Update on Diagnostics for *Phytophthora ramorum*[1]

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Abstract

Diagnostics used by the U.S. Department of Agriculture (USDA), to determine absence or presence of *Phytophthora ramorum* in plant samples, are based on a combination of tests and depend on collaboration with external laboratories. The system currently in place attempts to maximize limited resources and at the same time provide the most scientifically defensible, accurate, and rapid diagnostics available. Described here is an overview of the current system, followed by a discussion of future plans.

Plant tissue samples originating from trace-forward or trace-back surveys, from national surveys, or from certification surveys of nurseries are processed by state Department of Agriculture laboratories or by National Plant Diagnostic Network laboratories. This processing usually includes ELISA pre-screening (which we strongly recommend) to determine the presence of *Phytophthora* spp. Samples that are positive on the basis of ELISA are then subjected to further testing. Laboratories have the option, either before or after ELISA testing, to try to culture the organism. Presumptive positive cultures are then to be sent to Dr. Mary Palm (National Mycologist, Beltsville, MD) for confirmation. Samples where *P. ramorum* was not isolated, or if they are ELISA positive and no culture was attempted, are to have DNA extracted, and the DNA sent to the National Plant Germplasm and Biotechnology Laboratory (NPGBL) (Beltsville, MD), for PCR analysis. This system is summarized in *table 1*.

The current system was designed with a number of factors in mind. First, there are limits on the number of samples that can be processed by individual laboratories or on a national basis. Steps need to be taken to try to limit the number of samples, such as triage by ELISA. Secondly, one of the major bottlenecks in the diagnostic system is likely PCR, and particularly preparation of DNA from samples. Thus, anything that can be done to minimize the number of samples that must have DNA prepared and subjected to PCR would assist in reducing laboratory diagnosis turn around time. Thirdly, while successful isolation of *P. ramorum* is diagnostic for the presence of the pathogen, failure to isolate it does not allow one to conclude that it is not present. Furthermore, it can be difficult to isolate the pathogen from certain hosts because there are potential but unknown effects of fungicides and

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environmental effects can impact isolation efficiency. Therefore, a sensitive molecular test should provide a more reliable indication or absence of presence of *P. ramorum*. It is recognized that PCR also has limitations, but we believe that it represents the best compromise possible, when considering the entire program.

Animal and Plant Health Inspection Service, Plant Protection and Quarantine (APHIS PPQ) utilizes two PCR tests. The first is the multiplex PCR developed by Oregon State University. Although we have validated this method, because it is relatively insensitive compared to the nested PCR test (developed at University of California, Berkeley), it is used as a quality assurance procedure to ensure that DNA received by the NPGBL is of sufficient quantity and quality to be amplifiable in the nested PCR test. Any DNA samples that do not meet these criteria are not tested, and we request a new sample. DNA that passes this test is subjected to validated nested PCR test. Appropriate positive and negative controls are included in all tests performed. The nested PCR, while very sensitive, is technically more demanding than typical PCR assays, is more susceptible to sample-to-sample contamination, and has relatively low throughput. Nonetheless, the only validated test available to USDA APHIS PPQ at present is this method. There may also be cross-reactivity with a limited number of other *Phytophthora* spp. (and perhaps only one). We have noted that it (*P. hibernalis*) produces an amplicon in nested PCR about 20 bp larger than that produced by *P. ramorum*, and this difference can be detected by trained eyes and good laboratory technique. While any test has some rate of false positive or false negative results, the diagnostic tests currently used are such that they will more likely provide a false positive rather than a false negative, allowing the opportunity to obtain additional samples in the case of important potential detections.

At the end of June/beginning of July 2004, a Science Panel was convened, and among several topics substantial time was spent considering *P. ramorum* diagnostics. The recommendations or conclusions of the panel included: 1) Improve communications within the regulatory community as well as to stakeholders regarding diagnostics and associated issues; 2) It would be highly desirable to have an additional diagnostic test(s) to obtain confirmatory results, particularly a test that is based on a different genetic locus than that currently used. Such a test would also need to have acceptable throughput; 3) Obtain better or more complete information on the accuracy of diagnostic tests, and determine the confidence limit, rate of false positive and false negative results; 4) Culturing of the organism, while definitive when successful, is inefficient and can lead to a high rate of false negatives under certain circumstances; 5) A potentially major source of error is related to sampling methods, not necessarily the diagnostic tests used.

Much remains to be done and the program has room for improvement and/or modification. APHIS is in the process of finalizing the validation of a Real-time PCR assay for *P. ramorum*. This test is based on the test currently used in the United Kingdom and developed by the Central Science Laboratory (York, U.K.). It is robust and sensitive, and has higher throughput than current APHIS methods. However, it is still based on an ITS region. Work is underway in a highly collaborative effort involving several United States, Canadian and United Kingdom laboratories to identify the next test that will enter the validation process. Work will also be initiated to determine the effects of fungicide treatments on *P. ramorum* detectibility and to determine the correlation between ELISA, culture, and PCR detection. Finally, efforts are underway to approve laboratories external to APHIS on a provisional basis to

perform the validated diagnostic tests. The process involves inspection of laboratory facilities, determining that the laboratories seeking approval have appropriate instrumentation and training, and ensuring the proficiency of these laboratories using blind test samples. (APHIS must also pass the proficiency panel.) The system that has been developed is very similar to analogous programs used by the National Animal Health Laboratory Network. It is hoped that this program will increase national capacity for *P. ramorum* testing and decrease the time needed to process samples. The goal is to have as many as 10-15 laboratories approved by March 2005.

Table 1. *Summary of the Federal Phytophthora ramorum diagnostic system.*

Culture		Nested PCR	Action
+	Or	+	Yes
-	And	-	No
Not required	n/a	-	No
Not required	n/a	+	Yes
-	And	+	Yes
+	n/a	(optional)	Yes