USE OF A POLYMERASE CHAIN REACTION SYSTEM (BIO-PCR) FOR DETECTION OF 
ERWINIA RUBRIFACIENS IN TISSUES OF WALNUT TREES

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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 was to complete development of a PCR protocol to detect the deep bark canker bacteria in walnut tissue. Primers derived from the DNA sequence of the regulatory gene for exopolysaccharide synthesis produced successful detection of Erwinia rubrifaciens in symptomatic and asymptomatic walnut tissue. Deep bark canker incidence increased during 1995 in Chandler orchards mapped in Tulare County.

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.

A method to detect low populations of the pathogen in symptomless tissues used for propagation could be useful in development of a program to produce bacteria-free trees. The polymerase chain reaction (PCR) procedure, combined with Southern blot analysis, 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. A refinement of the PCR method, known as bio-PCR detects living cells instead of dead cells and is the method used here.

The progress of the disease in cv Chandler orchards in Kings and Tulare Counties was assessed by orchard mapping. Deep bark canker on the highly susceptible cultivar Hartley was first observed in the same area, thus we perhaps can shed some light on the potential for development in
Chandler by following the progress of the disease in orchards. If an increase in deep bark canker in Chandler orchards seems to parallel that experienced in Hartley orchards at the beginning of the epidemic some 30 years ago, then we could better assess the potential risk of Chandler to be affected by the disease.

Objectives

1. Test and standardize the viable cell bio-PCR protocol for \textit{E. rubrifaciens} \textit{in vitro} and in walnut tissue.

2. Examine bark tissues of branches and current season shoots of naturally-infected and inoculated Hartley walnut trees for the presence of \textit{E. rubrifaciens} to confirm usefulness of the bio-PCR as a detection method.


Results and Discussion

Objective 1. Test and standardize the viable cell bio-PCR protocol for \textit{E. rubrifaciens} \textit{in vitro} and in walnut tissue.

The “bio” portion of the bio-PCR system entails culture of the test sample on agar medium. This allows any viable bacteria present in the sample to multiply thereby increasing the amount of DNA available for PCR analysis, and making detection of very small numbers of target cells possible.

The success of the “bio” portion depends upon providing the target organism, in this case, \textit{E. rubrifaciens}, conditions in which it can grow rapidly and well. The combination of agar medium, incubation time and temperature should favor the growth of the target bacteria. We tested 14 strains of \textit{E. rubrifaciens}, representing isolations from infected Hartley walnut trees in seven counties, on five media and determined that 28 hr incubation at 30°C on yeast sucrose peptone medium produced the fastest and greatest growth of \textit{E. rubrifaciens}.

We identified a set of primers which were used in the bio-PCR system to specifically detect \textit{E. rubrifaciens}. These primers were derived from the DNA sequence of the regulatory gene for exopolysaccharide synthesis (\textit{RcsA}). Our tests showed that, using these primers in the PCR process, DNA of \textit{E. rubrifaciens} was specifically amplified whereas DNA of \textit{E. nigrifluens}, (causative agent of shallow bark canker of walnut), \textit{E. quercina}, and \textit{E. amylovora} (Erwinia species closely related to \textit{E. rubrifaciens}) was not. In addition, PCR successfully distinguished the deep bark canker organism from other organisms cultured from infected or healthy walnut tissues.

Southern blot analysis was used to further increase the sensitivity of detection of \textit{E. rubrifaciens}. Using this technique, PCR products at a concentration too low to be detected visually on an agarose gel by ethidium bromide staining and UV irradiation, can be identified.
**Objective 2.** Examine bark tissues of branches and current season shoots of naturally-infected and inoculated Hartley walnut trees for the presence of *E. rubrifaciens* to confirm usefulness of the bio-PCR as a detection method.

**Naturally infected trees:** This was not done because the completion of the development of the PCR was not finished until fall. We chose to concentrate on testing tissue from inoculated trees remaining from the previous year’s work as follows.

We inoculated Chandler (23 May 1994) and Hartley (17 June 1994) trees in orchards in Kings County to assess susceptibility of each cultivar to strains of the pathogen (see Annual Report to the Walnut Marketing Board, 1994, for results). We collected samples from the resultant infections on these trees on 23 October 1995 and tested them using the bio-PCR system. Tissues with symptoms (symptomatic tissue) were necrotic areas taken from fresh, active lesions (Numbers 1 and 2). Asymptomatic tissue was located within three inches of visible symptoms (Numbers 3 and 4).

Current-season shoots also were tested. Symptoms on the Hartley trees were limited to the trunk thus we could not determine which scaffolds (and therefore which shoots) were most likely to harbor the bacteria. Shoots from Hartley trees were collected from scaffolds that seemed closest to visible disease symptoms (Numbers 5-8). Chandler scaffolds that had visible symptoms were pruned in winter 1994-95 to force new shoot growth. New shoots from these pruned scaffolds were selected for these tests (Numbers 9-12). In both cases, a 1-inch long section of bark from the entire circumference of the shoot, approximately 1 inch from the shoot base, was removed and tested.

Controls included bark from a healthy Chico walnut tree (Number 13) and a Mission almond tree (Number 14), water blanks (15-17), *E. rubrifaciens* isolate 9505, 10^1 and 10^3 cfu/ml (Numbers 18 and 19) and *E. rubrifaciens* isolate 9515, 10^1 and 10^3 cfu/ml (Numbers 20 and 21). All samples were surface sterilized before testing.

<table>
<thead>
<tr>
<th>Bark tissue from Hartley</th>
<th>PCR</th>
<th>Controls</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Symptomatic tissue-A</td>
<td>+</td>
<td>Chico walnut</td>
<td>-</td>
</tr>
<tr>
<td>2. Symptomatic tissue-B</td>
<td>+</td>
<td>Mission almond</td>
<td>-</td>
</tr>
<tr>
<td>3. Asymptomatic tissue-A</td>
<td>-</td>
<td>Blank-A</td>
<td>-</td>
</tr>
<tr>
<td>4. Asymptomatic tissue-B</td>
<td>+</td>
<td>Blank-B</td>
<td>-</td>
</tr>
<tr>
<td>5. Hartley shoot-A</td>
<td>-</td>
<td>Blank-C</td>
<td>-</td>
</tr>
<tr>
<td>6. Hartley shoot-B</td>
<td>-</td>
<td>Isolate 9505 10^1</td>
<td>+</td>
</tr>
<tr>
<td>7. Hartley shoot-C</td>
<td>-</td>
<td>Isolate 9505 10^3</td>
<td>+</td>
</tr>
<tr>
<td>8. Hartley shoot-D</td>
<td>-</td>
<td>Isolate 9515 10^1</td>
<td>+</td>
</tr>
<tr>
<td>10. Chandler shoot-B</td>
<td>-</td>
<td></td>
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<tr>
<td>11. Chandler shoot-C</td>
<td>-</td>
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<td></td>
</tr>
<tr>
<td>12. Chandler shoot-D</td>
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</table>
To assure that the pathogen would be present in tissue close to current season shoots and thereby increase the possibility that bacteria might move into the shoots, we inoculated Hartley trees at Kearney Agricultural Center. On 30 October 1995, approximately 0.05 ml of a suspension containing $10^6$ cfu/ml was introduced into the bark of branches just below the point of attachment of ten current-season shoots. Eight inoculations were made such that the inoculation sites encircled to emerging shoot. Five shoots were collected on 14 December 1995 and tested as previously described. *E. rubrifaciens* was detected in one of the five shoots tested indicating that the bacteria can move into potential budwood shoots. The remaining five shoots will be similarly tested in January 1996.

The bio-PCR system performs well and can be used to test walnut tissue for presence of the deep bark canker pathogen. Much remains to be learned about sampling procedures and this is the subject of another proposal.

**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 15 December 1995. 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, 14, and 18 infected Chandler trees in 1993, 1994, and 1995, respectively in the Roberts orchard and 6, 11, and 16 infected Chandler trees in 1993, 1994, and 1995, respectively in the Whitendale orchard.

**Kings County** - One Chandler orchard (Vandergraaf orchard), planted in 1987, has not yet been mapped for 1995.