells were grown in Kings B broth, suspended at a final concentration of 20% glycerol, and stored at −70°C. Tomato plants
(Early Pak 7) were used as indicator plants. The terminal shoots were removed from ten to fifteen cm tall plants. Cells, grown on Kings B, were inoculated onto the cut surface. Plants were covered with a polyethylene bag and then incubated overnight so that they were never exposed to direct sunlight. After one month, plants were evaluated for gall. All pathogenic isolates were characterized for biovar using the 3-ketolactose test and the PDA-CaCO₃ acid-clearing test. Isolates with ambiguous results were also tested for motility, and for growth in 2% NaCl and in tyrosine broth. To test agrocin-sensitivity, K84 was grown in the center of a KB plate and incubated at 28°C for 5 days. A fresh culture of the strain to be tested was suspended in Mg SO₄ buffer, and then misted over the plate with K84. After incubation at 28°C for 2 days, the plates were rated for growth of the cells in the zone surrounding K84. rep-PCR was performed as described in Rademaker and DeBruijn (1997) and Rademaker et al. (1997). REP-PCR was performed at least two times, with reproducible results. Results from rep-PCR were analyzed using Gel-Compar software. Methods used with the lux-tagged strain are described in Kado (1996) and McElhaney et al. (1998).

RESULTS AND DISCUSSION

1. Characterization of all the isolates in the collection, with an emphasis on rep-PCR fingerprints. We collected a total of 518 pathogenic isolates from galls on 233 trees. The galls were on trees that were planted in eight counties in California in a region that extends from 40° 20' to 37° 10'. The counties from north to south and the number of galls from each county are as follows: Tehama, 6; Butte, 96; Sutter and Yuba, 62; Lake, 5; Yolo, 2; San Joaquin, 14; and Stanislaus, 48. Most of our data on pathogenicity on tomato, biovar, agrocin-resistance, and control by K-84 were shown in last year’s report and will only be summarized here. Of all the pathogenic isolates, 65% were biovar one, 34% were biovar two, and none of the isolates were biovar three. Thus, the data indicate that biovar one is more commonly isolated from walnut galls in California than is biovar two. None of the isolates were biovar three, i.e., there is no evidence of a systemic biovar.

Thirty-four percent of the isolates were biovar one and agrocin-resistant and 31% were biovar one and agrocin-sensitive. Thus, there is a 48% chance that biovar one will be sensitive to agrocin. In contrast, 22% of the isolates were biovar two and agrocin-resistant and 12% were biovar two and agrocin-sensitive. Thus, there is only a 35% chance that a biovar two isolate will be sensitive to agrocin. Whereas biovar is determined by chromosomal genes, resistance to agrocin is determined by plasmid-borne genes. Because agrocin is the antibiotic produced by the biocontrol agent K-84, it is often assumed that there will be a correlation between agrocin-resistance and an inability of K-84 to control crown gall. However, K-84 has never been effective against pre-existing infections. According to our agrocin-test, only 43% of the pathogenic isolates in our collection were agrocin-sensitive. In addition, agrocin-resistant isolates were obtained from all sampling locations, including places in which K-84 has never been used. Taken together, our data and the literature suggest that K-84 will only be effective in circumstances in which K-84 can be applied in sufficient concentration before the plants are exposed to Agrobacterium.
Using REP-PCR, a “DNA fingerprint” was obtained on 452 isolates. All REP-PCR fingerprints were determined at least twice and were highly reproducible. REP-PCR utilizes repetitive elements. For all 453 isolates, the REP-PCR fingerprint had from one band to 25 bands, depending upon the strain. From the 453 isolates from 205 galls, we obtained 241 unique REP-PCR fingerprints. Thus, using a definition of a strain as all the isolates that had the same REP-PCR fingerprint, in a collection with an average of 2.2 isolates per gall, we isolated an average of 1.2 unique strains per gall. These data indicate that the population of Agrobacterium on walnuts is highly diverse. In addition to the REP-PCR fingerprinting, we also determined DNA fingerprints of 231 isolates from 121 galls using ERIC-PCR and from 128 isolates from 69 galls using BOX-PCR. In all cases where REP-PCR indicated that the same strain was present in galls on different trees, the isolates were examined using ERIC and BOX. In all cases in which REP-PCR indicated that the same strain was present in galls from different trees, ERIC-PCR and BOX-PCR also indicated that the strains were the same. Thus, ERIC-PCR and BOX-PCR confirmed results obtained with REP-PCR. Conversely, REP-PCR revealed more differences between strains than ERIC-PCR or BOX-PCR. Collectively, REP-PCR, ERIC-PCR, and BOX-PCR are known as rep-PCR. With all rep primers, using GelCompar’s band comparison grouping analysis with the Ward clustering method and a Jaccard coefficient, biovar one and biovar two isolates were placed in the same clade. Thus we conclude that while the rep elements are excellent at producing DNA fingerprints, the rep elements appear to be a poor method for deducing the phylogeny of Agrobacterium.

In many of the galls, more than one strain was isolated. Indeed, from each gall, we isolated from one to five different strains. Nonetheless, as expected, the same strain was isolated at least twice from 46% of the galls. In addition, in 47 cases, the same strain was isolated from different galls in the same field. The same apparent strain was only isolated from different locations in five cases. In one case, we isolated the same strain one time from each of two growers in the Sutter-Yuba area. In four separate cases, we isolated the same strain one time from each of two nurseries in Stanislaus county.

Here, we classified a strain as having the same rep-PCR fingerprint and being in the same biovar. Thus, isolates in the same strain have similar chromosomal DNA. However, because agrocin-resistance is a plasmid-associated trait, isolates within a strain can be either agrocin-resistant or sensitive. From two galls, we had isolates with identical rep-PCR fingerprints, but differing agrocin-sensitivity. These data support the contention that rep-PCR is a profile of chromosomal DNA. In three additional cases, there were two isolates from different fields that had the same rep-PCR profile and the same biovar, but in which one isolate was agrocin-resistant and the other isolate was agrocin-sensitive.

Due to the surprising diversity in Agrobacterium strains, we cannot use this data to indicate whether or not there is transmission of strains from a nursery to a grower’s field. Nonetheless, an understanding of the diversity of strains is extremely important to McGranahan/McKenna’s Paradox Diversity crown gall project, in which the goal is to select walnuts with disease resistance. Overall, our results on strain characterization support the following conclusions. 1) As indicated by the chromosomal DNA fingerprints (rep-PCR), there is an incredible diversity of strains of Agrobacterium isolated from walnuts in California. In conjunction with the pathogenicity data presented last year, there is no evidence that there is a “super-strain” that is
causing the increased incidence and severity of disease on walnuts. 2) Experimentally induced galls on walnuts appear to develop as clusters of many small galls, with each individual gall having a limited maximal size. Because galls frequently contain multiple strains of Agrobacterium, our current hypothesis is that the massive and “aggressive” galls seen in cases with severe disease are actually caused by multiple infections around the crown region, rather than by a single infection by an aggressive strain. 3) According to our plate assay, resistance to the biocontrol agent K84 is extremely common, even in areas in which the isolates were never exposed to K84. 4) Based on the REP-PCR data, there is no evidence that K84 has become a pathogen.

2. Assessment of our epidemiological model. In our grant proposal, we proposed the following hypothetical model. In this scenario, Agrobacterium has an asymptomatic stage of replication, in which the bacterium survives on the root and crown surface; the root surface is called the rhizoplane. We postulated that in nursery production, with dense plantings, trees adjacent to trees with galls might have a greater probability of having a rhizoplane population than trees that have no disease incidence amongst their neighbors. Furthermore, in this scenario, survival of Agrobacterium in soil would be correlated with survival of previous host-biomass. We noted that fumigation in a nursery may not completely disinfect soil if there is a considerable population of Agrobacterium on deep, infested roots that were left after the last harvest. In this model, new, uninoculated tree roots become infested when they grow adjacent to infested biomass either from a previous susceptible crop that still harbors the pathogen, or from the roots of an adjacent tree in the nursery that is either infested or infected. Once inoculated onto a new plant, the bacteria would either swim along the host surface, or conceivably move via mass flow, and form colonies in new areas, including on the crown region. We note that in this hypothesis, the bacterium primarily grows as an rhizoplane inhabitant, and infection in the field (i.e., gall formation), generally only occurs on very susceptible tissue, i.e., on some modestly wounded tissue, on very young tissue (seedlings or rootlets), or on transplants. Conditions in the nursery that may favor replication of the pathogen may include free water along the below-ground plant surface, dense plantings of susceptible tissue (i.e., much free food), and relatively large inputs of nitrogen. After harvest from the nursery, the trees are in a highly susceptible state, and the epiphytic populations on the crown surface cause multiple galls infections around the crown. We note that if this model is true, it suggests numerous opportunities for disease control strategies, which we can pursue in further research.

To address this hypothesis, we used a lux-tagged isolate that bioluminesces to demonstrate that Agrobacterium multiplies and spreads on the Paradox root surface, called the rhizoplane. On walnut trees in the lath house, the crown region was wounded five cm below the soil line on the tap root and inoculated with 10⁷ cells. After 0, 15 and 30 days, the Agrobacterium cells on the root surface were assessed with a luminometer. After 15 days, we detected an increased population in the crown region of the tap root. In addition, cells were detected in the tertiary roots near the crown and on the middle of the tap root. After 30 days, the population on the crown region of the tap root and the middle region of the tap root appeared to be maintained at the 15 day level. In addition, at 30 days, cells were detected on the tertiary roots near the root tip. The data indicate that Agrobacterium can multiply on the Paradox rhizoplane in a situation in which no visible galls are not present. We have correlated luminometer readings with
spectrometric measurements of the Agrobacterium population, and will shortly be able to correlate the luminometer readings with the cell population.

We have a field experiment in progress in which the objective is to determine whether trees in a nursery adjacent to trees with a gall are in "hot spots" and thus more likely to have an rhizoplane population of Agrobacterium than trees that have no neighbors with galls. We obtained 280 trees that were located next to a culled nursery tree with a gall, as well as control trees that were not adjacent to a tree with a gall. The trees were stored in saw-dust beds. Before transplanting on March 15, 1999 at the University of California at Davis in a field with no history of crown gall, one-half of the trees in each treatment were wounded in the crown and roots. The trees were planted in a randomized block design. The trees will be removed from the field in fall 2000 and assessed for the presence of gall.

As noted in our report from last year, cultural practices vary amongst nurseries. Overall, the data from our collections and field observations support the conclusion that most nurseries in California effectively control crown gall. Our hypothesis is that if a grower purchases a tree without a rhizoplane population of Agrobacterium, and then plants into a field that does not have a history of crown gall, that the endemic population of Agrobacterium in the grower's field will cause little or no crown gall, and will have minimal impact on tree health. We further postulate that much of the severe crown gall in California can be controlled by maintenance of an effective control program at the nursery-level. We are continuing experiments to elucidate the epidemiology of crown gall, and to determine cultural practices that can be used, particularly in a nursery, to reduce the potential of transmission from nursery to grower.

3. An initial assessment of the impact of crown gall on tree health. In Gale McGranahan's "reservoir block" at the University of California at Davis, we selected 10 trees with crown gall and 10 control trees without crown gall. The trees were removed with a backhoe. The region through the crown was sliced into 15-cm-thick cross sections, sanded, and observed for wood rot and vascular disorganization in the xylem tissue. There was no wood rot observed in any of the sections in any of the trees. There was relatively little vascular disorganization in the trees with galls, and no differences were detected in the amount of vascular disorganization between trees with and without galls. While our initial assessment of the impact of crown gall on tree health was negative, we note the following. Some of the trees that we rated as "non-galled" may have had galls that dried up and fell off. Thus, the vascular disorganization that was observed may have actually been in only galled trees, rather than in both galled and non-galled trees. Although the sample size was small, and we did not measure yield, we saw no obvious impairment of tree health in these cases with gall.

LITERATURE CITED


