DEVELOPMENT OF A POLYMERASE CHAIN REACTION (PCR) PROTOCOL TO DETECT THE DEEP BARK CANKER PATHOGEN, *ERWINIA RUBRIFACIENS*, IN WALNUT TISSUE

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Abstract

Contaminated graftwood is a suspected means by which deep bark canker disease is spread. To investigate this possibility, and ultimately to develop a source of bacteria-free budwood from which to produce healthy trees, a highly sensitive method to detect the pathogen in walnut tissue is needed. A procedure known as polymerase chain reaction (PCR) would meet this requirement. The subject of this project is to develop a PCR protocol to detect the deep bark canker bacteria in walnut tissue. The research has progressed, and an important step, the ability to get reproducible results using two primers, has been achieved but an applicable system has not yet been attained. Deep bark canker was found recently on Chandler trees in one orchard in Kings County and three orchards in Tulare County. To estimate the relative susceptibility of Chandler to deep bark canker, 6-yr-old Hartley and 7-yr-old Chandler trees in separate orchards in Kings County were inoculated with three isolates of the deep bark canker pathogen. Average canker length was 34.1 inches on Chandler and 13.1 inches on Hartley. Similar inoculations of 11-yr-old Hartley, Chandler, Sinensis and Vina trees in a planting in Davis indicate that the disease is severe on Hartley, Chandler and Sinensis cultivars. The number of trees with deep bark canker increased from 4 to 14 and from 6 to 11 in each of two Chandler orchards in Tulare County mapped in 1993 and 1994.

Introduction

Deep bark canker of English walnut is caused by the bacterium *Erwinia rubrifaciens*. Although the disease is most prevalent on cultivar Hartley, most cultivars are susceptible and may develop symptoms when placed under exceptional stress such as inadequate water, hot summers, or poor soil. Chemical protection against deep bark canker is not presently, and most likely will not be, available.

The disease may be spread by mechanical harvesters if contaminated shaker pads inflict deep wounds in the bark. Also, previous research indicates that the pathogen may be transmitted via the graftwood. In the latter case, graftwood taken from infected trees, whether these trees displayed external symptoms or not, would result in symptomless but infected trees being distributed through nursery sales. Because trees ordinarily do not express active external symptoms of the disease until after they mature and bear fruit, the importance of deep bark canker in new releases is difficult to predict.
A method to detect low populations of the pathogen in symptomless tissues used for propagation could be useful in development of a bacteria-free program to produce healthy trees. The polymerase chain reaction (PCR) procedure is such a method. PCR is an in vitro enzymatic procedure in which a specific segment of a genome is exponentially amplified. It is possible to amplify a discrete DNA fragment many million-fold. Previous research has shown that PCR has allowed the detection of 10-12 cells per milliliter.

The recent appearance of deep bark canker in several cultivar Chandler orchards prompted the questions of relative susceptibility of Chandler and Hartley trees to this disease and the potential for damage to Chandler trees. Cultivar susceptibility was investigated by Kado et al. in 1974-75, and among those tested, cv Sinensis was the most susceptible, Hartley very susceptible and Vina nearly resistant based upon calculated daily rate of canker expansion. Chandler was not included in that experiment. In addition to an intrinsic level of susceptibility, the rate and amount of disease spread within infected Chandler orchards may further hint at the potential for serious outbreaks of the disease in this cultivar in the future.

Objectives

The original objectives of this project were:

1. Develop, test and standardize a polymerase chain reaction (PCR) protocol for *E. rubrifaciens* in vitro and in walnut tissue.

2. Examine buds and the resulting shoots of naturally-infected Hartley walnut trees for presence of *E. rubrifaciens*.

3. Test buds as entry sites for *E. rubrifaciens* after artificial inoculation.

4. Test random infection of walnut by *E. rubrifaciens*.


The following objective was added in 1994.


**Objective 1. Development of the PCR system**

We have been developing a PCR procedure for detection of small amounts of *E. rubrifaciens* in walnut tissue. Specific primers which are homologous to the discrete fragment are required in PCR to prime DNA synthesis. For this purpose we have tested a series of degenerate and non-degenerate primer pairs which would amplify different genes (rcsA, hpf, hrgD or hrgN) from the
*E. rubrificiens* genome. In preliminary experiments, based on the yield, the size and the specificity (as determined by Southern hybridization) of the amplification products, we have selected the *hrpN* gene for further study.

To date we have tested four different degenerate and four different non-degenerate primer pairs whose sequences were derived either from amino acid alignments of different *hrpN* gene products or from the nucleotide sequence of this gene from *E. amylovora*. Although we obtained encouraging results regarding the size and specificity of the amplification products, the cloning of the gene from *E. rubrificiens* has not been possible thus far because either the yield of the PCR product was low, or the PCR reaction yielded a population of different molecules (among which were *hrpN*-related PCR products). To facilitate our selection for *hrpN*-related PCR products for cloning and subsequent sequencing, we have recently obtained a plasmid clone containing the *hrpN* gene from *E. amylovora*. This plasmid will be used as a probe to identify *hrpN*-related PCR products and clones from *E. rubrificiens*. Sequencing of these clones will permit us to select specific primers which will be used in a nested PCR format for the detection specifically of *E. rubrificiens* in walnut material with low level infection.

Reproducible results have been obtained using one set of primers. This should permit completion of the PCR-development phase of the project to proceed rapidly.

**Objectives 1 through 4**

These objectives were dependent upon development of the PCR system thus aside from work on the PCR, research on these objectives was not initiated.

**Objective 5.** Monitor development of deep bark canker in Chandler orchards in Tulare and Kings Counties.

**Tulare County.** Two Chandler orchards (Roberts and Whitendale) in Tulare County were mapped for location of deep bark canker-infected trees on 18 October 1993 and 17 November 1994. Trees in the Roberts and Whitendale orchards were planted in 1982 and 1983, respectively. Infections in the Roberts orchard did not appear to be associated with injuries whereas many trees in the Whitendale orchard bore harvester injuries and deep bark canker infections often were associated with these injuries.

There were 4 and 14 infected trees in 1993 and 1994, respectively in the Roberts orchard and 6 and 11 infected trees in 1993 and 1994, respectively in the Whitendale orchard.

**Kings County.** One Chandler orchard (Vandergraaf orchard), planted in 1987, was mapped 9 December 1994 and 15 infected trees were found.

**Objective 6.** To compare canker expansion on Hartley, Chandler, Sinensis and Vina trees inoculated with pathogens isolated from naturally-infected Hartley and Chandler trees.

Experiments were conducted at Davis and in two orchards in Kings County.
**Inoculum** - One and two isolates, respectively, of *E. rubrifaciens* were obtained from naturally-infected Chandler and Hartley trees grown in Kings County. Bacteria for inoculum were grown on yeast dextrose peptone calcium-carbonate agar for 72 hours then washed from the culture plates with sterile phosphate buffer. The concentration was adjusted to approximately $10^6$ cfu/ml by serial dilution, and inoculum was kept on ice in the field while in use.

**Inoculation** - Trees were inoculated by inserting a large darning needle through the bark tangentially to create a small hole next to the cambium. Approximately 0.05 ml inoculum was delivered into the wound with a sterile hypodermic needle. Inoculation sites were located on the trunk or primary scaffolds and were wiped with 10% bleach before each wound was made. The darning needle was wiped with a cloth soaked in 10% bleach then the needle was immersed in 70% ethyl alcohol between each wounding event. Noninoculated controls were injected with sterile phosphate buffer. After inoculation, wound sites were covered with 2.0 by 2.0 cm squares of duct tape. A month later the duct tape was removed and each inoculation site identified with colored paint and a number.

**Experiment at Davis** - Walnut trees planted in 1983 in a cultivar selection block at University of California were used in the experiment. Four each Hartley and Sinensis and three each Chandler and Vina trees were inoculated on 24 May 1994. Trees in each cultivar were arranged in a 2 X 2 group. Two each Hartley, Sinensis and Vina trees and one Chandler tree were on *J. hindsii* rootstocks, other trees were propagated on Paradox hybrid rootstocks. All the Hartley trees had external symptoms of deep bark canker. We placed the inoculation sites such that extension from them could be distinguished from natural infections. None of the other test trees showed symptoms of the disease at the time of inoculation. There were two inoculation sites for each inoculum source and the noninoculated control on each tree.

The Chandler trees had thick, hard bark with numerous growth cracks and was difficult to penetrate with the darning needle whereas the bark of the other three cultivars was smooth, relatively thin and easily penetrated with the darning needle. The Chandler and Vina trees were located in an area of poor water penetration: water was present around these trees when none was apparent near the Hartley or Sinensis trees.

Canker extension was measured on 8 November 1994. Bark was removed with a draw knife along the path of necrosis emanating from the inoculation sites and the vertical length of the necrosis upward from the point of inoculation was measured. Cankers that exceeded 100 cm in length were scored as ‘greater than 100 cm in length’ because the ends of most such cankers could not be reached.

Canker extension exceeded 100 cm in length at most inoculation sites on all Hartley and Sinensis trees, all inoculation sites on one Vina tree, and all inoculations sites on all Chandler trees (Table 1). The extensively infected Vina tree had died recently of crown rot. The stress of the crown rot (perhaps a simulated physiological ‘drought stress’ brought on by the root rot) probably rendered the otherwise tolerant tree susceptible to deep bark canker. Canker length on the two healthy
Vina trees averaged 13.4 cm. The infections on the Chandlers appeared to be as aggressive and extensive as on the Hartley and Sinensis. No differences among inoculum sources were detected. On Hartley, three inoculation infections could not be distinguished from natural infections. One noninoculated control site on one Sinensis and the dead Vina tree were infected.

Data from most inoculations could not be analyzed because numerical values were not assigned to cankers. For cankers shorter than 100 cm, too few replications remained to allow valid statistical analysis to determine differences in canker length among the three isolates. Generally, among those shorter than 100 cm, the cankers from the Chandler isolate tended to be shorter than those from the two Hartley isolates (Table 1).

Kings County- Five apparently healthy 7-yr-old Chandler trees in a commercial orchard (Vandergraaf Orchard) were selected by the grower as trees destined to be removed when the planting density would be reduced. The trees were not adjacent but were within four rows and 10 trees of each other. Five adjacent apparently healthy 6-yr-old Hartley trees located in an outside row of a commercial orchard (Danelle Orchard) were used. The trees were generally low in vigor and water penetration was poor in the orchard.

Inoculum preparation and inoculation were as described above. There was one inoculation site for each inoculum source and the noninoculated control on each tree. Chandler and Hartley trees were inoculated on 23 May and 17 June, respectively, and disease evaluated as described above on 14 November 1994.

The end points of all cankers could be found so all were measured. Overall average canker length was 89.6 cm on Chandler and 33.4 cm on Hartley trees (Table 2). Generally, canker length was greater when the isolate source cultivar and test host cultivar were the same. On the Chandler test host, the average canker length from the Chandler isolate was significantly longer than from one Hartley isolate and numerically longer than that of the second Hartley isolate. On Hartley trees, the canker length from the Hartley isolates were significantly longer than that of the Chandler isolate. Similar results were obtained when rates of canker expansion (mm/day) were compared. All noninoculated control sites were healthy.

There were five single-tree replications of each treatment arranged as a randomized complete block. Means were separated by Duncan's multiple range test.

Discussion

Chandler clearly is susceptible to deep bark canker, and extensive cankers can form in one season. The conditions of the trees in each orchard likely affected the rate of canker expansion and extent of infection. The Chandler and Hartley trees in Kings County are not in the same orchard so the two cultivars in this situation cannot be fairly compared for relative susceptibility to deep bark canker. The trees at Davis were purposefully stressed by withholding irrigation for much of the summer. Thus, disease development was encouraged in trees there whereas the Kings County orchards are commercial operations and trees were not purposely stressed. This perhaps explains, in part, why disease was generally more severe in the Davis than in the Kings County orchards.
The differences in canker length found in infections from bacteria from Chandler and Hartley sources suggest possible differences in pathogenicity.

The number of infected trees increased from 1993 to 1994 in both mapped orchards in Tulare County. The pattern of infected trees appears to be generally random in all mapped orchards although half of the infected trees in the Tulare County Roberts orchard are located in edge rows.
Table 1. Relative susceptibility of four English walnut cultivars to three isolates of *Erwinia rubrifaciens*, Davis, 1994.

<table>
<thead>
<tr>
<th>Inoculum source$x$</th>
<th>Sinensis$y$</th>
<th>Hartley$y$</th>
<th>Chandler$z$</th>
<th>Vina$z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chandler</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Hartley A</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Hartley B</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average length of cankers less than 100 cm long, cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chandler</td>
</tr>
<tr>
<td>Hartley A</td>
</tr>
<tr>
<td>Hartley B</td>
</tr>
<tr>
<td>Average</td>
</tr>
</tbody>
</table>

$w$ Canker length measured 8 November 1994, 168 days after inoculation. All trees 11 years old.

$x$ Trees inoculated 24 May 1994, approximately 0.05 ml of $10^6$ cfu/ml suspensions of each isolate of *E. rubrifaciens*.

$y$ Total of eight inoculations per treatment, two per each of four trees.

$z$ Total of six inoculations per treatment, two per each of three trees. One Vina died this summer of root rot (on Black rootstock), and all cankers on this tree-2 each per inoculum source-were more than 100 cm long. Among the others, only one canker, inoculum source Chandler, was more than 100 cm long.
Table 2. Relative pathogenicity of three isolates of *Erwinia rubrifaciens* on English walnut cultivars Chandler and Hartley, Kings County, 1994.

<table>
<thead>
<tr>
<th>Treatment x</th>
<th>Canker length cm (inches) y</th>
<th>Canker expansion rate mm/day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inoculum source z</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(On Chandler)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chandler</td>
<td>98.6 (38.8) a</td>
<td>5.6 a</td>
</tr>
<tr>
<td>Hartley A</td>
<td>76.7 (30.2) b</td>
<td>4.4 b</td>
</tr>
<tr>
<td>Hartley B</td>
<td>84.6 (33.3) ab</td>
<td>4.8 ab</td>
</tr>
<tr>
<td>(On Hartley)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chandler</td>
<td>20.1 (7.9) b</td>
<td>1.3 b</td>
</tr>
<tr>
<td>Hartley A</td>
<td>41.7 (161.4) a</td>
<td>2.8 a</td>
</tr>
<tr>
<td>Hartley B</td>
<td>39.9 (15.7) a</td>
<td>2.7 a</td>
</tr>
<tr>
<td><strong>Average all isolates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>On Chandler</td>
<td>89.6 (34.1)</td>
<td>4.9</td>
</tr>
<tr>
<td>On Hartley</td>
<td>33.4 (13.1)</td>
<td>2.3</td>
</tr>
</tbody>
</table>

x Trees inoculated with 0.05 ml of $10^6$ cfu/ml suspensions of each isolate. One inoculation site for each isolate and non-inoculated control on each of five trees. Five each 7-yr-old Chandler and 6-yr-old Hartley trees inoculated 23 May and 17 June 1994, respectively.

y Cankers measured 14 November, 175 and 150 days after inoculation of Chandler and Hartley, respectively.

z One and two isolates, respectively, of *E. rubrifaciens* obtained from naturally infected Chandler and Hartley trees (Hartley A and B).