NEW HIGH-TECH METHODS TO SOLVE OLD DECAY PROBLEMS

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INTRODUCTION

This is an exciting time in history to be involved in agricultural research. Technology is opening up a whole new world that, even several years ago, we never dreamed possible. It is a world of high speed computers, geographic information system (GIS) web-based disease models, digital cameras, and powerful molecular techniques (DNA technology). DNA technology is especially useful and is now a major part of the research in fruit diseases at the Mid-Columbia Agricultural Research and Extension Center (MCAREC) in Hood River. Three years ago, I (Spotts) went back to school and took a hands-on course "Techniques on molecular and cellular biology". I followed up with a five-month sabbatical in Canada working in a molecular mycology lab with Peter Sholberg and Dan O'Gorman. About two years ago, the pear industry provided funding for a real time PCR machine. We already had machines for PCR, ELISA, and Fast Prep DNA extraction in the lab. This equipment has totally changed the kind of research that we are capable of doing.

PCR stands for Polymerase Chain Reaction, a method for amplifying DNA in a test tube. Four main ingredients are required for the PCR reaction. First, **target DNA** (template) is the specific fungal pathogen DNA. Second, **primers** are short pieces of DNA that are unique for each fungus and specifically bind to the target DNA. Primers are chemically synthesized. Third, **nucleotide precursors** (dNTPs) are the building blocks of the new DNA and include the bases adenine (A), guanine (G), cytosine (C), and thymine (T). Finally, **a DNA polymerase enzyme** is the engine that catalyzes the reaction. In real time PCR (qPCR), we are using a dye (SYBR® Green) that specifically binds to the amplified DNA and emits fluorescence in proportion to the amount of DNA in the sample.

Major decays of fruit in storage include blue mold (caused by *Penicillium* species), gray mold (*Botrytis cinerea*), mucor rot (caused by *Mucor piriformis*), bull's-eye rot (*Neofabraea* species), Phasidiopychnis rot (*Potyebniamyces pyri*), and side rot (*Phialophora malorum*). These, as well as other decays have been causing losses ever since the invention of cold storage. Losses are estimated to be several million dollars each year.

This paper gives an overview of what is happening in our lab at MCAREC using high tech methods and what this research means to the fruit industry.

Example 1. Biological control with CIM

Several years ago, we found a yeast (*Cryptococcus infirmo-miniatus* [CIM]) growing on the surface of a pear fruit in Yakima. We found that CIM controls many postharvest diseases of

apple, pear, and sweet cherry. For postharvest fungicides to be effective, a minimum residue on the fruit is required. Similarly, for CIM to be effective, a minimum concentration of viable yeast cells is needed on the fruit surface. While I (Spotts) was at the Summerland Research Center, I determined the nucleotide sequences for part of one of the CIM genes. Using this information, I was able to develop the primers required for the PCR reaction to determine the concentration of CIM DNA on the surface of apple and pear fruit following CIM application. This makes it possible for us to provide packinghouses with CIM "residue" information on the same day that the samples are collected.

Example 2. Predicting decay risk at harvest

The objective of this research is to predict the amount of gray mold and blue mold decay developing in fruit in long-term cold storage based on the surface populations at harvest of *B. cinerea* and *P. expansum* determined by either standard dilution plating or by qPCR analysis of ribosomal internal transcribed spacer region (ITS) DNA of the fungi. The project is being done in eight orchards in the Mid-Columbia district of Oregon and Washington and in six orchards in the Motueka district of New Zealand. Although the project is not complete, it appears thus far that the quantity of spores of *B. cinerea* on the fruit surface at harvest is a good predictor of decay. Prediction was successful when *B. cinerea* spore levels were determined with either standard dilution plating or rapid DNA analysis. While dilution plating results are not available for several days, DNA extraction and analysis can be completed the same day that the fruit are harvested. Spore numbers alone may not always accurately predict incidence of storage decay because fruit appear to vary from year to year in their level of internal resistance to decay. Thus, a test needs to be designed to measure the fruit resistance factor and include it in the prediction. However, using spore numbers on the fruit surface should allow ranking of orchards according to decay risk potential in any given year.

Example 3. Spore loads in packinghouse water and decay risk

The quantity of fruit infected in packinghouse water systems is related to the concentration of fungal spores in the water. In the past, this has been determined by diluting the water, then spreading a small sample on the surface of agar in a Petri dish. Colonies of specific decay fungi can be counted after about a week. Often, many nonpathogenic fungi and yeasts grow on the Petri dishes, making accurate counts difficult. We are using PCR primers for *M. piriformis* and *P. expansum* to measure the amount of DNA of these fungi in the packinghouse water. We have determined that sodium ortho-phenylphenate (SOPP), ethoxyquin, chlorine, and diphenylamine (DPA) do not interfere with the PCR. We still need to check the effect of flotation salts and fungicide residues on the PCR reaction. This qPCR test will enable packinghouse quality control (QC) personnel to assess the level of sanitation in the dump and flume water and make adjustments if necessary.

Example 4. Bull's-eye rot fungi and sensitivity to fungicides

The fungi that cause bull's-eye rot were first identified and studied in the Mid-Columbia area by United States Department of Agriculture (USDA) plant pathologist Jess Kienholz. Dr. Kienholz determined that two species of Neofabraea were involved and also caused anthracnose and perennial canker on apple trees. Recently, it was discovered that two additional species of Neofabraea cause bull's-eye rot. The species appear very similar to the original two, but using PCR, it is apparent that they have unique DNA. We are using a modified PCR test (multiplex

PCR) that enables us to identify all four species at the same time. It appears that one of the new species, *N. alba*, is common in the major production areas in Washington and Oregon. The other new species has not been named yet, but it is more common in the Medford area.

It is important to know which fungicides are effective against each of the four species of *Neofabraea*. We have developed a new, high-tech method to determine fungicide sensitivity. The standard method involves incorporating the fungicide into a fungal growth medium, adding the medium to Petri dishes, and then placing a piece of the fungus on the dish and measuring growth after several days. This technique is labor intensive and requires mountains of Petri dishes. The new, high-tech method uses a small ELISA plate with 96 wells. Each well is ¼-inch in diameter. This single plate replaces 96 Petri plates. In addition, growth of the fungus in the wells can be read in less than a minute with an ELISA plate reader while fungal growth on the 96 plates may take over an hour to measure.

Example 5. Gray mold (Botrytis cinerea) and sensitivity to fungicides

Recently, a study in France showed that *B. cinerea* is a more complex species than we thought and is made up of at least three groups. As with the *Neofabraea* species, each *Botrytis* group may have unique properties including sensitivity to fungicides. We have a collection of *B. cinerea* that includes over 1000 isolates from Wenatchee, Yakima, the Mid-Columbia, and Medford. We are in the process of using group-specific PCR primers to identify the group classification of each isolate. Following the group identification, we will test each isolate of *B. cinerea* for sensitivity to all of the commonly used postharvest fungicides with the ELISA plate test.

SUMMARY

We are using new DNA technology for rapid, accurate identification of pathogens and determination of their population levels, leading to development of disease risk models. We have developed an ELISA plate method to determine fungicide sensitivity of new species and groups of decay pathogens. This will lead to a customized decay control program for each unique pathogen complex. Finally, we can quickly disseminate information and risk models using web based technology. Can new, high-tech methods be used to solve old decay problems? Without a doubt, the answer is “YES”.

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