

## RAPID IMMUNOLOGICAL TESTS FOR THE DETECTION OF PHYTOPHTHORA CINNAMOMI IN AVOCADO ROOTS

Jyh-Dar Lei and Karen Roberts

Twyford International Inc.

### I. INTRODUCTION

#### A. Project Objectives

The objective of the research is to evaluate the feasibility of Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of P. cinnamomi in avocado roots. We will prepare polyclonal antisera against fractionated antigens of P. cinnamomi and evaluate the feasibility of using polyclonal antiserum in ELISA.

#### B. Description of Project - Plan and Procedures

##### 1. Identification of proteins unique to P. cinnamomi

We will first prepare reference antisera to total proteins of P. cinnamomi and P. citricola. The protein profile of each fungus will be analyzed by SDS-Polyacrylamide Gel Electrophoresis(PAGE). By this procedure, fungal proteins are driven by an electric current and are separated into bands in a gel medium. The distance of the movement of each protein is dictated by its size. The protein bands will again be transferred to a membrane by electric current and allowed to react with each reference antiserum. A protein band which does not react with the heterologous antiserum is identified as unique to itself.

##### 2. Production of specific antiserum to P. cinnamomi

Rabbits will first be immunized with total antigens of P. cinnamomi. Unique proteins of P. cinnamomi will later be used to boost the production of specific antibodies. Theoretically, only the antibodies which are specific to the booster antigens will be produced by the rabbits. Therefore, only antibodies to the unique proteins of P. cinnamomi will be produced.

##### 3. Formulation and evaluation of ELISA test

A specific Enzyme-Linked Immunosorbent Assay (ELISA) is the goal of the project. In an ELISA, specific antibody immobilized on plastic microwells is allowed to react with the fungus in the sample. Subsequently, the specific antibody, chemically conjugated to an enzyme, is used to react with the immobilized fungus which was previously trapped by the antibody. Finally, the substrate of the enzyme is added to detect the presence of the enzyme. A positive reaction usually will result in a color change of the solution.

Antibody will be purified from the specific antiserum and conjugated to an enzyme. Combinations of different concentrations of antibody and enzyme-conjugated antibody will be evaluated to formulate the ELISA. Other fungi which may occur in the root zone of avocado trees will also be tested to characterize the specificity of the ELISA. Microplate-based, double-antibody sandwich ELISA (Clark and Adams, 1967) will be the principle format, although other formats will not be excluded.

#### 4. Detection of *P. cinnamomi* in container-grown trees

Container-grown nursery stock will be inoculated with zoospore suspensions of *P. cinnamomi*. Healthy and infected root samples will be taken over time and tested by ELISA and by the selective medium method (Ko, et al., 1978) .

## II. PROGRESS AND ACCOMPLISHMENTS MADE NOVEMBER 1, 1989-FEBRUARY 23, 1990

### A. Fungal Isolates and Antigen Preparation

Two avocado isolates of *Phytophthora cinnamomi* (ATCC32992, 32993) and one isolate of *P. citricola* (ATCC32994) were acquired from the American Type Culture Collection. *Phytophthora* was grown in liquid GYP medium (Hall, et al. . 1969). The mycelia were harvested by drawing the solution through a Buchner funnel and Whatman #1 filter paper with an aspirator. The mycelial masses were washed three times with 0.8% saline by centrifugation, pulverized with a glass tissue grinder and fixed with one percent glutaraldehyde for one hour at room temperature. The mycelia were then dialyzed in phosphate buffered saline to remove the fixative. Protein content was determined by the BCA (bicinchoninic acid) assay (Pierce Chemical Co., Rockford, IL).

### B. Production of Antisera

Rabbits were first immunized with the fixed mycelia at the protein content of 0.5mg. Three rabbits were injected with *P. cinnamomi* and one rabbit was injected with *P. citricola*. After four weeks, one rabbit each, primed with each fungal antigen, received a booster injection of SDS-solubilized protein extracts of each antigen. A test bleed was performed on the two rabbits ten days after the booster injection. The other two rabbits remained primed with *P. cinnamomi*, awaiting the injection of the unique protein bands which are currently in the process of being determined.

### C. Analysis of the Reference Antisera

Antisera from the first test bleed were analyzed with the indirect ELISA. Mycelial antigens (unfixed) diluted in coating buffer were allowed to coat plastic microwells. Serial dilutions of the antisera were applied to the microwells to react with the antigens. An enzyme-conjugated secondary antibody was applied to detect the rabbit antibody which reacted with the antigens. Initial data indicated that antiserum to *P. cinnamomi* had high titer, but did not discriminate between *P. cinnamomi* and *P. citricola* (Figure 1) . We expect that the antisera will be more specific when the other two rabbits were boosted with specific protein bands. The antiserum to *P. citricola* had very low titer which may be due to the poor response of the rabbit.

The protein profiles of *P. cinnamomi* and *P. citricola* were also analyzed by SDS-polyacrylamide electrophoresis (PAGE). Initial results demonstrated similarity between

two isolates of *P. cinnamomi* and several distinct bands between *P. cinnamomi* and *P. citricola*.

### **III. FUTURE PROJECT OBJECTIVES**

#### **A. Obtain a Antiserum of Higher Titer to *P. citricola***

Another booster injection of the *P. citricola* will be performed to produce a better antiserum. The antiserum will be used to probe the protein profiles of *P. cinnamomi* and to identify unique protein bands.

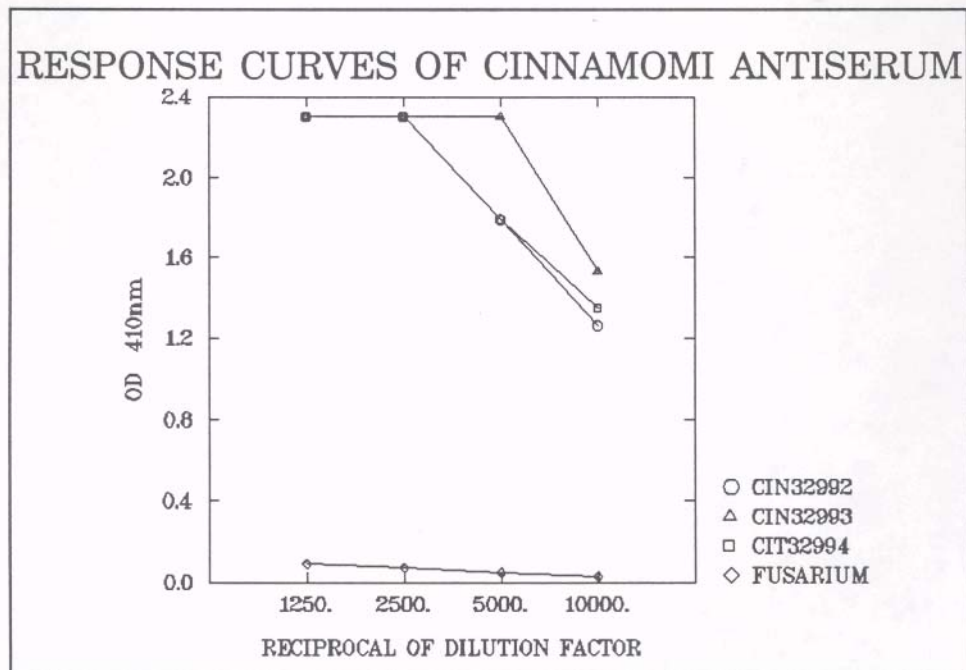
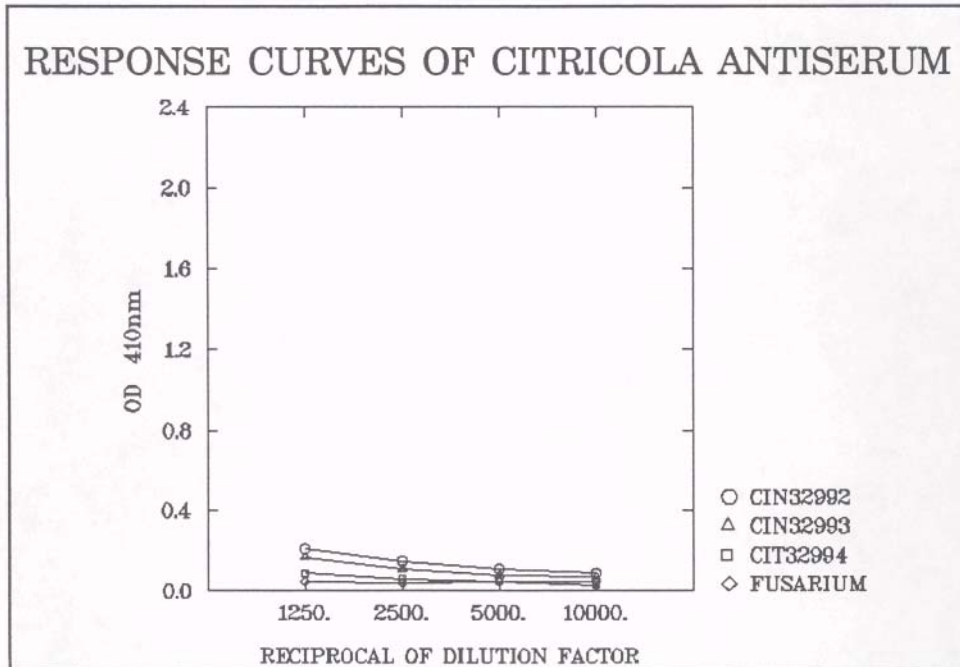


Figure 1. Response curves of *Phytophthora* reference antisera in indirect ELISA. Antigens were coated to ELISA plates at the protein content of 10ug/ml. CIN32992 and CIN32993, *P. cinnamomi*; CIT32994, *P. citricola*; FUSARIUM, *F. solani*.

### B. Identify Unique Proteins of *P. cinnamomi*

The immunological relationship of the proteins of the two fungi will be analyzed by

probing the protein blots with each antiserum. The bands which do not react with the antibody are considered unique and will be selected.

### **C. Produce Specific Antiserum and Formulation of ELISA**

Unique proteins, identified in the previous procedures, will be used for booster injection of the two rabbits which were previously injected with total antigens. ELISA reagents will be prepared from the antisera and formulated into a test.

### **D. Evaluation of Specific Antisera**

Test characteristics such as sensitivity, i.e. detection limit, and specificity, i.e. reaction with other soil fungi and different isolates of *P. cinnamomi* will be determined. Monoclonal antibodies to Phytophthora spp. will be obtained from Agri-Diagnostics Associates, Cinnaminson, N.J. Polyclonal antibody will be compared with the monoclonal antibody for their feasibility in double antibody-sandwich ELISA.

### **E. Detection of *P. cinnamomi* in Container-grown Trees**

If a specific ELISA can be formulated, the test will be evaluated for its ability to detect *P. cinnamomi* in avocado roots as outlined earlier.

## **IV. REFERENCES**

1. Clark, M.F., and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34: 475-483.
2. Hall, R., and Zentmyer, G.A., and Erwin, D.C. 1969. Approach to taxonomy of Phytophthora through acrylamide gel-electrophoresis of proteins. *Phytopathology* 59: 770-774.
3. Ko, W. H., Chang, H.S., and Su, H.J. 1878. Isolates of Phytophthora cinnamomi from Taiwan as evidence for an Asian origin of the species. *Trans. Br. Mycol. Soc.* 71: 496-499.