Assessment of the potential of *Phytophthora ramorum* to infect roots of red oak and balsam fir

by

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Abstract

To assess the susceptibility of roots of *Quercus rubra* (red oak) and *Abies balsamea* (balsam fir), to infection by *Phytophthora*, plants were grown in potting media infested with *P. ramorum* inoculum. Following the experimental period, necrosis, tissue vascular discoloration, and presence of the pathogen were evaluated. The susceptible *Rhododendron catawbiense* was used as a positive control. The European 1 (EU1) *P. ramorum* isolate resulted in significant root vascular discoloration in inoculated plants as compared to the negative controls for all species, in particular with high discoloration in rhododendron. There was no significant difference in the stem vascular discoloration in red oak and balsam fir. Detection of *P. ramorum* DNA using real time PCR was successful with the EU1 isolate for the inoculated rhododendron plants while mostly negative for red oak and balsam fir. Our results indicate that the roots of red oak and balsam fir appear to be weakly susceptible to *P. ramorum*.

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1. INTRODUCTION

1.1 Background

Phytophthora ramorum is a heterothallic fungus-like organism that belongs to the class *Oomycota*. *Phytophthora ramorum* causes sudden oak death mainly on trees in the Fagaceae family and ramorum shoot dieback and ramorum foliar blight on numerous woody horticultural species and shrubs (COMTF, 2003; Parke & Lewis, 2007).

Symptoms caused by *Phytophthora ramorum* were first observed on rhododendron in Germany and in the Netherlands in 1993 (Werres & Marwitz, 1997). At around the same time, the disease was observed in the wild on *Notholithocarpus* (formerly known as *Lithocarpus*) *densiflorus* (tanoak) and subsequently on *Quercus* species, leading to the term "sudden oak death" (McPherson *et al.*, 2000). Frankel (2008) reported that the causal agent was identified as a new and unnamed *Phytophthora* species in 2000. The pathogen was formally named *Phytophthora ramorum* Werres, de Cock, & Man in't Veld in 2001 (Werres *et al.*, 2001). In Canada, detection was first made in June 2003 (Hansen *et al.*, 2003; RAPRA, 2009) on imported rhododendrons in British Columbia.

With new developments each year and the list of hosts constantly expanding, the pathogen is now known to affect over 130 plant species, which comprise 75 plant genera in 37 different families (RAPRA, 2009). Such plant species as *Abies balsamea* (Balsam fir) and *Quercus rubra* (Red oak) are of importance both economically and in nature. Balsam fir is a

small to medium-sized tree that is among the more important conifers in Canada and in north-eastern United States. The species can be dominant in eastern Canada from Newfoundland and Labrador to northern Ontario and more scattered from Manitoba to Alberta. Balsam fir is mainly used for pulp, lumber and as Christmas trees (US Forest Service, 2012a). Red oak is a medium to large-sized tree that is found in eastern Canada from Nova-Scotia to southern Ontario. In the US, it is one of the more important lumber species of oak in the East. In addition to its importance as a commercial species, this species has been extensively planted as an ornamental and is an important food source for several animals (e.g. squirrel, deer and some bird species) (US Forest Service, 2012b).

In Europe, this pathogen had been found mostly on ornamental plants in nurseries. Outside of nurseries, the pathogen had also been found in a few managed gardens and semi-natural woodland areas on hardy shrubs as well as affecting a very small number of trees mainly in the UK and the Netherlands. In the US, the disease has been found in 14 California counties where a foliar host *Umbellularia californica* (bay laurel) on which *P. ramorum* abundantly sporulates was reported as being largely responsible for disease propagation in California (Davidson *et al.*, 2008). In Oregon, the disease is present mainly on tanoaks in one county in the south-west extremity of the State. Eradication efforts to eliminate the disease have been forsaken in California forests while quarantine area in Oregon forests has continually expanded since its first mention in 2001 (ODA, 2012; Rizzo *et al.*, 2005).

As a result of the disease and impacts caused, the US and Canadian authorities have enacted phytosanitary measures such as destruction of infected material, inspection, quarantine

and restriction of nursery stock movement in order to help eliminate or prevent the spread of the pathogen (APHIS, 2007).

1.2 Classification and characteristics

Phytophthora ramorum has been shown to possess a unique set of morphological characteristics that can be observed clearly on such laboratory media as V8-juice agar. The vegetative growth stage of *P. ramorum* consists of delicate, highly branched hyphae, as suggested in Figure 1. Optimal growth temperature for hyphae development has been determined to be 20°C (Parke & Lucas, 2008; Werres *et al.*, 2001). The most common reproduction for this organism is asexual in the form of sporangia or chlamydospores.

Figure 1: The characteristic growth of *Phytophthora ramorum* in culture showing the highly branched, delicate appearance of mycelia as seen on a culture plate (Left: Rizzo *et al.*, 2005) and hyphae on a cultured leaf sample (Right: www.apsnet.org).

Phytophthora ramorum sporangia are elongate and appear semi-papillate due to a short rounded tip. Sporangia of *P. ramorum* are significantly more elongate than those of its closest relatives. These sporangia are sympodial, with each of the successive sporangium developing on

a branch behind and to one side of the previous apex where growth has ceased. Sporangia, which measure 25-97 μm in length and 14-34 μm in width, are produced on a short pedicel and are caducous. Sporangia can either germinate directly or produce a large number of motile zoospores. At higher temperatures than optimal, such as 25°C, sporangia are more likely to germinate directly by the production of a germ tube (Parke & Lucas, 2008). Chlamydospores are important survival structures allowing the organism to endure extreme environmental periods, for instance associated with low temperatures, absence of host tissue or the presence of microbial antagonism. When conditions necessary to support vegetative growth are encountered, chlamydospores can grow germ tubes to create new colonies either vegetatively or through sporangial production and subsequent zoospore release. Chlamydospores also serve as a distinctive feature of *P. ramorum* as they are larger than those of their close relatives with an average size range of 40-80 μm. They are formed readily in V8 juice broth and agar. Chlamydospores produced by this oomycete are clear in color when they are young and become golden brown in older cultures. These chlamydospores are mostly terminal (Parke & Lucas, 2008).

1.3 Genetics and clonal lineages

Current infections by *P. ramorum* occur only in Europe and North America. Populations found on both of these continents are clonal and have been classified into three distinct lineages. Analyses based on population genetics have shown them to be distinct genetically as well as asexually reproducing, while yet still being clearly conspecific (Ivors *et al.*, 2004, 2006;

Prospero *et al.*, 2007). These three clonal lineages have thus been named as European 1 (EU1), North American 1 (NA1) and North American 2 (NA2). The European clonal lineage was the first to be discovered and identified in European nurseries on *Rhododendron* and sporadically on *Viburnum* since 1993 (Werres *et al.*, 2001). Although it is the only lineage found in Europe, it has now been regularly detected on the west coast of the US and in British Columbia, but mainly in nurseries (Grünwald *et al.*, 2008). There has been one report of the presence of the EU1 lineage in the wild after it was baited in a small stream in California in 2006 with a repeat detection in 2007 (COMTF, 2007). The NA1 clonal lineage is the one responsible for the natural spread and tanoak mortality in California and Oregon as well as many of the nursery infections in North America (Ivors *et al.*, 2006; Prospero *et al.*, 2007). The third clonal lineage, NA2, has been found to display a limited geographic distribution. According to Ivors and colleagues (2006), it has been isolated in a few nurseries in California and Washington in 2004. This lineage is apparently rarely found in California, but is more frequent in nurseries of Washington and British Columbia (Grünwald *et al.*, 2012).

There are two mating types assigned for *P. ramorum* isolates. The mating type of all NA1 and NA2 that have been isolated and tested has been determined as A2 while the EU1 is shown to be predominantly of mating type A1 (Brasier & Kirk, 2004; Werres & Kaminsky, 2005). However, identification of rare EU1 isolates of A2 mating type has been made in Belgium (Werres & De Merlier, 2003), but these isolates appear to have been completely eradicated (Grünwald *et al.*, 2012).

Phenotypic differences have also been observed among the three clonal lineages. The most obvious difference is the relative homogeneity among the EU1 isolates while in contrast the NA1 isolates show phenotypic variation and intrinsic instability. Variations in colony morphology, as well as vegetative growth rate among subcultures have demonstrated the intrinsic instability in NA1 isolates (Brasier *et al.*, 2006). Instability has also been suggested by the observation made of abnormal shaped sporangia in single colony segments and between subcultures (Werres & Kaminsky, 2005). Another factor of phenotypic difference is on the growth rates. The EU1 isolates have been shown to generally grow faster in culture accompanied with larger chlamydospores, though sometimes NA1 isolates growth rates can also equal that of EU1 isolates (Brasier, 2003; Brasier *et al.*, 2006; Werres & Kaminsky, 2005).

1.4 Hosts and symptoms

Phytophthora ramorum causes damage on over 100 plant species (Grünwald *et al.*, 2012), with the known host range continuing to expand. According to the RAPRA report (2009), over 130 plant species distributed in 75 plant genera and 37 different families have been reported as hosts for this pathogen.

Phytophthora ramorum has been shown to cause symptoms that can be placed into two classes consisting of the foliar phase or the bole canker phase. These symptom classes are considered to be separate and distinct.

1.4.1 Foliar phase symptoms

The foliar phase is mainly characterised by *P. ramorum* causing ramorum blight on foliar and twig hosts (Figure 2a). This form of disease is basically evidenced as shoot tip dieback, leaf necrosis or leaf spots. Hosts that have been shown to experience dieback include California bay laurel, Douglas fir (*Pseudotsuga menziesii*), evergreen huckleberry (*Vaccinium ovatum*), Pacific rhododendron (*Rhododendron macrophyllum*) and tanoak. Plant discoloration and wilting may also accompany the affected shoots. Lesions may also be found on the leaf tips, as is the case in California bay laurel where distinctive lesions are usually found on the downward pointing leaf tips where water usually puddles. Another evidence of the ramorum blight is water-soaked lesions on leaf margins and petioles which may become necrotic rapidly and spread to the whole leaf and the shoot. Sporangia are usually produced on the foliage and thus these hosts contribute to the spread of the pathogen (see e.g. review by Kliejunas, 2010).

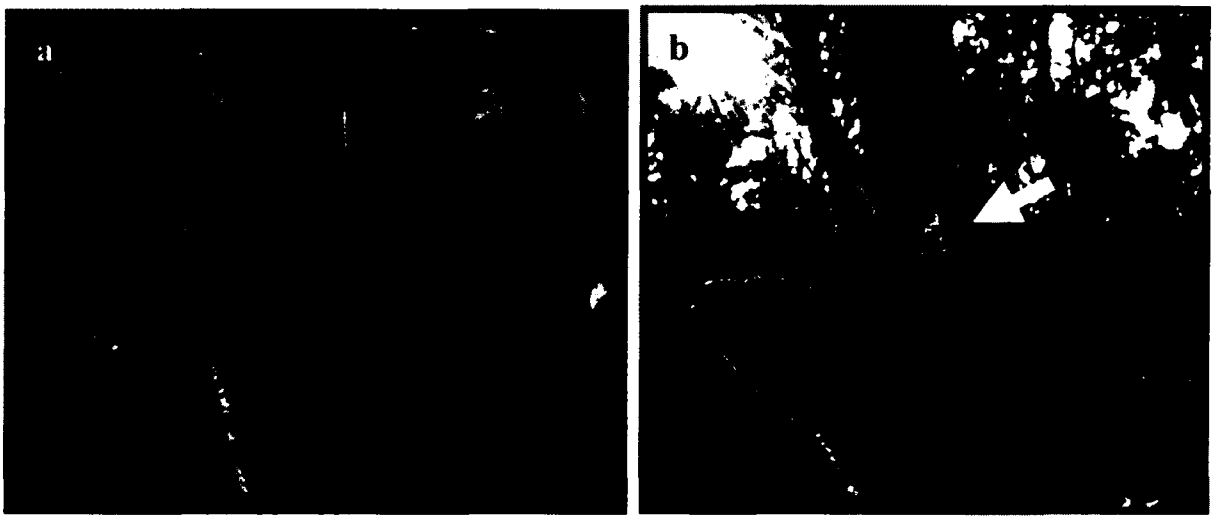


Figure 2: a) Leaf necrosis symptoms on California bay laurel caused by *Phytophthora ramorum* pathogen (courtesy: Grünwald *et al.*, 2008). b) Bleeding canker on the stem of tanoak tree caused by the *P. ramorum* pathogen (courtesy: D. Rioux, CFS).

1.4.2 Bole canker phase symptoms

The bole canker phase is characterized by bleeding cankers on the stem as depicted in Figure 2b. On such species as oaks, tanoaks and European beech, *P. ramorum* would cause bleeding cankers. Under the cambium, these cankers may only be visible after the removal of the bark. As a result of these cankers, the disease named sudden oak death has surfaced because these cankers cause oak mortality through the killing of the inner bark or phloem as well as girding of the tree. The pathogen usually colonizes the xylem and thereby reduces stem water transport (Parke & Lewis, 2007). This will often result in rapid wilting and death even though the plant might have been infected years before this apparent sudden death (Grünwald *et al.*, 2008). Except for tanoak, as it is often difficult to find spores on or around these cankers or even on their foliage, these trees are often considered as terminal hosts (Meentemeyer *et al.*, 2004).

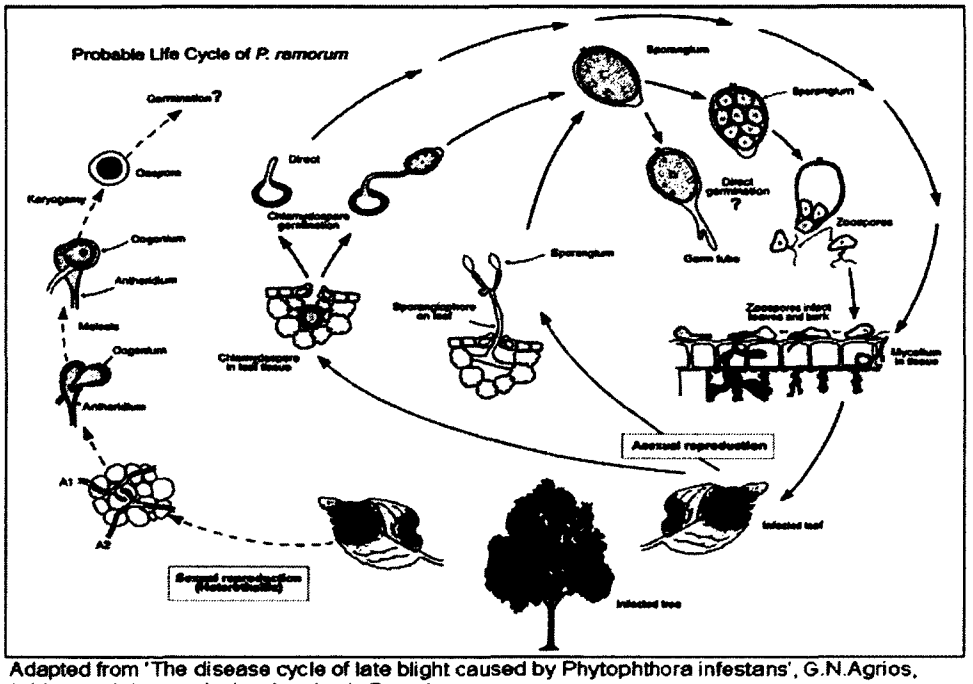
It should be noted that sometimes, infected material and plants may remain asymptomatic. For instance, sporulation of *P. ramorum* has been observed on asymptomatic leaves (Frankel, 2008) while another group (Parke & Lewis, 2007) has shown that rhododendron roots can remain asymptomatic while being infected from an infested potting media. Also plants can exhibit a wide range of susceptibility to *P. ramorum* by species as well as by cultivar within species (Grünwald *et al.*, 2008).

1.5 Dispersion and life cycle

The life cycle of *P. ramorum* is made of different stages where at each step the pathogen can still be dispersed through different media. A probable life cycle for *P. ramorum* is given in Figure 3. The pathogen usually produces sporangia on the surface of infected host plant foliage and twigs. These sporangia can then be dispersed to neighbouring hosts by splashing of water or be carried longer distances by wind and rain water (Davidson *et al.*, 2005). Dispersal by water has been shown where *P. ramorum* was baited from rivers and streams located nearby infected areas (Davidson *et al.*, 2005; Frankel, 2008). Inoculum dispersion can also be through soil or debris that is attached to boots of walkers and hikers as well as tyres when people move from infected area to a new location. Interestingly, in areas lacking *P. ramorum* hosts, a study indicated that the pathogen was present on trails whereas it was virtually absent off-trail (Cushman & Meentemeyer, 2005).

Once sporangia make contact with a suitable host, the sporangia can directly germinate or usually they release zoospores that will encyst and germinate on the host surface to initiate a new cycle of infection (Figure 3). Although *P. ramorum* mainly infect aerial plant parts, it has been shown to be able to survive in the soil and under snow cover (Fichtner *et al.*, 2007; Shishkoff, 2007) and has been recently shown to infect the roots of *Rhododendron* (Parke & Lewis, 2007). A preliminary report has also revealed that some eastern oak species in the US are susceptible to *P. ramorum* after exposing the roots to various zoospore concentrations for 24 h (Widmer *et al.*, 2010). *Phytophthora ramorum* readily produces chlamydospores in infected plant material, which can serve as resting structures for the pathogen thus allowing the pathogen to survive

severe conditions such as snow cover and may be important for survival in the soil (Shishkoff, 2007; Tooley *et al.*, 2008). Chlamydospores can also germinate, as depicted in Figure 3, to produce sporangia or can directly germinate and produce mycelium in host tissues. Because *P. ramorum* is heterothallic, sexual reproduction is theoretically plausible as shown in Figure 3. Oospores can be produced when two compatible mating types (e.g. A1 and A2) cross to give an oogonium that would produce these oospores. However, no such recombinants have been observed yet under natural conditions (Grünwald *et al.*, 2008) and even under special laboratory conditions, oospores are much more difficult to obtain than with other heterothallic *Phytophthora* species (Brasier & Kirk, 2004).



Adapted from 'The disease cycle of late blight caused by Phytophthora infestans', G.N.Agrios, (with copyright permission Academic Press)

Figure 3: A probable life cycle of *Phytophthora ramorum* showing the different stages involved in the asexual reproduction and sexual reproduction paths (source: http://rapra.csl.gov.uk/background/lifecycle.cfm)

1.6 Sudden oak death

Sudden oak death is a plant disease caused by *P. ramorum*. It is considered a threat not only to the biodiversity of native forests in North America, but also to the integrity of these forests due to its killing of oaks and other plant species. This disease was first observed around 1995 in California oak forests. The disease was first noted to be causing bleeding cankers on the tree stems and subsequently tree mortality on *Notholithocarpus densiflorus* (tanoak) and thereafter on *Quercus agrifolia* (coast live oak) in the coastal areas north and south of San Francisco (Rizzo *et al.*, 2005) and south western Oregon.

1.7 Economic impacts

Phytophthora ramorum can have several economic impacts, of which some have great importance. It has been shown to impact directly both the horticultural industry and the health of forest ecosystems. The emergence of this pathogen has brought to the forefront the complexities of balancing ecological and economic well-being (Rizzo *et al.*, 2005). The introduction of quarantine regulations has affected the ornamental nursery industries in both North America and Europe.

The nursery industry has endured costs due to the pathogen's regulatory impact, particularly for those nurseries that have been found to be infected with the pathogen (Goss *et al.*, 2011). Main costs for the industry occur when plants are destroyed, phytosanitary measures are implemented on the facility and sale opportunities are lost when plants are kept in quarantine.

Losses resulting from the impacts of *P. ramorum* in Washington State nurseries in USA as a result of destroying plant as part of the necessary phytosanitary measures for 2004 and 2005 have been estimated. Dart and Chastagner (2007) have estimated that plant destruction at 32 nurseries had a retail value of $423,043, with the most common plant genera involved being *Rhododendron* (89%), *Calluna* (4%) and *Camellia* (4%). Frankel (2008) concluded that there was a greater economic impact than just the value of the plants destroyed on the affected nurseries in Washington. In 2004, the USDA incurred $20 million costs in tracking and destroying all suspect stock after *P. ramorum* was found on two large southern California nurseries which had shipped to over 1200 nurseries in 29 different states potentially infected plants. Potential losses in southwest Oregon nursery industry as a result of *P. ramorum* are estimated to be between $79 million and $304 million per year (RAPRA, 2009).

1.8 Methods of detection

As a result of threat and impacts posed by *P. ramorum*, suitable, fast and accurate diagnostic tests are required so as to facilitate effective measures for the control, prevention of spread to non-infected areas as well as eradication of this pathogen. Interpretation of visual symptoms on host is usually the initial step. However, identification of *P. ramorum* based only on host symptoms is not enough due to variation in the expression of these symptoms based on the host as well as the range of other causes that can produce similar symptoms. Thus, a number of different tests are employed to help identify this pathogen in samples. These diagnostic tests include the conventional or traditional methods and the advanced molecular analysis methods.

One of the advantages of conventional methods such as culturing on media is that they are often cheaper to perform. However, such drawbacks as sensitivity and often taking longer time to complete where culturing is required makes them not desirable where rapid high throughput diagnosis are in need. Difficulty may also be experienced when making discrimination based on morphological characters only for organisms that are closely related.

On the other hand, nucleic acid-based diagnostic tests such as polymerase chain reaction (PCR) techniques provide the advantage of high sensitivity and specificity (Ward *et al.*, 2004). Confirmation of *P. ramorum* in a sample thus usually requires that the pathogen be cultured and identified through morphological characteristics and through DNA analysis by PCR.

1.8.1 PARP detection method

Plant tissues can be plated on selective media where the use of morphological characteristics allows for the identification of pathogenic oomycetes that grew in the media. *Phytophthora* species were known as being difficult to isolate from plant tissues until pimaricin was added to the selective media (Jeffers & Martin, 1986). A selective medium known as PARP is used for *P. ramorum*. This media contains pimaricin, ampicillin, rifampicin and pentachloronitrobenzene (PCNB). Pimaricin is a polyene macrolide antibiotic that suppresses almost all non-pythiaceuos fungi (Jeffers & Martin, 1986). The antibiotics in this medium, that is, ampicillin and rifampicin, as well as the antifungal agents PCNB and pimaricin are added to the media to prevent growth of competitive species of fungi and saprophytic bacteria (USDA, 2011) which would otherwise obscure and mask the morphological characteristics of pathogen in

the plate. However, isolation success has proven to be extremely variable as it may depend upon plant substrate and time of year. For instance, during dry summer months in California, the success rate for isolating *P. ramorum* on *U. californica*, one of the most susceptible hosts, drops by as much as four times (Garbelotto, 2003 reported in CFIA, 2009). Also the fungicides and antibiotics present in the selective media may have the possibility to suppress the development of hyphae from plant tissue (Davidson *et al.*, 2003).

1.8.2 ELISA

Enzyme linked immunosorbent assay (ELISA) is an immunodiagnostic assay. The main goal of immunodiagnostic assay is the detection or quantification of the binding of the diagnostic antibody with the target antigen. Detection of the resulting antibody-antigen binding involves coupling the antibody to an enzyme, which can act on another substrate and produce fluorescence. Thus ELISA makes use of an enzyme-mediated color change reaction as a way of detecting antibody binding. This assay is usually performed in a microtitre plate where the level of color change, which is normally measured in a computer-controlled plate reader, can be used as a way to determine the amount of pathogen present in the sample (Ward *et al.*, 2004). ELISA offers the advantages of being simple to perform, cheap and can process many samples at a time in one plate. For *P. ramorum*, ELISA is used as a pre-screen test especially useful when a larger number of samples need to be processed. A positive reaction indicates that a *Phytophthora* species is present in the sample and subsequent tests, using PCR for instance, are necessary to confirm the presence of *P. ramorum*.

1.8.3 PCR technique

PCR is a technique for rapid synthesis or amplification of millions of copies of specific DNA sequences. For *P. ramorum*, it has been shown to be the technique of choice for diagnostic purposes. This method involves steps where double stranded DNA is first denatured by heating to high temperatures of 95°C. To allow the binding of the two primers used to the target region being amplified, the temperature is lowered to a range of 40-66°C. The primers bind specifically to target DNA by complementary base pairing, therefore primers give the PCR its specificity. Extension from the primers, which usually occurs at a temperature of 72°C generates a second strand using DNA polymerase and deoxyribonuclease triphosphates such that at the end of the first cycle the number of target DNA is doubled. Thus, after many cycles, such as 25, a million copies of the sequence would have been produced. PCR takes advantage of the sequence divergence between *P. ramorum* and other *Phytophthora* species, for instance in the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (Tomlinson *et al.*, 2007).

Real time (RT) PCR is a variation of PCR method where DNA quantification is monitored as the products are being amplified. This method thus eliminates post-reaction processing that may be required for conventional PCR as the amplicons are detected by a built-in fluorescence as they accumulate. This detection is achieved by the use of non-specific DNA binding dyes such as SYBR Green, which are specific to the target DNA. The main principle for RT PCR is that when there is higher quantity of target DNA in the sample being tested, the reaction progresses faster and enters the exponential phase of amplification faster (Ward *et al.*,

2004). RT PCR methods that employ the use of TaqMan chemistry as is the case for *P. ramorum*, have the particular advantage of reduced risk of contamination because they require no post-amplification steps. As the PCR process occurs in a TaqMan reaction, the 5' exonuclease activity of Taq polymerase cleaves the dual-labelled probe, thus separating the reporter and quencher dyes causing an increase in the reporter fluorescence which is monitored in real time and the cycle threshold can be calculated. The cycle threshold (Ct) value is the cycle number at which a statistically significant increase in fluorescence signal is detected (Tomlinson *et al.*, 2005). In addition to *P. ramorum* specific primers and probes, RT PCR for detection of this pathogen involves the use of generic plant cytochrome oxidase (COX) specific primers and probes to detect plant host DNA. In this way, detection of host DNA provides confirmation of successful DNA extraction, thereby avoiding false negative results for *P. ramorum* (Tomlinson *et al.*, 2005).

1.9 Hypothesis and objectives

Although *P. ramorum* is generally considered as a pathogen that would spread through aerial plant parts, it has been recovered from soil and streams in infected area, in Germany from recirculating irrigation system in infected nurseries and also from container media, field soil and water from a pond at a nursery. In addition, this pathogen has also been recovered from the asymptomatic roots of rhododendron that had foliar symptoms and stem lesions (Parke & Lewis, 2007).

There is concern for the introduction and impact in eastern North America. Studies mainly conducted in the eastern North America have focused on mainly investigating foliage susceptibility, while potential for root infections remain poorly understood. A study by Parke & Lewis (2007) has experimentally shown that *P. ramorum* in potting media can infect rhododendron roots. This could present another pathway for the spread of this pathogen. This alternative pathway for spread of *P. ramorum* would be one that is not being currently tested in the process used for nursery stock certification (Parke & Lewis, 2007).

A study by Jinek *et al.* (2011) on foliage susceptible to infection by *P. ramorum* has shown the plant species *Quercus rubra* (red oak) and *Abies balsamea* (balsam fir) to be more susceptible to infection by this pathogen.

It is hypothesised that if *P. ramorum* were introduced in eastern North America, most of the infected leaves from the numerous potential hosts would drop to the ground in the fall season, survive winter under snow cover, and could have the potential to infect new plants in the spring through the root system.

The main objective of this study was first to determine whether plants native to Canadian eastern region, *Q. rubra* (red oak) and *A. balsamea* (balsam fir) grown in potting media infested with *P. ramorum* pathogen could become infected via the root system resulting in pathogen spread to parts above the potting mix surface. The second objective was to determine the pathogen aggressiveness of isolates representing the three clonal lineages of *P. ramorum* on detached *Rhododendron catawbiense* leaves.

2. MATERIALS AND METHODS

2.1 Plant material

The plant species chosen for the experiment were *Abies balsamea* (balsam fir), *Quercus rubra* (red oak). *Rhododendron catawbiense* cv. 'Roseum Elegans' was used as a positive control as it is known to be susceptible to *P. ramorum*. *Rhododendron roseum elegans* obtained from Phytoclone Inc. (Saint-Etienne-des-Grès, QC) were 2 years old and the *Quercus rubra* seedlings were in their first year of growth, the acorns having been provided by the Ministry of Natural Resources and Wildlife (Quebec City, QC). Balsam fir plants (Pepiniere Agrofor, Saint-Apollinaire, QC) per experimental treatment were in their third year of growth, except for two balsam fir plants per treatment in the last trial when using EU1isolate which were 5 years old and were obtained from the Ministry of Natural Resources and Wildlife (Quebec City, QC). Before being transported to Ottawa for inoculations, tree seedlings were grown in the greenhouse (Laurentian Forestry Centre, NRCan, Quebec City, QC) at temperature settings of 20°C day and 18°C night and a 14 h photoperiod. With the exception of the last trial which involved using EU1isolate, Plants were moved to a laboratory in Ottawa (see below) at least one week before the assays to help their acclimation to a new environment. Plants were in pots with soil mixture consisting of sand, peat moss and vermiculite (by volume, 3:1:1).

2.2 *Phytophthora ramorum* isolates

Isolates used in this study were chosen from the three clonal lineages (Table 1) and supplied from the culture collection of Plant Pathology Laboratory of the Canadian Food Inspection Agency (Ottawa, Ontario, Canada). To improve virulence, the isolates were inoculated onto detached *R. catawbiense* leaves. The inoculated leaves were grown on PARP agar plates at 20.5°C for 7 days in the dark for pathogen re-isolation. The re-isolated pathogen was transferred to new V8 agar plates and incubated at 20.5°C under 12 h diurnal lighting cycle for 10 days for inoculum production.

Table 1 : Isolates of *Phytophthora ramorum* species used in this work.

Isolate Number	Clonal Lineage[1]	Isolate Number	Clonal Lineage[1]
SOD03-105	EU1	SOD06-31653	NA2
SOD03-107	EU1	SOD07-636	NA2
SOD03-110	EU1	SOD07-34599	NA2
SOD04-2231	NA1	SOD07-46063	EU1
SOD04-2232	NA1	SOD08-1352	NA2
SOD04-17036	EU1	SOD08-3443	EU1
SOD04-25165	NA2	SOD08-3469	EU1
SOD05-12977	NA2	SOD08-6286	NA2
SOD05-17401	NA2	SOD08-7045	EU1
SOD06-18462	NA1	P11-263	NA1

[1] Clonal lineage: NA1 = North American 1; NA2 = North American 2; EU1 = European 1.

2.3 Effect of *Phytophthora ramorum* on trees

2.3.1 Production of zoospore suspensions

Production of *P. ramorum* zoospore suspension was carried out using a method similar to that described by Parke & Lewis (2007). Ten-day-old plates of *P. ramorum* incubated at 20.5°C under 12 h diurnal lighting cycle were inspected under a microscope to verify that a large number of sporangia with the correct characteristic morphology for *P. ramorum* had been produced. Ten ml of cold distilled water was poured on the surface of each plate and the sporangia were dislodged by gentle scraping of the hyphae using a sterile glass rod. Sporangial suspensions were poured into a clean collecting beaker on ice and placed at 5°C for 60 min to induce zoospore release. The sporangial suspensions were then placed at room temperature for 60 min to allow the complete discharge of zoospores (Parke & Lewis, 2007). Zoospore suspension was gently stirred for 30 s and filtered through 2 layers of sterile cheesecloth to remove mycelial fragment, zoosporangia and chlamydospores (Parke & Lewis, 2007). An aliquot of the suspension was vortex to induce encystment of the zoospore. The encysted zoospores were counted on a haemocytometer and expressed as zoospores/ml.

2.3.2 Plant inoculations

Plants were subjected to three different treatments: non-wounded, wounded, and wounded-dipped for both control and inoculated plants. In the non-wounded treatment, 15 ml of

the inoculum or distilled water (control) was directly added to the potting mix at a distance of 2 cm around the stem.

Wounded treatments were performed by depotting the plants and sectioning one large root and five small roots around 1 cm from the apex of the plants with sterilized scissors. The wounded treatment plants were repotted and received 15 ml of inoculum or distilled water around the stem as previously described.

Wounded-dipped treatment plants were wounded as described above and roots were dipped into the inoculum or distilled water for 5 min. Wounded-dipped plants were then incubated 1 h in a plastic bag to allow for the zoospore to encyst onto the plant roots prior to repotting.

2.3.3 In vivo experimental trials

In an initial experiment, plants were inoculated with a 40,000 zoospores/ml suspension of NA2 isolate SOD05-17401. This isolate was known to have the potential to cause symptoms and recently, it was reported to cause necrosis on foliage of various tree species commonly found in eastern Canadian forests (Jinek *et al.*, 2011). The experimental design was a factorial design consisting of three plant species (*A. balsamea*, *Q. rubra*, *R. catawbiense*), three wounding treatments (non-wounded, wounded, wounded-dipped), and two inoculation treatments

(inoculated, non-inoculated (control)). Each treatment had five replicates and the experiment was repeated twice. The experimental unit consisted of one plant in an individual pot.

In a second experiment, plants were inoculated with a 30,000-40,000 zoospores/ml suspension of EU1 isolate SOD08-3469. The experimental design was a factorial design consisting of three plant species (*A. balsamea*, *Q. rubra*, *R. catawbiense*), three wounding treatments (non-wounded, wounded, wounded-dipped), and two inoculation treatments (inoculated, non-inoculated (control)). Each treatment had 5 replicates. The experimental unit consisted of one plant in an individual pot. The experiment was repeated twice.

For all trials, the pots were placed in a growth chamber with a photoperiod of 12 h dark and 12 h light for 8 weeks at 20°C with watering being done every 5- 7days. Plants were watered individually in their pots around the stem and care was taken to prevent splashing water onto the stems or foliage so as to reduce contamination chances. Following the incubation period, disease symptoms were noted using the following measures. Wilting and leaf necrosis were used as phenotypic plant symptoms and scored as no wilting, slight wilting or severely wilted and necrotic. For biological data, plant heights were measured three times during the experimental period, that is, at week 0, week 4 and week 8 to monitor the growth of plants. The stem and root discoloration were scored based on the degree of brown discoloration on the root and stem sections of the plants. A scale of 0-4 was used to generate numerical data for discoloration with 0 representing no discoloration and 4 representing severe discoloration.

In addition to the evaluation of symptoms, plant samples were taken for re-isolation of *P. ramorum* on a selective PARP medium and detection of pathogen DNA by RT-PCR. To get tissue samples, plants were removed from their pots and most of the potting mix was gently removed from the plant roots by hand. The roots were then surface sterilised in 0.6% sodium hypochlorite solution for 90 seconds then rinsed in distilled water (modified from Parke &Lewis, 2007). Samples of the plant stem, large roots and small roots for each plant were plated under the PARP media and *P. ramorum* mycelium growth and sporangia formation was assessed after incubation in darkness at 20°C for 7 days.

To complement the re-isolation of *P. ramorum* from infected plants, RT PCR was performed with specific primers to confirm the presence of *P. ramorum* in the plant samples. For each plant, 0.15 g of the stem, the large roots and the small roots were individually collected. DNA extraction was performed using the KingFisher 96 (Thermo Fisher Scientific, Asheville, NC, USA) according to standard procedure currently used by the Plant Pathology Laboratory (CFIA, Ottawa, Canada). Briefly, the tissues were lysed and homogenized by adding 800 µl Lysis Buffer A (MP Biomedicals LLC, Solon, OH) and adding the optional second ceramic sphere. Samples were then homogenized at a speed of 5.0 for 20 s in a Fastprep instrument (MP Biomedicals LLC). Tubes were then centrifuged for 5 min at 12,800 rpm. Then 150 µl of the supernatant from the tubes were added to each well of the 96-well plate. To the supernatant in the well, 35 µl of Magnesil particles (Promega Biosciences, LLC, CA, USA) were added, which would allow the magnesil beads to bind the DNA and the plate was placed in position in the KingFisher 96. In the automated steps, 250 µl Lysis Buffer B was added. This step was followed by washing the bound DNA in 250 µl of 70% ethanol twice to wash off proteins and cell debris.

The extracted and purified DNA was then eluted in 100 µl ultra-pure distilled water and the eluted DNA was stored at -20°C until use.

2.3.4 Real time PCR

RT PCR of plant tissue DNA extracted was performed using the procedure outlined below. First the PCR master mix was prepared in 5 ml tubes and was composed of ITS primers, Cox primers, Probe TaqManITS and Probe TaqManCox all at a concentration of 0.2 µM as well as RNase/DNase free water and the iQ SuperMix/ABgene (Biorad Laboratories, CA, USA). Sequences for the primers used targeting the *P. ramorum* ITS region are: ITS622U Forward (5''-AATGACTGGTGAACCGTAGCTG-3'), ITS755L Reverse (5'-CGAAGCCGCCAACACAAG-3') and Probe TaqMan ITS GB651L R([6~FAM]AACACCGTCGATTCAAAAGCCAAGC[BHQ1~Q]) (Bilodeau *et al.*, 2007). Sequences for primers targeting the Cox region are: COX Forward (5'-GTATGCCACGTCGCATTCCAGA-3'), COX Reverse primer (5'-GCCAAAACTGCTAAGGGCATTC-3') and for the TaqManCox probe (5'TET/ATC CAG ATG CTT ACG CTG G/3'BHQ-2) (APHIS, USDA, 2011). After preparing the master mix, the CAS-1200 automated PCR setup (Corbett Life Science, Mortlake, Australia) was programmed to perform 1:5 and 1:10 automated dilutions of the DNA extractions such that final reaction for RT PCR would contain 20 µl of master mix and 5 µl of diluted DNA. Undiluted and serial diluted (10^{-1} to 10^{-4}) *P. ramorum* (CBS 101553) and also at were used as positive controls while Rhododendron leaf DNA at a dilution of 10^{-1} and water were used as negative controls. RT PCR

was performed on the Rotor Gene 6000 (Corbett Life Science, Mortlake, Australia) with cycling conditions set at 95°C for 15 min, followed by 36 cycles of 95°C for 15 s and 60°C for 60 s.

2.4 Effect of *P. ramorum* isolate on detached leaves

Disease severity caused by nineteen isolates from the three clonal lineages of *P. ramorum* was assessed on detached *R. catawbiense* cv. 'Roseum Elegans' leaves. Detached *R. catawbiense* leaves were washed in water and pat dried. Leaf wounding was carried out by making a 1 cm cut across the midrib with a sterile scalpel. Twenty-five µl of water was placed on the wound (to facilitate movement of the pathogen) and an agar plug (7 mm in diameter) taken from the margin of an actively growing *P. ramorum* culture plate was placed on the leaf top with the mycelium side touching the leaf. V8 agar plugs without mycelium served as controls. The leaves were placed in humid boxes and incubated for 7 days at 20.5°C under photoperiod of 12 h light:12 h dark. Following incubation, lesion size and percent lesion/leaf area was measured using ASSESS software (APS store, St Paul, MN, USA). The experiment was conducted according to a randomized complete bloc design with four replicates. The experiment was repeated twice.

2.5 Statistical analysis

In the in vivo trials, analysis of variance (ANOVA) was performed on main effects (plant species, wounding treatment, and inoculation treatments as well as interactions) using SAS software (SAS Institute, Cary, NC). In the detached leaf assay, ANOVA was performed and, when significant ($P < 0.05$), means were separated using a Tukey test at $P = 0.05$.

3. RESULTS

3.1 Plant inoculations

The root vascular discoloration caused by the NA2 *P. ramorum* isolate on the tested plant species was only significantly different (P = 0.05) for *Rhododendron* plants between controls and inoculated plants. The total root vascular discoloration of the inoculated *Rhododendron* plants received a score of 0.8 out of 4.00 while the controls had no discoloration (Figure 4).

Whole plant symptoms for wilting and leaf necrosis as well as stem discoloration were found to be not significantly different between the controls and inoculated plants. Inoculation method (inoculated compared to water control) were found to have no significant difference on plant heights (data not shown).

Comparing plant treatment method (non-wounding, wounding and wounded dipped) was found to be not significantly different from each other. The overall wounding treatment in rhododendron plants was significantly different but not for balsam fir or red oak.

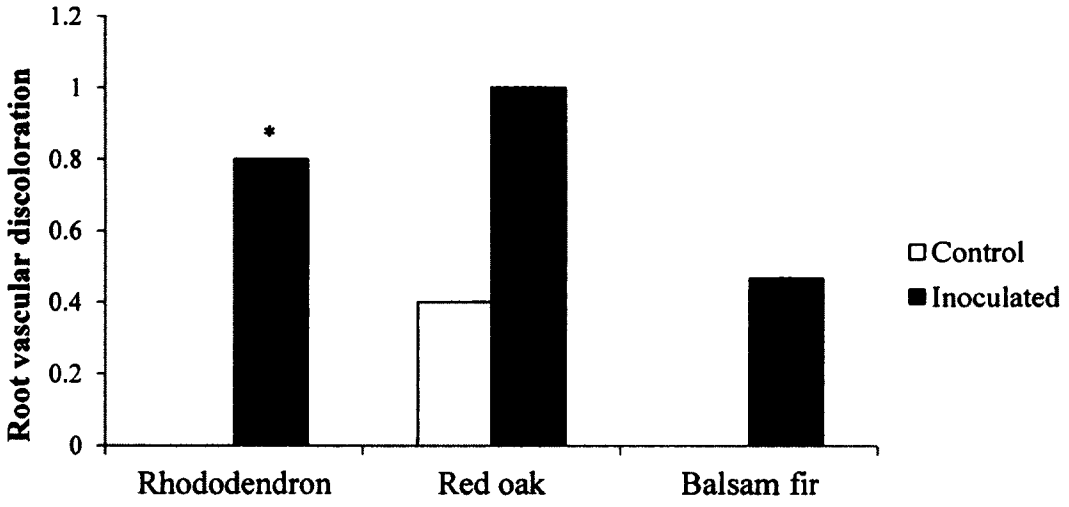


Figure 4: Root vascular discoloration caused by *Phytophthora ramorum* NA2 isolate on the tree species rhododendron, red oak and balsam fir after 8 weeks.

* Significant difference between control and inoculated plants.

Results obtained when using the EU1 *P. ramorum* isolate are shown below. *Rhododendron* showed a significant difference (P = 0.05) in stem vascular discoloration between control and inoculated plants (Figure 5), while there were no significant differences in red oak and balsam fir.

There was no significant difference for whole plant symptoms when looking at wilting and leaf necrosis for the tested plant species under the different treatment methods (data not shown).

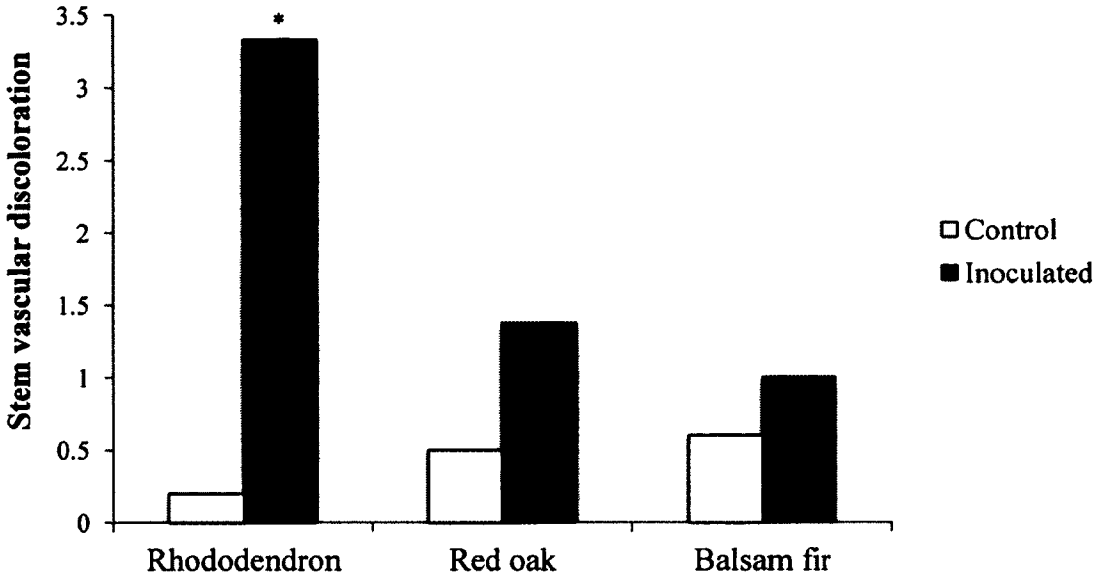


Figure 5: Stem vascular discoloration of rhododendron, red oak and balsam fir caused by *Phytophthora ramorum* isolate EU1 SOD08-3469 following 8 weeks growth period.

* Significant difference between control and inoculated plants.

The total root vascular discoloration observed on all three tree species when using the EU1 *P. ramorum* isolate was found to be significantly different (P = 0.05) between the control and the inoculated samples. Rhododendron showed the largest root vascular discoloration in comparison to the control and other species with a score of 3.6 out of 4 (Figure 6).

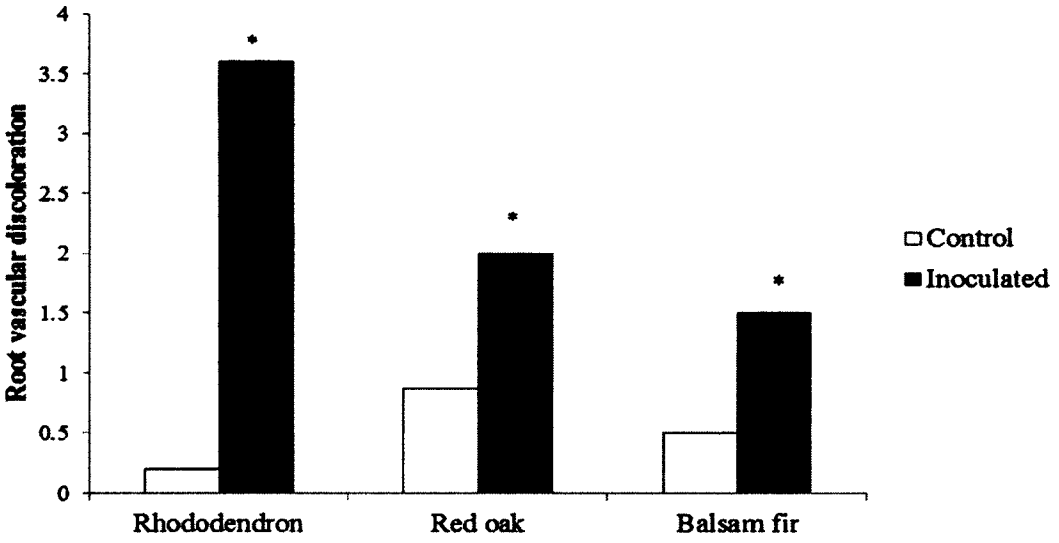


Figure 6: Root vascular discoloration of rhododendron, red oak and balsam fir trees at week 8 caused by EU1 *Phytophthora ramorum* isolate SOD08-3469.

* Significant difference between control and inoculated plants.

3.2 Re-isolation on PARP and detection by Real Time PCR

Detection of *P. ramorum* once the experimental period was over was done by using PARP media and RT PCR.

Tissue samples were transferred onto PARP plates and allowed to incubate. After 7 days, recovery of *P. ramorum* throughout this study was found to be negative.

Results for the RT PCR were analysed according to the cycle threshold (Ct) values. A Ct value of < 34 cycles was regarded as a positive result in this study while a Ct value equal or above 34 were considered negative.

Table 2: Number of both inoculated and control plants subjected to different treatment methods that were positive for the detection of the NA2 *Phytophthora ramorum* DNA (n = 10).

Plant Species	Treatment	Inoculated	Control
Rhododendron	Wounded dipped	6	0
	Wounded	5	0
	Non-wounded	3	0
Red oak	Wounded dipped	0	2
	Wounded	0	0
	Non-wounded	0	0
Balsam fir	Wounded dipped	0	3
	Wounded	0	0
	Non-wounded	0	0

Detection of the NA2 isolate DNA in rhododendron plants inoculated with the zoospore suspension resulted in positive results in all treatment methods (Table 2). Out of ten plants, six of the wounded dipped rhododendron plants were positive for DNA presence. Five of the wounded plants and three of the non-wounded treated plants had the pathogen DNA present in their tissues. None of the control rhododendron plants that received water only tested positive for DNA presence. None of the red oak and balsam fir plants that were inoculated with the zoospore suspension tested positive for pathogen DNA. Unexpected results were obtained when two of the control (water only) red oak plants and three of the control balsam fir plants were positive for *P. ramorum* DNA in the wounded dipped treatments (Table 2).

Using the EU1 isolate SOD08-3469 resulted in more rhododendron plants inoculated with the zoospore suspension being positive for the detection of *P. ramorum* DNA. As shown in Table 3, nine out of ten of the inoculated rhododendrons that were wounded dipped tested positive for presence of *P. ramorum* DNA. In addition, eight wounded rhododendron plants and three non-wounded rhododendron plants that had been inoculated were also positive for *P. ramorum* DNA in their tissues. None of the control (water only) rhododendron plants tested positive for pathogen DNA. Of the test species red oak and balsam fir, only one plant for each species that was wounded-dipped and inoculated with zoospore suspension tested positive DNA presence. The control plants that received only water for these two same species were negative for pathogen DNA presence.

Table 3: Number of inoculated and control plants subjected to different treatment methods that were positive for the detection of the EU1 *Phytophthora ramorum* DNA (n = 10).

Plant Species	Treatment	Inoculated	Control
Rhododendron	Wounded dipped	9	0
	Wounded	8	0
	Non-wounded	3	0
Red oak	Wounded dipped	1	0
	Wounded	0	0
	Non-wounded	0	0
Balsam fir	Wounded dipped	1	0
	Wounded	0	0
	Non-wounded	0	0

3.3 Detached leaf experiments

Experiments on the virulence of the *P. ramorum* among the three clonal lineages of the pathogen on the *Rhododendron catawbiense* leaves showed a significant difference between the controls and the pathogen-inoculated leaves (Table 4). However, there were no significant differences in the lesion area caused when comparing the clonal lineages EU1, NA2 and NA1. Similarly, comparing the % lesion per leaf area caused by the different clonal lineages shows no significant difference for virulence amongst the lineages.

Table 4: Lesion area (mm^2) and % lesion per leaf area on *Rhododendron catawbiense* leaves following application of *Phytophthora ramorum* isolates of clonal lineages EU1, NA1 and NA2.

Clonal lineage	**Lesion area (mm^2)** [a]	**% lesion/leaf area** [a]
EU1	719.6 a	47.0 a
NA2	708.2 a	43.2 a
NA1	647.5 a	42.5 a
CONTROL	2.8 b	0.2 b

[a] Within a column, means with different letter are statistically different according to a Tukey test (P = 0.05)

The necrotic areas rated as lesion size and % lesion per leaf area caused by specific *P. ramorum* isolates on the inoculated wounded leaves were significantly different when compared to the control wounded leaves (Table 5). Comparisons among the different isolates for the lesion size and % lesion per leaf area caused showed that there were no significant differences among

the isolates. The necrotic area ranged from 577.6 mm^2 to 815.3 mm^2 while the % lesion/leaf area ranged from 34.9 to 53.3 % (Table 5).

Table 5: Lesion area (mm^2) and % lesion per leaf area caused by the different *P. ramorum* isolates on the rhododendron leaves

Isolate #	**Clonal lineage**	**Lesion area (mm^2)** [a]	**% Lesion/leaf** [a]
P11-263	NA1	722.9 a	50.0 a
SOD03-105	EU1	580.1 a	34.9 a
SOD03-107	EU1	815.3 a	53.2 a
SOD03-110	EU1	694.4 a	46.2 a
SOD04-17036	EU1	698.3 a	45.7 a
SOD04-2231	NA1	584.4 a	37.9 a
SOD04-2232	NA1	705.0 a	44.8 a
SOD04-25165	NA2	674.0 a	40.3 a
SOD05-12977	NA2	667.8 a	41.8 a
SOD06-18462	NA1	577.6 a	37.3 a
SOD06-31653	NA2	736.9 a	38.9 a
SOD07-34599	NA2	752.9 a	47.4 a
SOD07-46063	EU1	812.1 a	53.3 a
SOD07-636	NA2	709.6 a	46.8 a
SOD08-1352	NA2	675.6 a	44.5 a
SOD08-3443	EU1	763.5 a	49.0 a
SOD08-3469	EU1	801.5 a	52.9 a
SOD08-6286	NA2	740.7 a	43.0 a
SOD08-7045	EU1	592.0 a	40.8 a
CONTROL		2.8 b	0.2 b

[a] Within a column, means with different letter are statistically different according to a Tukey test (P = 0.05)

4. DISCUSSION

This study was mainly conducted to determine the susceptibility of the roots of *Q. rubra* and *A. balsamea*, two important Canadian native forest species, to *P. ramorum* and to evaluate the severity of necrosis on detached *Rhododendron* leaves caused by isolates from different clonal lineages.

The lineage NA1 is still the most abundant in North America according to Prospero *et al.* (2009) and this wide distribution is mainly attributed to shipments of infected material from the three west coast states, Oregon, Washington and California (Goss *et al.*, 2011), but this is particularly related to a large shipment of infected *Camellia* plants from a California nursery in 2004 (Frankel, 2008). Due to this abundance and wide distribution, the *P. ramorum* isolate A2 SOD 05-17401 was used. At the beginning of the study, this isolate was previously determined to be of NA1 lineage, but during the course of the study, new information confirming that this isolate was in fact of NA2 lineage surfaced after genotyping and sequencing tests were performed on this isolate (Bilodeau, personal communication). A study investigating the genotypic diversity of *P. ramorum* in Canadian nurseries found that all three clonal lineages were present but the NA2 lineage appears now to be the more common (Goss *et al.*, 2011). To ensure virulence, this NA2 isolate was first inoculated on *Rhododendron catawbiense* leaves and then re-isolated from the necrotic parts on V8 plates.

Results obtained for whole plant symptoms were not significantly different for both the NA2 and the EU1 isolates used in this study. The wilting symptoms and necrosis on the plants

were not conclusive as there were no significant differences between the control and inoculated plants. One would have expected the positive control tree species rhododendron to display more wilting and necrotic symptoms than the other species. Some inoculated rhododendron plants exhibited wilted and collapsed leaves, symptoms that were in agreement with those observed by Parke and Lewis (2007). A plausible reason for this result is that the disease may have not progressed enough to result in sufficient visual symptoms. The disease symptoms that were observed on plants became visible in 4 to 6 weeks after inoculation. This was in agreement with the observations reported by Parke and Lewis (2007) where symptoms became apparent in weeks 3-6 after inoculation. One of the problems encountered in this project was that some of the non-inoculated plants, in particular oak, were not in a good health at the end of the assays and some were even dead. Of interest, is the findings about *Larix kaempferi* (Japanese larch) in which mature plants of 25-30 m tall and juvenile plantation plants experienced extensive dieback and mortality at various sites in south west England. Such symptoms as wilting, senescence and needle loss were observed (Weber *et al.*, 2010). Considering that Japanese larch is a susceptible conifer, this prompted the testing of the conifer balsam fir in this study.

Our results showed that there was no significant difference between the wounding methods. Considering that from non-wounded to wounded to wounded-dipped, severity of the treatment was increased, we would have expected the wounded dipped treatments to cause greater vascular discolorations. This is because after wounding, which presumably gave the pathogen entry point into the plant tissue, the plants were incubated for one hour in a plastic bag to allow the zoospores to encyst on the roots before they were repotted. However, increased severity of symptoms due to wounding methods was not found in our trials.

The vascular discoloration in roots and stems of plants were assessed and the results are shown in Figure 4 for the North American isolate and Figures 5 and 6 for the European isolate. For the NA2 isolate used, a significant difference was only observed on the rhododendron plants for root vascular discoloration between the control plants and the inoculated plants. This result was somewhat expected as the rhododendron served as positive controls and that rhododendron was already potentially susceptible to infection following root inoculations (Parke & Lewis, 2007).

When using the European isolate, a significant difference was observed between the control plants and inoculated plants for root vascular discoloration in all the plant species used in this study. The largest difference between the control and inoculated plants was observed in rhododendron species (Figure 6). Assessing the root vascular discoloration, scores among the plants species in Figure 6 reveals that this EU1 isolate of *P. ramorum* has higher pathogenicity to the rhododendron plant species than to the other two species. This is illustrated by the high score of 3.6 out of 4, which represented a severe discoloration of the root vascular tissues.

In terms of stem vascular discoloration, the use of EU1 isolate had a significant difference when comparing the control plants and inoculated plants only for rhododendron (Figure 5). The large score of discoloration in the stem vascular system of inoculated rhododendron plants implies that the discoloration caused by *P. ramorum* infection from the roots, which also had high discoloration, was apparently related to continuous colonization primarily in the xylem, which is rather unusual for any *Phytophthora* spp., as also reported in

Parke and Lewis (2007). In spite of that latter result, it is worth mentioning that *P. ramorum* has the capacity the extensively invade the xylem of red oak and balsam fir, as revealed by recent microscopic examinations of shoot tissues after stem inoculations (Simard *et al.*, 2010; Rioux, unpublished results). Another study by Brown & Brasier (2007) suggested that *Phytophthora* species, including *P. ramorum*, may be present in the xylem more often than previously thought and could even use this tissue to colonize the tree more rapidly. They had sampled the phloem and xylem from the stem of several tree species, mostly from the Fagaceae family, and in 23% of their samples, the isolation of *P. ramorum* was achieved from the xylem while it was negative from the phloem. Parke et al. (2007) also showed that *P. ramorum* can colonize the xylem of naturally-infected tanoak trees.

Comparing the North American with the European isolate, the results obtained reflect the fact that the European isolate is more virulent than the North American isolate. More vascular discoloration in the stem and root tissues of inoculated rhododendron plants was observed with EU1 than with NA2. The EU1 isolate was also able to cause significantly more discoloration in the roots of red oak and balsam fir whereas the discoloration caused by the NA2 isolate in the roots of the same species was not significantly different. This difference in virulence of the two isolates is in agreement with the results observed in rhododendron by Parke and Lewis (2007). In their study, they reported that both of the isolates they used, which represented the North American and European lineages, were pathogenic on rhododendron cv. "Nova Zembla", but the European genotype caused more symptoms than the North American genotype. Another study indicated that an isolate obtained from *U. californica*, likely in nature and thus part of the NA1 lineage, caused symptoms after stress and inoculation of the root of a *Rhododendron* hybrid

"Cunningham's White" (Roubtsova & Bostock, 2009). Parke and Lewis (2007) even reported mortality at 7 weeks post-inoculation using a zoospore concentration quite similar to ours in all the 12 seedlings inoculated with the European isolate while only one out of 12 seedlings died after inoculation with the North American isolate. In line with this preliminary report on *P. ramorum*, that is before its populations were divided into three lineages, other studies had already revealed that the European isolates were on average a little more aggressive than the North American isolates. For instance, following artificial inoculations of *Q. rubra* and rhododendron stems, more necrosis was noted after inoculating with European than with North American isolates (Brasier, 2003).

Re-isolation assays of the pathogen on PARP media plates were all negative. This was a rather surprising result as PARP is known to be selective for oomycete growth and seeing that the EU1 isolate resulted in significant root vascular discoloration in all inoculated plant species as compared to controls. An explanation may be that not enough of the pathogen was in the samples to result in visible growth on the media plates. Also, *P. ramorum* is not a very competitive oomycete (Lee *et al.*, 2011) and as most of the plates had different mold forms after incubation, these could have out-competed *P. ramorum* and hence masked its re-isolation on PARP. This observation was not expected because the antibiotics and chemicals in the PARP media are supposed to suppress growth of fungi and bacteria. The use of 0.6% sodium hypochlorite solution for surface sterilising the roots prior to sampling for PARP re-isolation could have in the process killed the *P. ramorum* present on the roots resulting in no growth on the plates. Although in this study exposure to the sodium hypochlorite solution was lengthier (90 sec versus 30 sec), Parke & Lewis (2007) had also used the same concentration of sodium

hypochlorite to sterilise plant tissue samples without negative effects. An alternative would be the use of the Teepol, a less harsh detergent that allowed detection of the pathogen when using sodium hypochlorite do not afford detection (Levesque, personal communication)

Real time PCR technique was used in this study as a method to detect the presence of *P. ramorum* DNA in tissue samples. In this technique, the ITS and COX region was tested and it has been noted by Bilodeau *et al.* (2007) that the TaqMan ITS assay is the most sensitive method for *P. ramorum*. The technique itself worked efficiently as shown by the successful detection of the COX region when using the plant internal positive control primers and probe for the cytochrome oxidase gene. This is a gene that is present in all plants (Tomlinson *et al.*, 2005) and thus served as an internal control to indicate successful DNA extraction. In addition, the fact that the known PCR standards (positive controls for *P. ramorum* and negative control) gave the expected results is another indication of the efficiency of the technique.

However, the NA2 real time PCR results were disappointing because in the first trial our results were invalidated by detection of pathogen DNA in control plants that received no zoospore inoculum. There might have been a cross contamination during DNA extraction step or a likely mislabelling although care was taken that none of this should occur. A retrial with the same isolate gave only one positive for the inoculated rhododendrons which was also disappointing as it was expected that the inoculated positive control species would show positive results that would allow for comparison with the test species. Consequently, conclusions from the NA2 isolate could not be drawn and thus the option to use the EU1 isolate was chosen.

Using the EU1 isolate gave more prominent results from which conclusions could be made. In the first trial with this isolate, the inoculated rhododendron plants gave expected results as most of the plants (three out of five for both non-wounded and wounded plants, and four out of five for wounded dipped plants) were positive for the presence of *P. ramorum* DNA. In a re-trial of this experiment, all five plants representative of the wounded and the non-wounded treatment that were inoculated with the zoospore suspension had positive detection for pathogen DNA in their stem and big roots tissue samples. This was an expected result as it is known that rhododendron is susceptible to infection by *P. ramorum* and these results were in agreement to those obtained by Parke and Lewis (2007) who also isolated *P. ramorum* DNA from the roots of rhododendron after being grown in an artificially infested potting medium. Detection of *P. ramorum* DNA in roots and stem suggests that the pathogen was taken up through the vascular system and was able to move up to the stem as water travelled up to aerial parts in the xylem (Parke & Lewis, 2007). Using the EU1 isolate, pathogen DNA was not detected frequently (2 positive out a total of 60 plants) in the roots of red oak and balsam fir even though significance differences in discoloration occurred between control and inoculated plants.

Lesion size in terms of area (mm^2) and % lesion per leaf area on detached rhododendron leaves was measured as a way to determine the pathogenic aggressiveness or the ability of the pathogen to colonize the host once infection had occurred. In this experiment, nineteen *P. ramorum* isolates that were genotypically confirmed as representing the three clonal lineages of the pathogen were used. In this experiment, some level of necrosis was expected following wounding of the leaves and inoculation with a plug of the pathogen mycelium because *P. ramorum* hyphae have been shown to be attracted to wounds. In a study by Lewis and Parke

(2005), observations were made using scanning electron microscope where hyphae from germinating cysts were attracted to a wound in the rhododendron leaf whereas no such observation was made on a nearby stoma. Wounded tissues are known as being more susceptible to *P. ramorum* infection. Recently, wounding of foliage was also reported to cause more necrosis when compared to unwounded leaves (Jinek *et al.*, 2011).

In our study the only significant difference in the lesion area and % lesion per leaf area obtained was between the pathogen-inoculated and the control leaves, which had received no mycelium. This was expected because the *Phytophthora* hyphae in the plugs would invade the leaves through the wound cuts made on the leaves whereas for the control there would be no hyphae to cause necrosis. The only slight necrosis observed in the control leaves around the wound cut was the background necrosis that occurred as a form of plant response to the wounding effect. Our results show that there is no significant difference in lesion area caused among the *P. ramorum* isolates used. Consequently, grouping the isolates according to their clonal lineages and analysing the lesion area and % lesion per leaf area also gives no significant difference among the clonal lineages. This implies there is no difference in virulence among NA1, NA2 and EU1 isolates used. This result contradicts that obtained by Elliot *et al.*, (2011) where their study on phenotypic differences among three clonal lineages of *P. ramorum* showed isolates from the NA2 and EU1 lineages as being the most aggressive and NA1 being the least in virulence. Another study on virulence between the *P. ramorum* clonal lineages by Manter et al. (2010) showed that the isolates representing EU1 and NA2 were more virulent than NA1 as they produced significantly greater lesions than those from NA1.

5. CONCLUSION

Assessment of the potential of *P. ramorum* to infect the roots of red oak and balsam fir was successfully achieved after the use of the EU1 isolate. It can thus be concluded based on the results obtained in this study that the roots of the eastern Canada tree species *Q. rubra* and *A. balsamea* are of low susceptibility to infection by *P. ramorum*.

Based on the root vascular discoloration results, it can also be concluded that the EU1 isolate appeared more aggressive than the NA2 isolate. The low disease severity in the inoculated controls made it difficult to assess the susceptibility of the plants with the NA2 isolate.

Of the different isolates representing the three clonal lineages used in this study, no lineage seems more aggressive than the other. However, more studies are still required as knowledge on *P. ramorum* root infection is still in its infancy stage. Microscopic studies to investigate the infection process through the roots are required and are currently being conducted at the Laurentian Forestry Centre (Quebec, Natural Resources Canada). Also, such studies as those that try to address the question of which hosts are more susceptible to which clonal lineage should be carried as one host may show varied susceptibility to different clonal lineages of *P. ramorum*.

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