Identification of Control Agents and Factors Affecting Pathogenicity of *Phytophthora ramorum*[1]

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Abstract

A collection of 67 isolates of *Phytophthora ramorum* from the United States (U.S.), European Union (EU), and Canada was screened using differences in phenotypic traits (pathogenicity, growth rate at several temperatures, and sensitivity/resistance to metalaxyl, dimethomorph, and streptomycin) and for presence of cytoplasmic elements (dsRNA and plasmids). Results of these tests showed a high level of variation among *P. ramorum* isolates. Isolates which differed by +/- two standard deviations from the mean are being examined for presence of dsRNA viruses and DNA plasmids. Plasmids were extracted from some of these isolates and are being characterized. To date, no dsRNA viruses have been isolated.

Key words: *Phytophthora ramorum,* plasmid, dsRNA virus, cytoplasmic elements, pathogenicity.

Introduction

Several hosts for *Phytophthora ramorum* are present in forested and urban areas in Canada. These are primarily foliar hosts that can serve as potential reservoirs for *P. ramorum* inoculum. Establishment of *P. ramorum* on these hosts creates the risk of disease spread to more susceptible hosts in other locations, especially through the nursery trade. In 2004 the Canadian Food Inspection Agency (CFIA) confirmed the presence of *P. ramorum* in imported plants found at a number of retail garden centres in the Vancouver area. These sites tested negative in 2005 but became positive when tested again in September 2006. This was the only incident where *P. ramorum* was detected in Canada. The potential impacts of *P. ramorum* establishment in Canada are estimated to include direct and indirect losses to the horticulture industry and could jeopardize Canada's export trade for rhododendrons, which is valued at over $5 million annually.

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A collaborative project between the Canadian Forest Service – Pacific Forestry Centre and CFIA was initiated to further understand *P. ramorum* pathogenicity and explore potential control measures. The objectives of this research are 1) to examine a large collection of *P. ramorum* isolates from the U.S., Canada, and Europe and screen for the presence of dsRNA viruses, plasmids, and other cytoplasmic elements that affect pathogenicity and 2) screen and test the efficacy of chemical fungicides and biocontrol agents *in vitro* and on *P. ramorum* infected leaves of several plant hosts commonly found in British Columbia nurseries, landscapes, and forests. Preliminary results from the first objective are presented here.

Methods

Phenotypic Characters

Pathogenicity to detached *Rhododendron* leaves, growth rates at 2, 20, and 28 °C, and sensitivity to the fungicides Acrobat (dimethomorph) (0.3 ppm), Subdue Maxx (metalaxyl) (0.2 ppm), and streptomycin (110 ppm) were used to screen a collection of 67 *P. ramorum* isolates representing North American forests and nurseries and European nurseries. Isolates which differed by +/- two standard deviations from the mean values are being examined further for presence of dsRNA and plasmids.

dsRNA and Plasmid Isolation From Various P. ramorum *Isolates*

Mycelial suspensions were prepared by inoculating 500 ml 20 percent V8 (1 percent $CaCO_3$ w/v) with 15-20 mycelial plugs and grown for two weeks at 20-22 °C. Mycelia were harvested by gravity flow filtration and tissues were frozen and stored at -80 °C prior to extraction. To screen for dsRNA, Morris and Dodd's (1979) protocol using CF11 as well as modified protocols from Tooley and others (1989) and Newhouse and others (1992) are being tested. Protocols for plasmid extraction were adapted from Boeke and others (1985), which involves suspension in a sorbitol:EDTA:mercaptoethanol buffer, enzymatic digestion with zymolase (lyticase), and purification with HiPure Plasmid DNA purification kit (Invitrogen).

Results and Discussion

Phenotypic Characters

High variability in pathogenicity was observed among isolates, especially in those from Washington (WA) nurseries (fig. 1). Isolates from Europe (EU), Canadian (CDN) nurseries, and some from California (CA) nurseries were the most aggressive while those from CA forests were the least aggressive. A similar level of variability among isolates was seen in other phenotypic characters (Table 1) but none of these were correlated with pathogenicity (data not shown). Canadian nursery isolates showed low phenotypic variability, suggesting they may belong to the same clone.

Preliminary screening of isolates based on pathogenicity to *Rhododendron* and growth rates at various temperatures has shown great variability among both European and North American isolates. Isolates exhibiting high, moderate, and low pathogenicity are being examined for dsRNA viruses and plasmids. These will be compared to isolates behaving more within the normal range. If dsRNA viruses or

plasmids are detected that are associated with pathogenicity, RT-PCR methods will be developed to screen large numbers of isolates for these elements. In addition, controlled experiments will be conducted to elucidate the biological significance and the role of plasmids and dsRNA viruses in *P. ramorum* and their effects on pathogenicity.

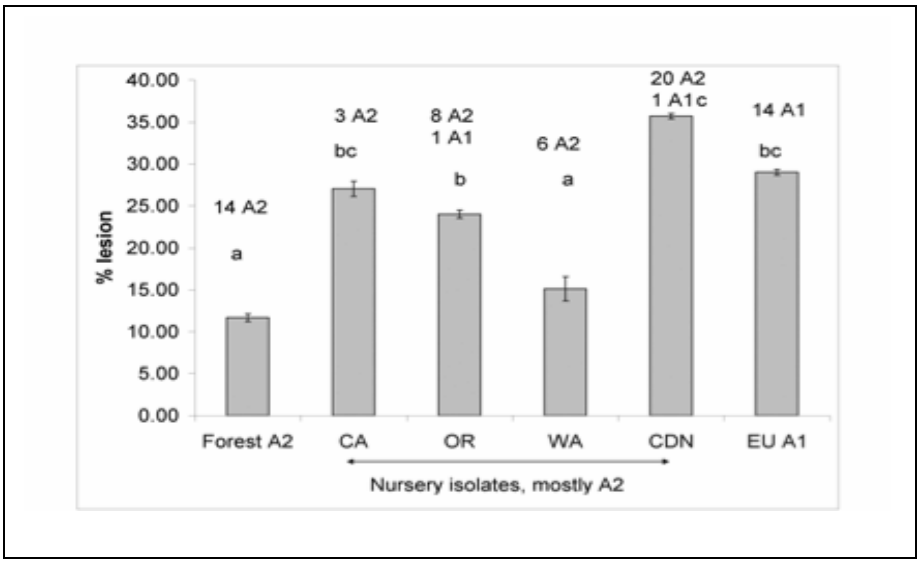

Figure 1.

Table 1—Further information about selected *P. ramorum* isolates. These isolates are being examined for dsRNA and plasmid DNA. Some isolates behaving within the normal range are included for comparison. Heat intolerant – slow growth at 28°C; cold intolerant – slow growth at 2°C.

Isolate number	Strain number	Origin	MT	Characters
0002	MSOD03-0002	Canada	A1	Heat intolerant
5041	Pr-102	OR, USA	A2	Heat intolerant, dimethomorph sensitive
5044	1033.1	OR, USA	A2	Streptomycin, dimethomorph sensitive
5047	4284	OR, USA	A2	Streptomycin resistant
5052	03-156-6	OR, USA	A2	Aggressive
5058	wsda4175	WA, USA	A2	Weakly pathogenic, cold intolerant, fast growing at 20°C
5060	wsda964	WA, USA	A2	Cold intolerant
5061	wsda1839	WA, USA	A2	Weakly pathogenic, cold intolerant, fast growing at 20°C
5063	Wsda3765	WA, USA	A2	Dimethomorph resistant
5064	Pr 0-4	CA, USA	A2	Streptomycin resistant, cold intolerant, fast growing at 20°C
5067	Pr 106	CA, USA	A2	Cold intolerant, heat intolerant
5073	RHCC-23	CA, USA	A2	Aggressive
5074	RHCC-4	CA, USA	A2	Dimethomorph resistant
5078	P1363	UK	A1	Metalaxyl resistant
5079	P1357	UK	A1	Dimethomorph sensitive
5084	CSL2266	Germany	A1	normal
5091	CSL1659	Germany	A1	Metalaxyl resistant
16207	SOD05-16207	Canada	A2	normal
16391	SOD05-16391	Canada	A2	normal
17017	SOD05-17017	Canada	A2	normal
18753	SOD05-18753	Canada	A2	normal

dsRNA and Plasmid Isolation From Various P. ramorum *Isolates*

To date, only the Canadian isolates have been screened for dsRNA. No bands nor viral-associated sequence data has been obtained. Work is ongoing to screen isolates for dsRNA using various protocols. Putative plasmids were found in 4 of five Canadian isolates tested as well as in five other isolates (1 from Germany, 1 from the United Kingdom, and 3 from the U.S.). The approximate size of the putative plasmid is between 14-20kb and the size appears to be variable between isolates. Putative plasmids were found in both A1 and A2 mating types. Work is ongoing to generate sequence information from the plasmid DNA as well as determining the exact size of the plasmids and performing restriction profiling. Further work will examine the relationship of plasmids to virulence and pathogenicity.

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