Stress Physiology of *Phytophthora*-canker Pathogens in Landscape Trees: impacts, mechanisms, and mitigation through biochar amendment

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Abstract

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Soil-amendment with biochar is thought to confer multiple benefits to plants including induction of systemic resistance to plant pathogens. Pathogens in the genus *Phytophthora* cause damaging diseases of woody species throughout the world. The objectives of the experiments described in the following chapters were to determine 1) what are the physiological impacts of stem cankers caused by *Phytophthora cactorum* in *Acer rubrum* seedlings, 2) if biochar amendment induces resistance to canker causing *Phytophthora* pathogens and how this resistance is related to the amount of biochar amendment in two common landscape tree species: *Quercus rubra* (L.) and *Acer rubrum* (L.), and 3), how does biochar improve resistance against stem lesions.

Inoculation of *A. rubrum* seedlings with *P. cactorum* resulted in a dramatic and sustained reduction in carbon assimilation rates and stomatal conductance, and a temporary reduction in photosynthetic efficiency compared to non-inoculated control plants. Foliar starch levels did not support the hypothesis that the reduction of photosynthesis was the result of reduced carbohydrate transport and subsequent feedback inhibition. Water use efficiency and stem water potential data suggested a slight moisture stress in the inoculated group compared to the control group, which may have contributed to the photosynthetic decline. Early reductions were probably not due to water shortage and are most likely caused by pathogen-derived signals, or hormonal responses in the plant caused by wounding and pathogen inoculation that led to reduced stomatal aperture and a carbon dioxide supply-related reduction in photosynthesis.

Amendment of potting media with 5 % biochar by volume significantly reduced the expansion of necrotic cankers in both test species combinations. Further, in *A. rubrum* seedlings, biochar amendment was shown to significantly improve photosynthetic performance compared to non-amended, inoculated seedlings. Analysis of foliar defense compounds did not clarify the potential mechanism underlying the reduction in disease impact in amended seedlings. However, combining two years of data clearly indicated that amendment of potting mix with 5 % biochar by volume significantly improved plant physiological performance and reduced the size and impact of necrotic lesions caused by *P. cactorum* in *A. rubrum* seedlings.

Further studies will be needed in order to determine the exact mechanism underlying the induced resistance against lesion development in biochar amended seedlings. Analysis of defense compounds immediately adjacent to the infection site, rather than a foliar analysis, will help to clarify the impact of biochar amendment on the plant defense response. Analysis of plant phenolic content over time as opposed to at the conclusion of the experiments may also shed light on the process of resistance induction following biochar amendment.

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CHAPTER 1

INTRODUCTION

1.1 *Phytophthora* disease in woody plants

It has long been known that plant diseases caused by organisms in the genus *Phytophthora* can be devastating to field crops, tree crops, and forest systems (Erwin and Ribeiro, 1996; Hansen et al., 2008; Zentmyer, 1980). In addition, these diseases can be widespread and damaging to woody plants that are commonly found in the managed landscape (Erwin and Ribeiro, 1996). In landscape trees, *Phytophthora* pathogens can cause a variety of disease types, including fine root diseases, root collar or crown rots, and trunk lesions that are often referred to as 'bleeding cankers'(Erwin and Ribeiro, 1996). Recently, interest in 'bleeding cankers' has increased due to the discovery of *Phytophthora ramorum* as a lethal pathogen to several tree and many woody ornamental species (Hansen et al., 2005; Rizzo et al., 2002; Weres, 2001), the discovery of *P. ramorum* and *P. kernoviae* causing cankers and mortality of hosts in the United Kingdom (Brown and Brasier, 2007), and the illumination of the extent of damage caused by *P. cactorum* and *P. citricola* on *Fagus* species in the Northeastern U.S. (Cerny et al., 2009; Jung et al., 2005; Weiland et al., 1996). *Phytophthora* pathogens are also known to cause stem cankers or lesions worldwide in several oak species (*Quercus* spp.) (Moralejo et al., 2009; Robin et al., 2001; Robin and Desprez-Loustau, 1998; Tainter, 2000; Wood and Tainter, 2002), maple species (*Acer* spp.), (Drilias et al., 1982; Wang, 2008), and other tree species (Erwin and Ribeiro, 1996).

In addition to acting as lethal primary pathogens, *Phytophthora* stem-canker diseases often leave host plants more vulnerable to secondary insect or pathogen attack. For example, trees infected with *P. ramorum* are more likely to be attacked by several species of ambrosia beetle (Mcpherson et al., 2008), and beetle attacks in combination with decay fungi lead to more rapid tree death and increase the potential for structural failure (Mcpherson et al., 2010). In the northeastern United States, European beech (*Fagus sylvatica*) is a very common and often high-value component of the landscape, and surveys have indicated that potentially 40% or more of mature beech trees are infected with lethal, canker-causing *Phytophthora* species (primarily P*. cactorum, P. citricola,* and *P. plurivora*) (Weiland et al., 1996). Mortality of mature landscape trees related to chronic *Phytophthora* diseases and secondary pests also results in significant economic loss. In the case of *P. ramorum* in California, loss of mature oaks due to disease is estimated to reduce home values by up to 15% (Kovacs et al., 2011a) and cost cities and homeowners millions of dollars for treatment, removal, and replacement of dead and dying trees (Kovacs et al., 2011b).

Phytophthora cactorum and *P. cinnamomi,* the pathogens under investigation in the experiments described in the ensuing chapters, have been reported from an extensive variety of host plants. These pathogens have been reported causing root rot, crown rot, or bleeding cankers on many common landscape trees including species in the genera *Abies, Acer, Aesculus, Alnus, Arbutus, Betula, Carya, Castanea, Cedrus, Citrus, Cornus, Corylus, Crataegus, Eucalyptus, Fagus, Fraxinus, Juglans, Larix, Liquidamber, Liriodendron, Malus, Nyssa, Picea, Pinus, Prunus, Pseudotsuga, Pyrus, Quercus, Rhododendron, Syringa, Tsuga, Tilia,* and *Ulmus*, to name just a few (Table 11-1 in (Erwin and Ribeiro, 1996)) . *Phytophthora cinnamomi* is known to cause damage to over 1,000 host plants (Zentmyer, 1980), while reports of bleeding cankers or oozing lesions on red maple (*Acer rubrum*) caused by *P. cactorum* were published as early as 1940 (Caroselli, 1940), and have since been reported on *A. rubrum* (L.) and several other maple species (Cerny et al., 2009; Drilias et al., 1982; Hong and Marsten, 2005; Nelson and Hudler, 2007; Schwingle and Blanchette, 2008; Wang, 2008).

In light of the tremendous host range of *Phytophthora cinnamomi and P. cactorum* in landscape plant species and the widespread use of red maple and members of the red oak group (*Quercus* section *Lobatae*) in managed landscapes, investigations of the underlying physiological mechanisms and potential treatment options in these host-pathogen combinations are warranted. Information obtained from these studies will provide valuable information or applicable mitigation strategies for other *Phytophthora* disease situations.

1.2 Physiological effects of *Phytophthora*-canker on woody species-

Due to the importance of root rot-causing *Phytophthora* species in agricultural systems, much research has been published regarding the infection, disease progression, resistance, treatment, and physiology relative to this type of disease. There is significantly less research published on these aspects of canker-causing *Phytophthora* species. In a search of relevant scientific literature (ISI Web of Science, v. 4.10, searched on 11/20/2012 for publications in previous 20 year span), very few research articles were found that investigated the effects of stem inoculation or natural infection of a canker-causing *Phytophthora* species on gas exchange or photosynthetic efficiency in woody plant species (Clemenz et al., 2008; Fleischmann et al., 2005; Fleischmann et al., 2002; Luque et al., 1999; Manter et al., 2007). Furthermore, results of these experiments show some discrepancies in the effects of inoculation, the timing of the effects, and the underlying mechanisms.

A reduction in assimilation of carbon dioxide and transpiration of water vapor, decreased stomatal conductance, and reduced photochemical efficiency of the photosynthetic apparatus have commonly been shown in these experiments, but this may be caused by several factors. Impaired water transport due to occlusion of xylem elements, feedback inhibition due to starch accumulation caused by destruction of phloem tissue, or the production and recognition of pathogen-derived elicitins have all been reported as potential mechanisms for the observed responses.

For example, Luque et al. (1999) showed a gradual decline in photochemical efficiency of photosystem II (dark-adapted Fv/Fm) following stem inoculation of *Quercus suber* with *P. cinnamomi*, but did not investigate the underlying mechanisms. Manter et al. (2007) showed that inoculation of *P. ramorum* in three different woody species resulted in reduced photosynthetic Rubisco capacity (Vcmax) which occurred prior to any detectable loss in water transport capacity. Exposure of foliage to *P. ramorum*-derived protein elicitins caused a similar reduction in photochemical efficiency and an increased production of metabolites that are commonly regarded as part of a hyper-sensitive defense response, indicating that the effects in this case were related to elicitins and not water status (Manter et al., 2007). Fleischmann et al. (2005) showed that in *Fagus* spp. inoculated with *P. citricola*, reduction in photosynthesis occurred prior to the reduction in photochemical efficiency, and also prior to detectable differences in leaf water potential and plant water consumption. In this study elicitins produced by *P. citricola* were isolated and characterized, and these metabolites caused a hyper-sensitive reaction in tobacco leaves and cell suspensions, but did not cause any differences in beech plants or beech cell suspensions. Maurel et al. (2004) also showed that root infection by *P. cinnamomi* in *Castanea sativa* seedlings reduced stomatal conductance, and thus reduced assimilation and transpiration. They, however, concluded that this was due to moisture stress and not the action of elicitins, because application of purified elicitins to foliage did not result in reduced stomatal conductance in leaves while application of a drought related hormone (ABA) did. Finally, Clemenz et al. (2008) found that basal inoculation of *Alnus glutinosa* with *P. alni* subsp. *alni* led to reduced carbon assimilation and transpiration, and increased foliar starch content, but had no effect on water use efficiency or minimum leaf water potential. These authors suggest that the accumulation of starch in foliage was due to impaired movement in damaged phloem tissue, and this led to feedback inhibition of photosynthesis prior to moisture stress. In the discussion of these results, the authors discount the role of elicitins due to prior findings (Fleischmann et al., 2005; Maurel et al., 2004), but did not test elicitins or fully examine the starch feedback mechanism. It appears that the exact mechanism causing the reduction of carbon assimilation and water vapor transpiration in *Phytophthora*-canker stricken trees is not clear, or varies depending on the specific host/pathogen combination**.**

1.3 Biochar and disease resistance

Biochar, or black carbon, is the product of pyrolysis, a process of heating plant biomass in the absence of oxygen (Keiluweit et al., 2010; Lehmann, 2007b). The process of pyrolysis becomes exothermic after a relatively small amount of activation energy is applied, producing heat that can offset the use of fossil fuels. The process also drives gasses (syngas) and oils from the plant material which can be collected and serve as an additional alternative to the use of fossil fuels (Laird, 2008; Lehmann, 2007a; Mathews, 2008). In addition to the reduced carbon dioxide emissions resulting from biomass-derived energy production, the product of pyrolysis, biochar, which is often considered a by-product in bio-energy production systems, is a carbon-rich solid product that is highly recalcitrant to microbial decay. While the exact soil half-life of biochar is variable and the source of some debate, it is generally accepted that the carbon in biochar can be stored in soils for hundreds or thousands of years before eventually breaking down and releasing carbon dioxide back to the atmosphere (Harvey et al., 2012; Keiluweit et al., 2010; Lehmann, 2007b). Many scientists have argued that simple reduction of fossil-fuel use and subsequent reductions in gaseous emissions is not enough to prevent catastrophic climate change, and carbon sequestration, such as the addition of biochar to soil, will also be necessary to address this issue (Keiluweit et al., 2010; Laird, 2008; Lehmann, 2007a; Mathews, 2008).

In addition to sequestration of carbon dioxide, soil application of biochar has been studied extensively in regard to soil physical and chemical properties, plant growth, agricultural productivity, soil microbial communities, and many other factors. The exact chemical nature of biochar is dependent on many factors, including the biomass source (parent material), the temperature of pyrolysis, the style of pyrolyzing kiln or oven, and the age of biochar material (Harvey et al., 2012). By extension, the effects of biochar on soil properties and plant performance are highly variable. A recent statistical meta-analysis of data derived from many different experiments showed that biochar addition to soils resulted in a grand mean increase in plant growth of 10%, with greater positive growth effects seen under certain soil conditions (Jeffery et al., 2011). The specific benefits found through research on biochar in soils are numerous and wide-ranging. Biochar has been shown to increase soil fertility and nutrient retention, in part due to the high cation exchange capacity and liming effect of biochar (Glaser and Zech, 2002; Novak et al., 2009). Biochar or charcoal incorporation has also been shown to improve the plant-beneficial soil microbial community, including bacteria that are known to promote plant growth, and mycorrhizal fungi (Ishii and Kadoya, 1994; Kolton et al., 2011; Robertson et al., 2012; Warnock et al., 2007), and has been studied as a potential carrier of microbial inoculants such as nitrogen-fixing bacteria (Beck, 1991). Biochar can limit movement and plant uptake of pesticides in soil (Yu et al., 2009), and has been studied in regard to remediation and restoration of contaminated soils (Beesly et al., 2011). There is also emerging evidence that biochar incorporation can help to prevent or limit damage caused by plant diseases (Elad et al., 2011). The potential for biochar incorporation as a disease management option has only recently been suggested and research to date is limited (Elad et al., 2011; Lehmann et al., 2011). Induction of systemic resistance against a range of plant pathogens may be important co-benefit to biochar amendment of landscape soils.

Prior studies have shown that the incorporation of biochar in the root zone of plants may limit plant disease through various potential mechanisms, many of which are related to overall plant vigor. These include reduction of soil bulk density, increased water holding capacity, altered soil chemical status, increasing mycorrhizal associations, harboring beneficial soil bacteria, and potentially inducing systemic resistance in the plant (Elad et al., 2010; Laird, 2008; Lehmann, 2007b; Novak et al., 2009; Warnock et al., 2007; Yu et al., 2009). Addition of various other forms of organic carbon such as sewage sludge or wood mulch have also been shown to suppress various *Phytophthora* diseases, at least in part by increasing soil microbial activity (Downer et al., 2002; Downer et al., 2001; Leoni and Ghini, 2006). Recently, Elad et al. (2010) showed that incorporation of biochar into potting mix of pepper (*Capsicum annuum* cv. Maccabi) and tomato (*Lycopersicum esculentum* cv. 1402) reduced the disease severity caused by two foliar pathogens with very different life histories and damage from a broad mite pest (Elad et al., 2010). In addition, Harel et al. (2012) showed that incorporation of biochar reduced the damage caused by three foliar pathogens of strawberry (*Fragaria* x *ananassa* cv. Yael). Due to the reduction in disease caused by pathogens exhibiting both necrotrophic and biotrophic strategies, and reduction of damage caused by an arthropod pest, the authors in both studies attributed these results to systemic induced resistance (SIR) in response to biochar incorporation. These effects may have been caused by direct interactions between the plant and the biochar, or may be the result of biochar-related alterations in the soil microbial community (Elad et al., 2011; Kolton et al., 2011; Warnock et al., 2007). If SIR was the cause of the observed decrease in disease severity, similar results may be possible in defense against *Phytophthora* pathogens.

1.4 Managing *Phytophthora* disease

Mitigation or management strategies for *Phytophthora* diseases in the managed landscape depend on repeated chemical treatments (Garbelotto et al., 2009; Weiland et al., 2009) and/or changes to the environmental conditions under which the plants are growing (cultural methods). Repeated chemical treatments of *Phytophthora* pathogens have led to resistance in several different species against various management options, including the common active ingredients mefenoxam and salts of phosphorous acid (Dobrowolski et al., 2008; Hill and Hausbeck, 2008; Jeffers et al., 2004). Frequent and repeated maintenance or treatment needs will also lessen the benefits of landscape trees related to reduction of carbon dioxide and other greenhouse gases (Nowak, 2002) .

The apparent systemic resistance-inducing effects of biochar incorporation are potentially similar to the effects of potassium phosphonate, a chemical material commonly used for treatment of *Phytophthora* disease which appears to have complex and varied modes of action (Daniel and Guest, 2006; Daniel et al., 2005; Garbelotto et al., 2009; Jackson et al., 2000). In high concentrations, the pathogen is directly affected by the material, while at lower concentrations plants have been shown to mount a quicker and more intense defense reaction after treatment upon exposure to *Phytophthora* pathogens (Daniel and Guest, 2006; Daniel et al., 2005; Jackson et al., 2000). Multiple modes of action are an ideal quality in any pest management material. However, isolates of *P. cinnamomi* from areas with a long history of phosphonate use have shown reduced sensitivity to phosphite *in vitro* and were able to colonize treated plants to a greater extent than isolates from non-treated areas, while colonization of untreated plants was the same for isolates from either group (Dobrowolski et al., 2008). The ability of biochar to substitute or supplement chemical treatment would add a useful option in disease management efforts, and would help slow or prevent the development of resistant strains.

1.5 Purpose of Research

The goals of the experiments detailed in the following chapters are to a) investigate the effects of a canker-causing *Phytophthora* species on several physiological parameters in red maple (*Acer rubrum*) (Chapter 2), b) clarify the mechanisms underlying the observed effects of infection (Chapter 4), c) determine if the use of biochar as an organic soil amendment can help to mitigate the damaging effects of two *Phytophthora* pathogens on different host plants as compared to a standard chemical treatment (Chapter 3), and d) to illuminate which physiological factors are affected by this potential mitigation strategies (Chapter 4). Biochar amendment is a novel but promising approach to mitigating the impact of these diseases in the managed landscape that may serve as a valuable substitute or supplement to chemical control options. Also, understanding the physiological mechanisms underlying both the impact of disease on plant physiology and behind the induction of resistance will provide valuable information for future work on plant disease-resistance in many host-pathogen systems. Secondary potential benefits stemming from this research include the development of a management strategy for any *Phytophthora*-related disease situation in the managed landscape that reduces dependence on chemical treatments, and may offer several other benefits. These include a potential reduction in use of fertilizers, reduced leaching of fertilizer, increase in water holding capacity, reduction in soil bulk-density, and permanent storage of carbon that would otherwise return to the atmosphere and contribute to global warming.

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CHAPTER 2

The effects of *Phytophthora cactorum* stem lesion on leaf physiology in *Acer rubrum*

2.1 Introduction

A number of studies have been conducted examining the relationship between *Phytophthora* root disease of woody species, photosynthetic performance, and plant water status. In most of these studies, infection of root systems by *Phytophthora* pathogens resulted in decreased photosynthesis (net carbon assimilation, A_n). The reduction in A_n as a result of root infection is often related to a reduction in stomatal conductance to water vapor (g_s) and reduced stem or leaf water potential is also commonly observed in infected plants (Dinis et al., 2011; Fleischmann et al., 2002; Maurel et al., 2001; Robin et al., 2001). Both of these factors are common in plants subjected to drought stress, and in studies where the responses to infection were compared to drought response, similar patterns were seen in stomatal conductance and stem water potential between well-watered inoculated plants and drought stressed non-inoculated plants (Maurel et al., 2001; Maurel et al., 2004). In addition, physiological indicators of drought stress such as decreased carbon isotope discrimination (Maurel et al., 2001) and increased water use efficiency (Fleischmann et al., 2002) have been shown to be similarly affected by drought and *Phytophthora* root disease.

In contrast to studies involving root-disease, very few studies have investigated the effects of stem inoculation or natural infection of a canker-causing *Phytophthora* species on gas exchange or photosynthetic efficiency in woody plant species (Clemenz et al., 2008; Fleischmann et al., 2005; Fleischmann et al., 2002; Luque et al., 1999; Manter et al., 2007). Furthermore, while reduced photosynthetic performance is a common result, these experiments show some discrepancies in the physiological effects of inoculation, the timing of the effects, and the underlying mechanisms.

For example, in *Quercus suber* seedlings stem-inoculated with *Phytophthora cinnamomi*, diseased plants showed a reduction in stomatal conductance and a gradual decline in photochemical efficiency of photosystem II (dark-adapted Fv/Fm) compared to control seedlings (Luque et al., 1999). In contrast, inoculation of *P. ramorum* in three different woody species resulted in reduced photosynthetic Rubisco capacity (Vcmax) which occurred prior to any detectable loss in water transport capacity. Exposure of foliage to *P. ramorum*-derived protein elicitins caused a similar reduction in photochemical efficiency, indicating that the effects in this case were related to pathogen-derived elicitins and not water status (Manter et al., 2007). In *Fagus* spp. inoculated with *P. citricola*, reduction in photosynthesis occurred prior to the reduction in photochemical efficiency, and also prior to detectable differences in leaf water potential and plant water consumption. In this study elicitins produced by *P. citricola* were isolated and characterized, but had no effect when applied to beech plants or beech cell suspensions (Fleischmann et al., 2005). In another study, application of purified elicitins from a *Phytophthora* pathogen to foliage did not result in reduced stomatal conductance in leaves while application of a drought related hormone (ABA) did (Maurel et al., 2004). Finally, Clemenz et al (2008) found that basal inoculation of *Alnus glutinosa* with *P. alni* subsp. *alni* led to reduced carbon assimilation and transpiration, and increased foliar starch content, but had no effect on water use efficiency or minimum leaf water potential. These authors suggest that the accumulation of starch in foliage was due to impaired movement of photosynthates in damaged phloem tissue, and this led to feedback inhibition of photosynthesis prior to moisture stress. It appears that the exact mechanism causing the reduction of carbon assimilation and stomatal conductance in *Phytophthora*-canker stricken trees is not clear, or varies depending on the specific host/pathogen combination.

The objectives of this study were a), to characterize the effects of *P. cactorum* stem-infection on photosynthetic performance in *A. rubrum* (L.) (red maple) seedlings in comparison with findings from previous studies on different combinations of host plant and *Phytophthora* spp., and b), to suggest potential underlying mechanisms by examining the photosynthetic physiology of diseased and healthy seedlings.

2.2 Materials and Methods

2.2.1 Plant materials

Acer rubrum seedlings used in this experiment were seed-grown 2-year-old plants (15-30 cm bare-root seedlings) obtained from Lawyer Nursery, Olympia, WA, and planted on 15 June, 2011 at the University of Washington Center for Urban Horticulture, Seattle, WA. Root systems were washed, cut to a uniform length of 15-cm, and planted in 2.54-L tree pots containing Sunshine Professional Growing Mix #2 (75-85% Canadian sphagnum peat moss, horticultural grade perlite, dolomitic limestone; Sun Gro Horticulture, Canada Ltd.). Maple seedlings in the infected group were inoculated on 18 July, and all seedlings were grown under greenhouse conditions with average temperature and average relative humidity maintained at 20.7°C and 53.09%, respectively, until harvest 108 days post-inoculation. Maple treatment groups each consisted of 20 seedlings, arranged on a greenhouse table in a completely random design. All plants were monitored daily, watered by hand as needed at 1-2 day intervals to maintain near-constant field capacity conditions, and fertilized weekly with Hoagland's #2 nutrient solution mixed at half strength as described in (Kinmonth-Schultz and Kim, 2011).

2.2.2 Pathogens

The isolate of *P. cactorum* used in this experiment was isolated through direct plating of phloem tissue from the margin of necrotic lesions that were associated with symptoms of 'bleeding cankers' typical of *Phytophthora* spp. infections on a red maple (*Acer rubrum,* Indian Land, SC). The isolate was identified by morphology by D. Zwart (Erwin and Ribeiro, 1996; Stamps et al., 1990). Briefly, the isolate produced distinctly papillate, variable shaped sporangia in culture, terminally borne singly or in clusters.

Hyphal-tip subcultures of the isolate readily produced oospores (homothallic) which were pluerotic and smooth-walled. Antheridia were single celled and paragynous.

2.2.3 Inoculation

A stem-wound technique was employed to introduce the pathogen into seedlings in the inoculated treatment. A 5-mm diameter disc of bark and phloem was peeled back from the lower stem using a sterile cork borer and razor blade. Immediately following wound creation, a 5-mm diameter agar plug was taken from the margin of an actively growing colony of *P. cactorum* and placed on the wound with the upper surface facing inwards. A sterile agar plug was placed on the wound of non-inoculated control treatments. All inoculation sites were immediately wrapped with moist sterile gauze followed by a layer of parafilm in order to maintain adequate moisture for infection and then covered with a layer of aluminum foil to exclude light.

Cultures of the pathogen were isolated and maintained in the dark on a clarified V-8 juice agar (PARP) selective for the culture of Oomycete organisms (Jeffers and Martin, 1986).

2.2.4 Physiological responses

Gas exchange parameters were assessed using a LiCor LI-6400 portable photosynthesis system (LiCor, Lincoln NE) at bi-weekly intervals. At each sampling date, a 6 cm^2 portion excluding the mid-vein of the uppermost fully expanded green leaf was assessed for maximum net carbon assimilation rates (A_{max} or A_n) and stomatal conductance (g_s). Gas exchange measures were made under set conditions of 400 ppm CO_2, 25 °C (block temperature), 45-65 % relative humidity, flow rate of 300 μmol s^{-1}, and photon flux density of 1500 μmol photon m^{-2} s^{-1}, as regulated by the LI-6400 unit. Measurements were made between 700-1300 PDT, and all plants were allowed to stabilize for at least 10 minutes prior to data recording.

Measures of chlorophyll fluorescence (dark adapted Fv/Fm) were taken to monitor the quantum yield and photochemical efficiency of photosystem II (PS-II), and as an indicator of overall plant stress as described in Percival (2005). Briefly, at each sampling date, leaves were adapted to darkness for at least 40 minutes using light exclusion clips placed on the uppermost fully expanded green leaf, in most cases the same leaf used in gas exchange measures. Chlorophyll fluorescence was measured with a HandyPEA portable fluorescence spectrometer (Hansatech Instruments Ltd., King's Lynn, U.K.), and data were collected up to 1 s with a data acquisition rate of 10 µs for the first 2 ms and 1 ms for the remainder. The fluorescence responses were induced by a red (peak at 660 nm) light with photon flux density of 1500 µmol photon m^{-2} s^{-1} provided by an array of six light-emitting diodes.

Analysis of foliar starch content was assessed on upper, fully expanded leaves that were collected prior to harvest at the end of the experiment. Samples were placed in small sealable plastic bags and immediately frozen at -80° C. Samples were packed in dry ice, and shipped overnight to the laboratory of Dr. Richard Sicher (ARS-USDA, Beltsville, MD) where samples were freeze-dried and analyzed for starch as mg per gram of fresh weight in hexose equivalents. Starch levels were assessed using a solvent extraction procedure, as described in previous studies by Dr. Sicher (Sicher and Bunce, 2011) and others (Hendrix, 1993).

Lesion size and biomass were assessed at harvest which took place 108 days after inoculation. Vertical and horizontal advance of the pathogen, as evidenced by expansion of the necrotic lesion, were measured at the conclusion of the experiment. Lesion measurements were made at the edge of discolored, intact bark tissue. Vertical expansion was recorded as total length of the necrotic region, and horizontal expansion was recorded as the percentage of stem circumference girdled by the necroses. Removal of bark and examination of necrotic phloem tissue confirmed that the margin of discolored bark was indicative of the margin of the underlying necrotic lesion originating from the original inoculation point. Following harvest, 5 plants from each treatment group were assessed to confirm pathogen colonization. Tissue from the margin of the necrosis was placed on PARP-V8 agar

(Jeffers and Martin, 1986) and isolate identity was verified morphologically by D. Zwart (Stamps et al., 1990).

At the conclusion of the trial following lesion measurements and re-isolation, all seedlings were dried at 65° C for at least 72 hr. Root and stem portions were separated and weighed. Leaf weight was not included because senescence and dropping of leaves occurred during the course of the experiment but was not recorded.

2.2.5 Statistical analysis

All statistical analyses were conducted using SigmaPlot 12.0 for windows statistical software (Systat Software Inc., San Jose, CA). The type I error rate (α) was set at 0.05 for all treatment comparisons. Lesion size, biomass, gas exchange parameters, starch content, and chlorophyll fluorescence data were analyzed using the Student's t-test, with inoculation status as the main factor. When data failed the Shapiro-Wilk test for normality, treatment group means were compared using the Mann-Whitney rank sum test (Zar, 1999). Physiological data were analyzed in two ways: as raw values of treatment group means, and by assessing the percent reduction in various parameters compared to pre-inoculation levels on each plant. In this manner, seedlings were used as their own controls to determine the mean change in assimilation, conductance, or photosynthetic efficiency in plants compared to pre-inoculation levels in the same plant using the formula:

$$[(\text{value at time } t - \text{Pre-treatment value}) / \text{Pre-treatment value}]$$

Each treatment group was comprised of 20 seedlings, but due to time constraints, bi-weekly gas exchange measures usually included only 16-18 seedlings from each treatment group.

2.3 Results

Attempts to re-isolate *P. cactorum* from margins of necrotic lesions were successful in all cases where necrosis was evident. *Phytophthora cactorum* was not isolated from any non-inoculated plants, nor did necrotic lesions form on these control plants. Callus tissue had overgrown the wound site on all control (non-inoculated) plants. The mean lesion size on the inoculated plants was 30.2-mm of vertical expansion and 80.0 % girdling of the stem at the point of inoculation.

2.3.1 Effects of inoculation on biomass accumulation

Seedlings in the inoculated group were reduced in mean biomass accumulation compared to the control seedlings. The total biomass of all seedlings was normally distributed (Shapiro-Wilk: $p = 0.866$) and equal in variance ($p = 0.959$) among groups. Seedlings in the control group produced significantly more total biomass ($p < 0.001$; t = 4.528, 38 d.f.), with a mean in control seedlings of 26.62-g (s.e. ± 1.31) compared to 17.56-g (s.e. ± 1.51) in the inoculated treatment. Mass of stem portions alone was also recorded because root system growth may have been altered by contact with pots in all seedlings. Mean stem mass of all seedlings was normally distributed (Shapiro-Wilk: $p = 0.183$) but was not equal in variance ($p < 0.05$). Mean stem mass among treatments was significantly different ($p < 0.001$; Mann-Whitney U-statistic = 54.0), with control plants (8.23-g ± s.e. 0.30) producing significantly more stem mass than inoculated seedlings (5.22-g ± s.e. 0.53).


Figure 2.5. Typical callus over wound site of control (left), and non-callused inoculation site (right) with necrotic discoloration.

2.3.2 Effects of inoculation on carbon assimilation

For carbon assimilation data, comparisons were made on means of raw values (Figure 2.1) and mean percent reduction in A_{max} compared to pre-inoculation levels. Prior to inoculation, mean values for maximum rates of assimilation of carbon dioxide (A_{max}) did not differ among treatment groups ($p = 0.906$; t = 0.119, 37 d.f.) and were 8.44 µmol $m^{-2} s^{-1}$ and 8.379 µmol $m^{-2} s^{-1}$ for control and inoculated treatment groups, respectively. One week after treatment, A_{max} was reduced in both groups compared to pre-treatment, but significantly more so in the inoculated seedlings. The control treatment was reduced by 17.1 % compared to a reduction of 35.8 % in the inoculated treatment ($p = 0.0236$), with mean A_{max} values of 7.195 µmol m^{-2} s^{-1} in the control treatment and 5.294 µmol m^{-2} s^{-1} in the inoculated treatment ($p = 0.008$; t = 2.803, 37 d.f.).

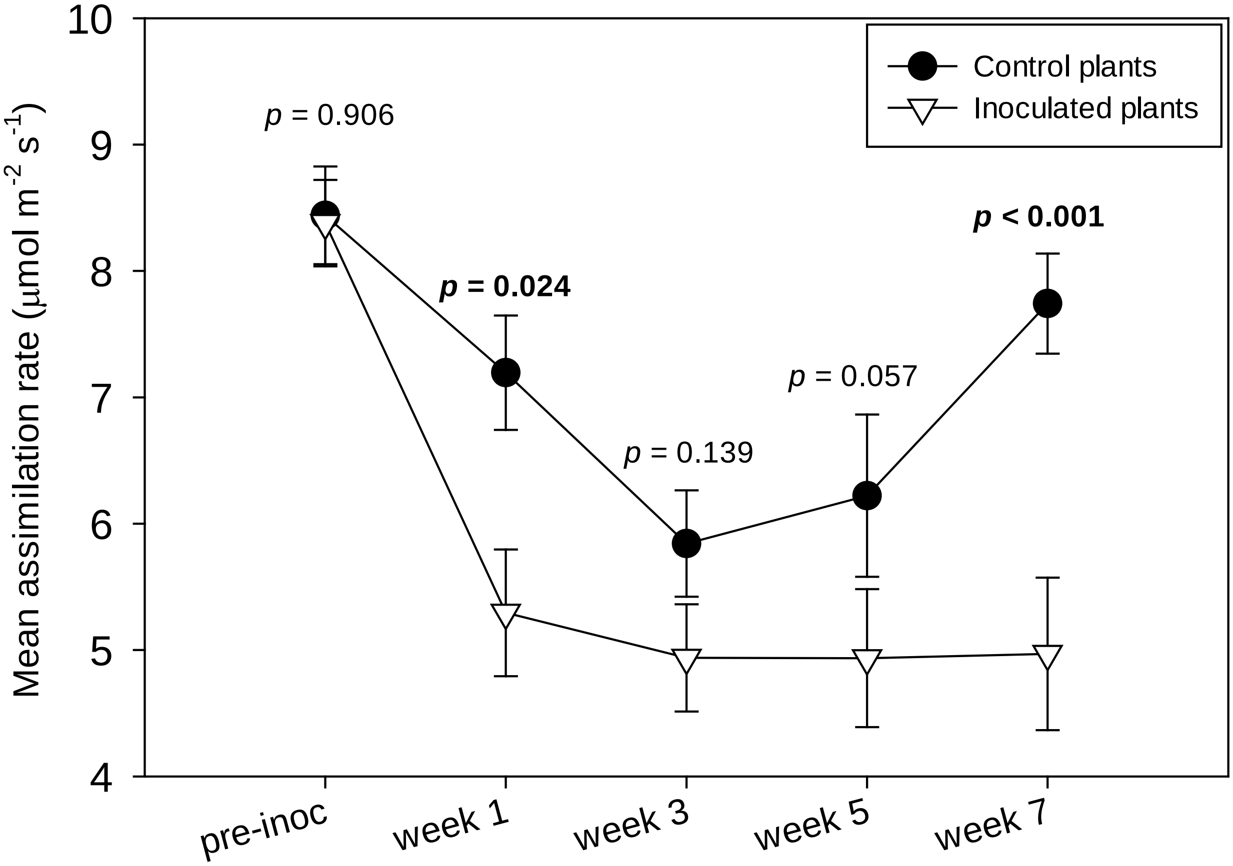

Figure 2.1. Mean maximum assimilation rates of carbon dioxide (µmol m^{-2} s^{-1}) over time in *Acer rubrum* seedlings stem inoculated with *Phytophthora cactorum* (inoculated plants) or mock-inoculated with a sterile agar plug (control plants). *P* – values are the result of Student's t-test between treatment groups for that week. Bars are on standard error.

Three weeks after inoculation, A_{max} rates were reduced by 26.2 % and 39.5 % compared to pre-inoculation levels in the control and inoculated groups, respectively ($p = 0.0998$), with mean assimilation rates of 5.842 µmol m^{-2} s^{-1} in control plants and 4.938 µmol m^{-2} s^{-1} in inoculated plants ($p = 0.139$; t = 1.512, 37 d.f.). At five weeks post-inoculation, mean A_{max} rates in control plants were reduced by 20.4 % while inoculated plants mean A_{max} was reduced by 38.4 % compared to pre-inoculation levels ($p = 0.136$)

with group means of 6.594 µmol m^{-2} s^{-1} in control plants and 4.936 µmol m^{-2} s^{-1} in inoculated plants (p = 0.0567; t = 1.975, 33 d.f.). At the last date of data collection, seven weeks following inoculation, mean A_{max} rate in the control treatment was reduced by 7.59 % while mean A_{max} in inoculated group was reduced by 36.4 %, compared to pre-inoculation levels (p = 0.0236), with mean rates of 7.741 µmol m^{-2} s^{-1} in the control group and 4.969 µmol m^{-2} s^{-1} in the inoculated group (p < 0.001; t = 3.839, 34 d.f.). At the conclusion of the experiment, mean A_{max} rates in control seedlings were not significantly different from pre-treatment values (p = 0.215), while mean A_{max} rates in inoculated seedlings remained significantly lower than pre-treatment values (p < 0.001), indicating that assimilation had recovered in control plants, but not in inoculated plants.

2.3.3 **Effects of inoculation on stomatal conductance**

For stomatal conductance, comparisons were made on means of raw values as pre-treatment levels of this parameter were not different between treatments (Figure 2.2). Prior to inoculation, stomatal conductance to carbon dioxide (g_s) did not vary among treatment groups, with mean values of 0.0895 µmol m^{-2} s^{-1} and 0.0913 µmol m^{-2} s^{-1} in control and inoculated groups, respectively (p = 0.85; t = -0.191, 37 d.f.). One week after inoculation, g_s in control plants was significantly greater than in inoculated plants, with mean of 0.0843 and 0.0599 µmol m^{-2} s^{-1} respectively (p = 0.0066; t = 2.877, 37 d.f.). At three weeks and five weeks post inoculation, mean values of g_s were slightly higher in the control group compared to the inoculated group, however these differences were not significant (week 3: p = 0.786; week 5: p = 0.677). At seven weeks post-inoculation, mean g_s in the control group (0.11 µmol m^{-2} s^{-1}) was significantly greater than in the inoculated group (0.0731 µmol m^{-2} s^{-1}) (p = 0.003). At this time mean g_s in the control seedlings had surpassed pre-treatment values, but not significantly so (p = 0.084), while mean g_s in inoculated seedlings remained lower than pre-treatment levels (p = 0.048), indicating a full recovery of conductance in control seedlings but not in inoculated seedlings.

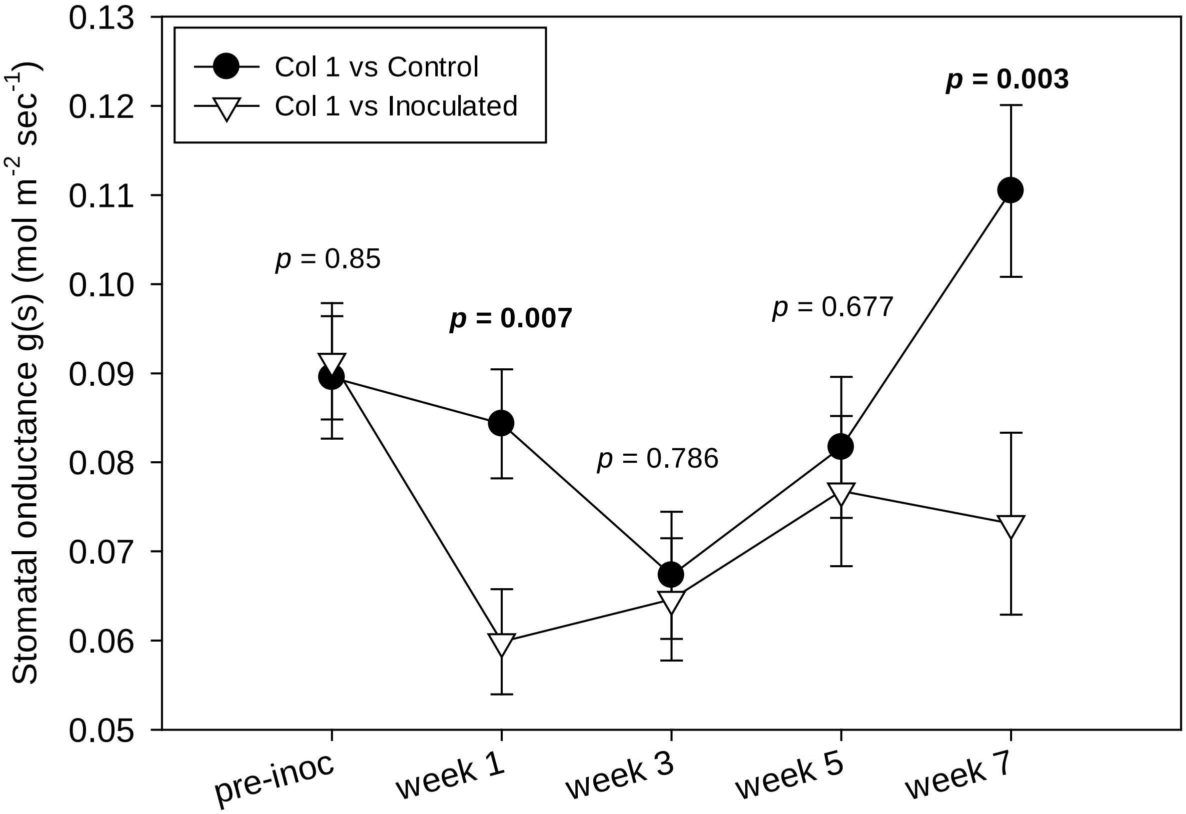

Figure 2.2. Mean stomatal conductance (mol m-2 s-1) over time in *Acer rubrum* seedlings stem inoculated with *Phytophthora cactorum* (inoculated plants) or mock-inoculated with a sterile agar plug (control plants). *P* – values are the result of Student's t-test between treatment groups for that week. Bars are on standard error.

2.3.4 Relationship between carbon assimilation and stomatal conductance

The ratio of stomatal conductance to assimilation rate, an indicator of intrinsic water use efficiency (iWUE) was also examined (Figure 2.3, Table 1). This parameter was highly variable, particularly in the inoculated treatment group. Overall, there was a significant effect of treatment (inoculation) on iWUE (p = 0.019), but this effect did not change over time (p = 0.631), nor was there an interaction between treatment and time after treatment (p = 0.858) (Table 1). Mean values for iWUE were higher in inoculated plants than control plants in all weeks of data collection, but due to high variability, these differences were not significant between treatments at any time (t-test: Week 1, *p* = 0.182; Week 3, *p* = 0.595; Week 5, *p* = 0.125; Week 7, *p* = 0.912) (Figure 2.3).

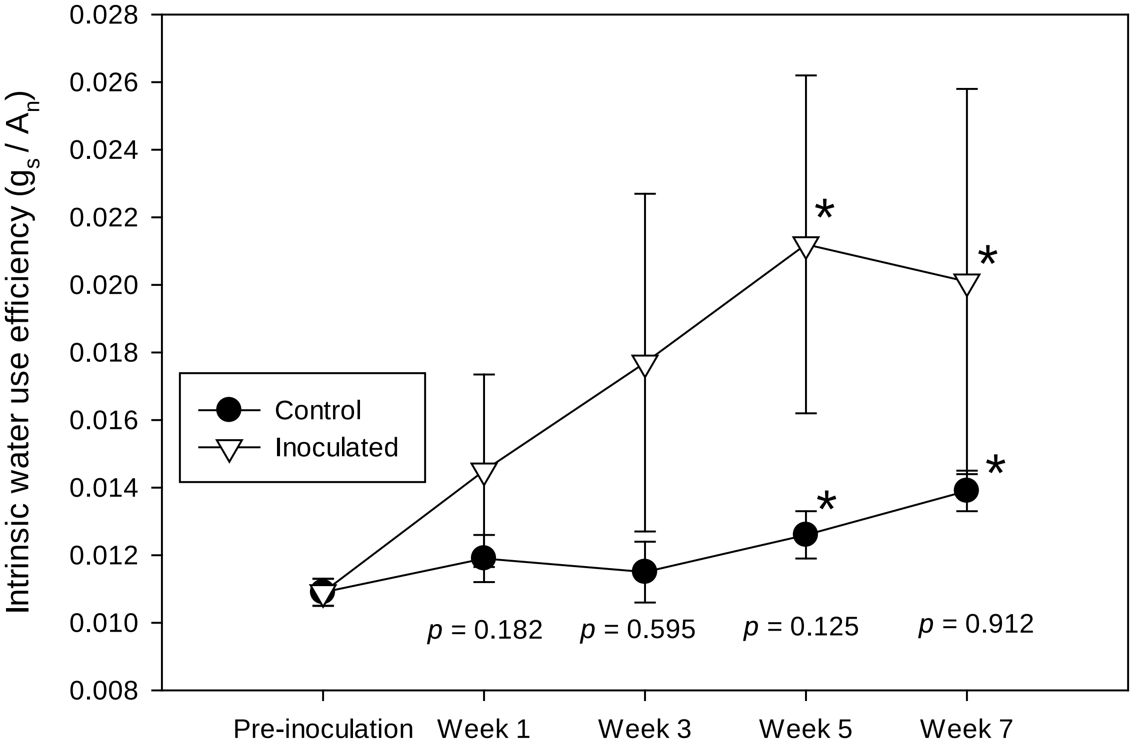

Figure 2.3. Intrinsic water use efficiency (conductance / assimilation) over time of *Acer rubrum* seedlings that were stem-inoculated with *Phytophthora cactorum* (inoculated) or mock-inoculated with an agar plug (control). *P*-values are the result of Student's t-test between treatments for each week. Asterisks indicate that the data point is different from pre-treatment values for that treatment group ($p \leq 0.05$).

Table 2.1. Two-way analysis of variance in iWUE between *Acer rubrum* seedlings inoculated with *Phytophthora cactorum* or non-inoculated control. Inoculation status was the 'treatment' variable and weeks post-inoculation was the 'week' variable.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Week	3	0.0004	0.00013	0.577	0.631
Treatment	1	0.0013	0.00127	5.642	**0.019**
Week x Treatment	3	0.0002	0.00005	0.254	0.858
Residual	138	0.031	0.00022		
Total	145	0.033	0.00023		

2.3.5 Efficiency of photosystem II

Dark adapted Fv/Fm values, a measure of maximum photosynthetic efficiency, were reduced in both groups following treatment (inoculation or wounding for control). Because mean Fv/Fm values were significantly different between treatment groups prior to inoculation (control = 0.773, inoculated = 0.790; $p = 0.041$), the differences in reduction of photosynthetic efficiency between treatment groups were compared by calculating the percent reduction from pre-treatment means for each week and treatment (Figure 2.4).

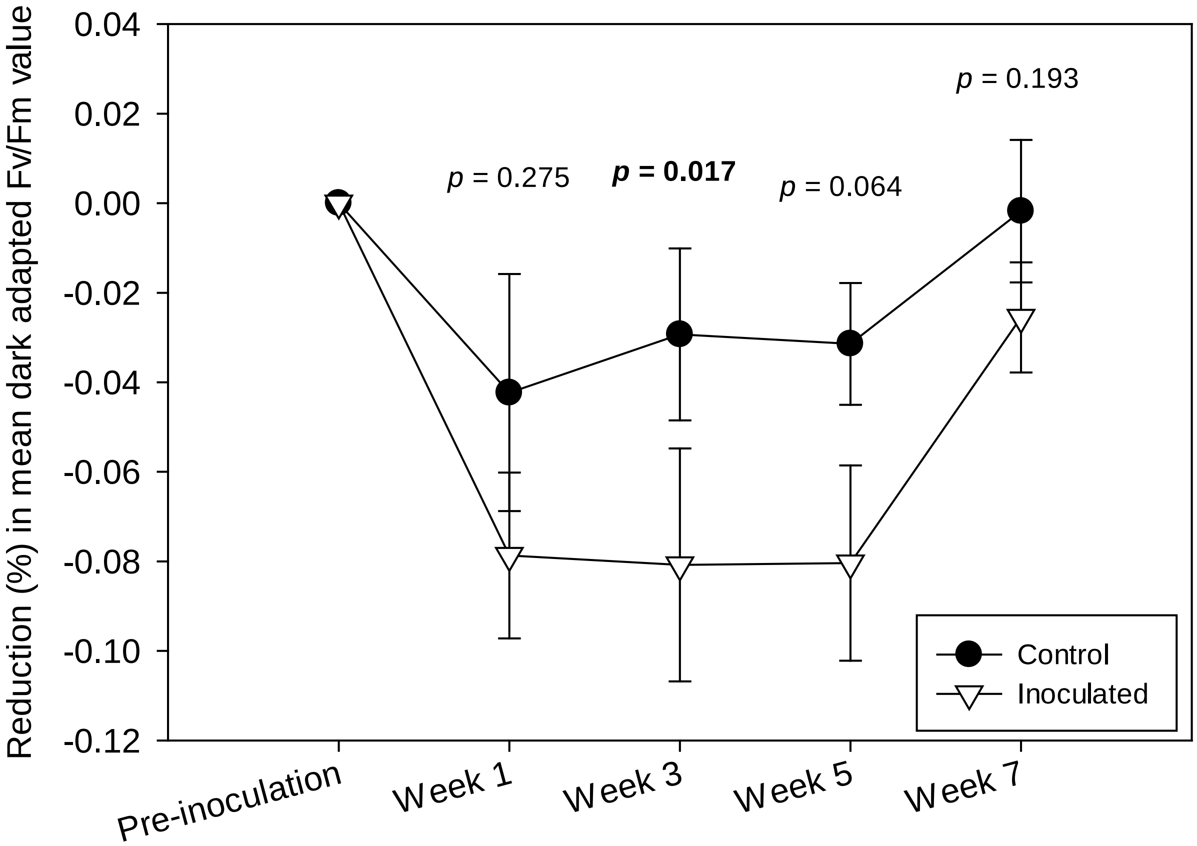

Figure 2.4. Mean reduction (%) in photosynthetic efficiency of PS-II (dark adapted Fv/Fm) over time in *Acer rubrum* seedlings stem-inoculated with *Phytophthora cactorum* (inoculated) or mock-inoculated with an agar plug (control). *P* – values are results of Student's t-test between treatment groups for that week. Bars = one standard error.

One week after treatments, mean Fv/Fm values were significantly reduced in both treatment groups compared to pre-treatment levels (control *p* = 0.017, inoculated *p* < 0.001); mean Fv/Fm was reduced by 4.23 % in control seedlings and 7.87 % in inoculated seedlings, but these reductions were not significantly different between treatments (*p* = 0.275; t = -1.126, 18 d.f.). Three weeks after treatment, mean Fv/Fm values were significantly reduced in both treatment groups compared to pre-treatment values for that group (control *p* = 0.025, inoculated p < 0.001); mean Fv/Fm was reduced in control seedlings by 2.93 % and by 8.08 % in inoculated seedlings, and this reduction in inoculated seedlings was significantly greater than in control seedlings (*p* = 0.017, Mann-Whitney U test statistic = 18.0). Five weeks after treatment, mean Fv/Fm values were significantly reduced in both treatment groups compared to pre-treatment values for that group (control *p* = 0.031, inoculated *p* < 0.001); mean Fv/Fm was reduced by 3.14 % in control seedlings and by 8.04 % in inoculated seedlings, and this reduction in inoculated seedlings was marginally significant compared to control seedlings (*p* = 0.064, Mann-Whitney U test statistic = 25.0). Seven weeks after treatment, mean Fv/Fm in control seedlings was 0.175 % higher than the pre-treatment mean for that group, but the difference was not statistically significant (*p* = 0.94), indicating a full recovery of PS-II efficiency. The mean raw-value for Fv/Fm of inoculated seedlings at this time remained significantly lower than the pre-inoculation mean for that group (*p* = 0.04), and was reduced by 2.55 % compared to the mean pre-inoculation value. However, the slight increase (+ 0.175%) in Fv/Fm value at week seven in control plants compared to pre-treatment levels was not significantly different than the slight reduction (- 2.55%) in inoculated plants compared to pre-treatment levels (*p* = 0.193).

2.3.6 Foliar starch analysis

Based on leaf samples taken at the termination of the experiment, mean foliar starch content of leaves in the control group was 120.08 mg/g DW (± s.e. 19.5) while mean foliar starch content of leaves in the inoculated group was 67.45 mg/g DW (± s.e. 8.2). Foliar starch content values were not normally distributed ($p < 0.05$; Shapiro-Wilk normality test). Based on the Mann-Whitney rank sum test, foliar starch levels were significantly higher in the control seedlings compared to the inoculated seedlings ($p = 0.025$; Mann-Whitney U test statistic = 110.0).

2.4 Discussion

The results of this study are consistent with several other studies on canker-causing *Phytophthora* species in tree seedlings (Clemenz et al., 2008; Fleischmann et al., 2005; Luque et al., 1999; Manter et al., 2007), demonstrating a reduction in carbon assimilation, stomatal conductance, and/or maximum photosynthetic efficiency of PS-II following inoculation, in comparison to control plants (wounded but non-inoculated) and in comparison to pre-inoculation levels in the same plants. Comparison to pre-inoculation levels of each parameter helps to determine the effect of pathogen ingress on these functions, while comparison to control plants is necessary to separate the effects of the pathogen from any alterations to these parameters resulting from the stem-wounding inoculation technique.

The conductance of stomata to carbon dioxide is considered to be the most important factor in limiting the rate of photosynthesis (Flexas et al., 2004), and is controlled by stomatal aperture. Stomatal aperture also controls the rate of transpiration, or water loss, and consequently is tightly linked with soil or plant water status (Lambers et al., 2008). Intrinsic water use efficiency (iWUE), or the ratio between conductance and assimilation, provides a measure of the relationship between these two factors and can give insight into plant carbon and water relations. In addition to these parameters of gas exchange, dark adapted Fv/Fm values represent the maximum efficiency of PS-II, and can also be used as a comparative indicator of overall plant stress (Baker, 2008; Maxwell and Johnson, 2000; Percival, 2004). In the present experiment, all physiological factors under investigation were affected to some degree by wounding or inoculation. Comparison of these effects between treatment groups revealed notable differences in the temporal pattern and magnitude, and these differences give some insight into the underlying physiological mechanisms.

One week after treatment, maximum carbon assimilation rates (A_{max}) and stomatal conductance (g_s) were reduced in both treatment groups compared to pre-treatment levels, but significantly more so in the inoculated plants. Also, at one week after treatment, the reduction in maximum photosynthetic efficiency of PS-II compared to pre-treatment levels in inoculated plants was not different from the reduction in control plants ($p = 0.275$), indicating that the cause of reduction in Fv/Fm was not related to the presence of the pathogen. The wounding process, which damaged roughly 20 % of the stem circumference, possibly in combination with a slight increase in temperature as summer progressed, may have caused some of the reduction in A_{max}, g_s, and Fv/Fm in both treatment groups. The hormonal response of plants to wounding may also have played a role in the reduced photosynthetic efficiency, A_{max}, and g_s. Previous studies have shown that wounding may result in rapid decreases in photosynthetic efficiency (Barron-Gafford et al., 2012), and a methyl jasmonate signal-related reduction in stomatal conductance and carbon assimilation (Suhita et al., 2004). Methyl jasmonate and ethylene are well studied plant hormones that act as signals following physical damage, and can reduce stomatal aperature independently of the drought-related hormone, abscisic acid (ABA) (Cho et al., 2012; Delaney, 2012). The relation between g_s and A_n (iWUE) was not significantly different between treatments after one week, though this value was increased throughout the experiment in the inoculated group, as would be expected if hydraulic signals resulting from xylem damage or colonization caused the reductions in A_n and g_s. The fact that inoculation significantly increased the relative reduction in A_{max} and g_s compared to wounding alone one week after inoculation, but did not alter the reduction in Fv/Fm or significantly affect the relationship between A_n and g_s (iWUE) after one week indicates that some factor associated with the pathogen reduced conductance and assimilation but did not impact photosynthetic function or damage photosynthetic apparatus.

While care was taken to remove bark and phloem tissue exclusively, it is unlikely that xylem was completely undamaged and this was likely a factor in the decline of g_s, A_{max}, and Fv/Fm in both treatment groups, in addition to the plant wound response. In addition, one week after inoculation it is unlikely that the pathogen had colonized xylem tissue or progressed beyond the point of inoculation, which implicates pathogen-derived elicitins as a potential cause of the greater relative effect on A_{max} and g_s but not photosynthetic function in inoculated plants when compared to wounded, non-inoculated plants. *Phytophthora cactorum* is known to produce a variety of elicitin and effector proteins (Dubery et al., 1994; Orsomando et al., 2001) which may either induce or prevent plant resistance reactions. The induction of defense responses by Phytophthora-derived elicitins has been shown to quickly but temporarily reduce photosynthesis (Scharte et al., 2005).

At three weeks post-treatment, mean A_{max} in plants of both treatment groups were reduced compared to pre-treatment levels, and remained marginally higher in control than in inoculated plants ($p = 0.139$). Similarly, the decline in g_s in both treatment groups continued into week three, and at this sampling time mean g_s in control plants was slightly higher than in inoculated plants, but not significantly so ($p = 0.786$). Intrinsic water use efficiency three weeks after treatment was not different from pre-treatment levels in control or inoculated seedlings, nor did the groups differ from each other, though iWUE was slightly higher in inoculated plants. However, three weeks after inoculation, in comparison with pre-treatment values, Fv/Fm values in control seedlings had recovered slightly (-4.23 % to -2.93 %), while values in inoculated seedlings declined slightly (-7.87 % to -8.08 %), resulting in a significant difference in reduction of Fv/Fm between treatments ($p = 0.017$). These data further support the idea that wounding caused much of the reduced assimilation in week 1, and that elicitins further reduced g_s and A_{max} in inoculated plants without damaging overall photosynthetic capabilities. By week

3, wounding was affecting assimilation equally in both treatment groups, and at this time the slight differences in Fv/Fm may have been related to pathogen signals and the negative effects on water relations caused by the increasing development of necrotic lesions.

At five weeks after treatment, A_{max} rates and g_s levels had started to recover toward pre-treatment values in control plants while A_{max} rates in inoculated plants remained steadily depressed and g_s remained lower in inoculated plants compared to control, though the difference was not significant (p = 0.677). In addition, mean g_s in inoculated plants was slightly increased compared to week 3 (+0.122 µmol m^{-2} s^{-1}, p = 0.268), while mean A_{max} in these plants was virtually unchanged from the previous sample date (-0.002 µmol m^{-2} s^{-1}, p = 0.997). The reduction in efficiency of PS-II compared to pre-treatment was also marginally greater (p = 0.064) in inoculated seedlings (-8.04 %) than in control seedlings (-3.14%). Taken together, these data indicate that by five weeks after treatment, recovery from wounding was occurring in control seedlings, as evidenced by recovery in g_s, A_{max}, and Fv/Fm values. Recovery likely included replacement of damaged xylem tissue and replacement of phloem or re-routing of assimilates around damaged phloem. In inoculated seedlings, the rate of assimilation remained depressed. The fact that iWUE was marginally higher at this time in the inoculated group compared to the control group (p = 0.125) indicates that the inoculated seedlings were slightly moisture-stressed compared to controls, which might be due to xylem colonization and blockage, or lack of repair of previously damaged tissue. In addition, defense metabolism instigated by the presence of the pathogen is another likely cause of reduced assimilation as production of defense compounds has been shown to reduce net assimilation rates (Scharte et al., 2005).

At the final sampling date seven weeks after treatment, g_s and A_{max} in control seedlings had returned to pre-treatment levels, while g_s and A_{max} in inoculated seedlings remained significantly lower than pre-treatment levels, and at this time both g_s and A_{max} in inoculated seedlings was significantly lower than the control seedlings. However, neither Fv/Fm nor iWUE differed between treatments although iWUE in inoculated seedlings continued to increase in comparison to control seedlings, indicating moisture stress, but due to high variability, this difference was not significant.

The reduction and subsequent recovery of all physiological parameters in control plants supports the implication of slight physiological stress, possibly as a result of xylem damage and subsequent hydraulic signals controlling assimilation in control seedlings, and in response to physical wounding and subsequent chemical signaling. Notably, wound sites on all control plants had callused over by the end of the experiment, and these data reflect a replacement or return to function of damaged xylem and phloem tissue. A similar recovery of Fv/Fm in inoculated plants, along with a lack of recovery in assimilation and stomatal conductance in inoculated plants and the increased magnitude of the initial reductions compared to control plants, suggests that some factor in addition to wounding and environmental conditions was affecting these parameters. Recovery of photosynthetic efficiency assessed by Fv/Fm in inoculated plants was not concomitant with recovery of assimilation rates, and the trend in the relation of g_s to A_{max} as shown in iWUE would suggest some moisture stress. In contrast to the wound-only control plants, all inoculated plants developed some degree of necrosis around the inoculation area and callus tissue formation was limited or absent. In fact, on average 80% of the stem circumference in inoculated seedlings was necrotic. Considering that inoculation did increase iWUE in the inoculated group compared to control over the entire experiment, but iWUE was not significantly different between treatments in any given week, it is likely that the pathogen was predominantly killing phloem tissue, and had only affected xylem conductivity slightly. Clemenz et al. (2008) also found that WUE did not change in response to stem infection while other factors such as A_n and g_s were reduced. This may lead to the conclusion that the reduction of assimilation without parallel damage to the photosynthetic process was caused by a feedback inhibition of g_s and A_{max} in inoculated seedlings as a result of phloem blockage and a lack of transport of carbohydrates to the roots. Increased non-structural carbohydrates in *Acer* spp. foliage as a result of girdling has been shown previously (Murakami et al., 2008), and reduced assimilation as a result of girdling and increased foliar non-structural carbohydrates has been shown in many woody plant species (Batista et al., 2012; Cheng et al., 2008; Domec and Pruyn, 2008; Vemmos et al., 2012). However, foliar starch analysis did not support this hypothesis, with higher levels of starch found in control plants, exactly opposite of the expected results if feedback inhibition were the underlying mechanism causing reduced photosynthesis. The starch content of leaves at the conclusion of the experiment was more clearly linked to the elevated assimilation rates in the control group as opposed to phloem blockage or death as a result of the pathogen activity in the inoculated treatment, indicating that the ability of the chloroplasts to store starch had not been exceeded to the point of feedback inhibition.

These results are more consistent with an initial reduction in photosynthesis caused by the wound response as a result of the inoculation process, followed by sustained reduction in photosynthesis caused by the lingering presence of the pathogen in the inoculated group and the related hormonal signals. After just one week following treatment, the data suggest that the major cause of reduced assimilation in both treatment groups was wounding involved in the inoculation or mock-inoculation process, which likely damaged xylem tissue and caused a systemic wound-response, resulting in hydraulic effects related to reduced water-transport, and direct wound-related hormonal signaling to stomata. The greater magnitude of reduced assimilation in inoculated seedlings may be attributed to a more rapid signal, such as pathogen-derived elicitins, as suggested by Manter et al. (2007), who demonstrated that severed stems submerged in an elicitin solution showed reduced Fv/Fm values after just 48 hours. Previous research has also demonstrated that assimilation is reduced in tobacco cells upon introduction of *Phytophthora*-derived elicitins and this shift from assimilation to respiratory activity is necessary for the induction of the defense response (Scharte et al., 2005).

In conclusion, we have shown that stem inoculation of *A. rubrum* seedlings with *P. cactorum* leads to a rapid and sustained reduction in carbon assimilation and stomatal conductance, while inoculation caused an ephemeral reduction in photosynthetic efficiency of PS-II, and had slight effect on intrinsic water use efficiency. In addition, the rapid reduction in A_n and g_s following inoculation was of a greater magnitude than the reduction caused by wounding alone, indicating that a pathogen derived signal was probably involved. The sustained reduction in A_n and g_s in inoculated plants, in comparison with the recovery of those factors in wounded plants, support additional mechanisms playing a role in reduced assimilation later in disease development. The possibility of feedback inhibition of photosynthesis due to phloem death and reduced transport was not supported by foliar starch data. Slight moisture stress resulting from damaged or blocked xylem tissue, combined with reduced stomatal conductance as a result of pathogen derived elicitins or pathogen induced hormonal signaling is the likely cause of longer term sustained reductions of assimilation in inoculated seedlings.

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CHAPTER 3

Biochar amendment increases resistance to stem-lesions caused by *Phytophthora* spp. in tree seedlings

3.1 Introduction

Plant diseases caused by organisms in the genus *Phytophthora* negatively impact nursery stock, field crops, tree crops, and forest systems (Erwin and Ribeiro, 1996; Hansen et al., 2008). *Phytophthora* diseases are also widespread and damaging to woody plants that are commonly found in managed landscapes. In trees, *Phytophthora* pathogens can cause fine root disease, root collar or crown rots, and trunk or stem lesions that are often referred to as 'bleeding cankers' (Erwin and Ribeiro, 1996). Physiologically, stem canker-causing *Phytophthora* species (e.g., *P. cinnamomi* and *P. cactorum*) are known to kill phloem, leading to plant death through girdling, and also to colonize and block xylem, leading to altered plant water relations (Brown and Brasier, 2007).

Chemical and non-chemical management options are+ increasingly being sought to preserve valuable infected specimen trees and protect non-infected hosts. Chemical products containing phosphorous acids or derivatives have often proven most effective and are widely recommended for use against *Phytophthora* bleeding cankers (Garbelotto et al., 2009; Weiland et al., 2009). Systemic induced resistance (SIR) is the mechanism underlying *Phytophthora*-disease reduction or prevention following treatment with phosphorous acids (Daniel and Guest, 2006; Daniel et al., 2005; Jackson et al., 2000). As a systemic treatment, this material has also proven effective in reducing plant damage caused by several fungal pathogens (Agostini et al., 2003; Elliott and Edmonds, 2008; Percival and Noviss, 2010). Recently, Elad et al. (2010) showed that incorporation of biochar into potting mix of pepper (*Capsicum annuum* cv. Maccabi) and tomato (*Lycopersicum esculentum* cv. 1402) reduced the disease severity caused by two foliar pathogens and damage from a broad mite pest (Elad et al., 2010). In addition, Harel et al. (2012) showed that incorporation of biochar reduced the damage caused by three foliar pathogens of strawberry (*Fragaria* x *ananassa* cv. Yael). Results in both studies were attributed to biochar-induced systemic resistance due to the reduction in disease caused by pathogens exhibiting both necrotrophic and biotrophic strategies, and reduction of damage caused by an arthropod pest. These effects may have been caused by direct interactions between the plant and the biochar, or may be the result of biochar-related alterations in the soil microbial community (Elad et al., 2011; Kolton et al., 2011; Warnock et al., 2007). If SIR was the cause of the observed decrease in disease severity, similar results may be possible in defense against *Phytophthora* pathogens. The potential for biochar incorporation as a disease management option has only recently been suggested and research to date is limited (Elad et al., 2011; Lehmann et al., 2011).

The objective of the present study was to determine if biochar amendment of a soilless potting media can reduce the development and impact of stem lesions caused by *Phytophthora* spp. on common nursery and landscape hosts, and to determine if an optimal rate of biochar amendment exists beyond which benefits are reduced. By testing an above-ground disease and providing all plants with ample nutrients and moisture, any reduction in disease severity due to biochar incorporation can reasonably be attributed to an increased systemic resistance in the plant rather than a direct effect of biochar on the pathogen. Two separate experiments on two host-pathogen systems were conducted to test the hypothesis of induced resistance and to determine the potential effectiveness for this soil amendment treatment on two combinations of plant host and *Phytophthora* pathogen.

3.2 Materials and Methods

3.2.1 Treatments

Treatments consisted of varying levels of biochar amendment by volume to potting mix (5, 10, 20, or 0% control) in inoculated plants in both experiments, a non-inoculated control (no biochar amendment) in the maple experiment, and a chemically treated and inoculated treatment (no biochar amendment) in the maple experiment. In the non-amended treatments, inoculated plants growing in 0% biochar amended potting mix are referred to as 0%+ while non-inoculated plants growing in 0% biochar are referred to as 0%-. The chemically treated seedlings were planted in potting mix without biochar and each pot was drenched with 1 pint (473-ml) Agrifos® (mono- and di-potassium salts of Phosphorous acid; Liquid Fertiliser PTY. LTD., Queensland, Australia) 6 days prior to inoculation according to labeled rates for ornamental applications (0.3549 L per 378.54 liters of water).

The biochar used in this experiment was produced from pine parent material (*Pinus taeda, P. palustris, P. echinata, P. elliotti*), which was pyrolyzed for 1 hour between temperatures of 550° C and 600° C in a pyro-torrefaction style kiln. The biochar was ground to create particle sizes between 4 and 6 mesh. The biochar contained (% dry weight): 1.0 % mobile Carbon (C), 63.1% resident C, 0.1 % mobile Nitrogen (N), 0.3 % resident N, 17 % mobile Hydrogen-Oxygen (H-O), 6.8 % resident H-O, 8.6% soluble ash, 3.7% non-soluble ash (analyzed July 2011 by Control Laboratories Inc., Soil Control Lab, Watsonville, CA). The biochar was obtained from New Earth Renewable Energy, a commercial producer that is no longer in operation.

3.2.2 **Plant materials**

Red maple (*Acer rubrum*) seedlings used in this experiment were seed-grown 2-year-old plants (15-30 cm bare-root seedlings) obtained from Lawyer Nursery (Olympia, WA) and planted on 15 June, 2011 at the University of Washington Center for Urban Horticulture, Seattle, WA. Root systems were washed, cut to a uniform length of 15-cm, and planted in 2.54-L tree pots containing Sunshine Professional Growing Mix #2 (75-85% Canadian sphagnum peat moss, horticultural grade perlite, dolomitic limestone; Sun Gro Horticulture, Canada Ltd.), amended with 0, 5, 10 or 20% biochar by volume. Maple seedlings were inoculated on 18 July, and grown under greenhouse conditions with average temperature and average relative humidity maintained at 20.7°C and 53.09%, respectively, until harvest 108 days post-inoculation. Maple treatment groups each consisted of 20 seedlings, randomly arranged on adjacent greenhouse tables.

Red oak (*Quercus rubra*) seedlings used in this experiment were seed-grown 2 year old plants (small plugs) obtained from Heritage Seedlings (Salem, OR) and planted on 5 Mar. 2011 in Seattle, WA. At planting, root systems were washed, and seedlings were planted in Sunshine Professional Growing Mix #2 amended with 0, 5, 10, or 20% biochar by volume in 455-ml pots (10.36-cm top, 6.95-cm bottom, 8.71-cm depth; T.O. Plastics, Minneapolis, MN). All oak seedlings were maintained under greenhouse conditions for one month, moved outdoors, inoculated on 14 June, and grown under ambient weather conditions (Seattle, WA) for the duration of the experiment (65 days). There was no non-inoculated control or chemical treatment in the oak experiment. During the experiment, average daily temperature ranged from 7.5°C-18.78°C (single day high 30.55°C, single day low 0.556°C), with 25.22-cm of rain over the duration, over 90% of which occurred April through June (National Weather Service Data, Seattle-Tacoma International Airport station). Treatment groups each consisted of 14 or 15 randomly arranged seedlings.

All plants were monitored daily, watered by hand as needed to maintain near-constant field capacity conditions, and fertilized weekly with Hoagland's #2 nutrient solution mixed at half strength as described in (Kinmonth-Schultz and Kim, 2011).

3.2.3 **Pathogens**

The isolates of *P. cactorum* and *P. cinnamomi* used in this experiment were isolated through direct plating of phloem tissue from the margin of necrotic lesions that were associated with symptoms of 'bleeding cankers' typical of *Phytophthora* spp. infections on a red maple (*Acer rubrum,* Indian Land, SC), and shingle oak (*Quercus imbricaria*, Washington Park Arboretum, Seattle, WA), respectively. The isolates were identified by morphology, and identification of the *P. cinnamomi* isolate was confirmed at the University of Washington DNA Sequencing Facility based on the ITS-1 and ITS-2 rRNA sub-units using PCR techniques.

3.2.4 Inoculation

A stem-wound technique was employed to introduce the pathogen into seedlings of both plant species in the inoculated treatments. A 5-mm diameter disc of bark and phloem was removed from the lower stem using a sterile cork borer and razor blade. Immediately following wound creation, a 5-mm diameter agar plug was taken from the margin of an actively growing colony of the pathogen and placed on the wound with the upper surface facing inwards. A sterile agar plug was placed on the wound of non-inoculated control treatments. All inoculation sites were immediately wrapped with moist sterile gauze followed by a layer of parafilm in order to maintain adequate moisture for infection and then covered with a layer of aluminum foil to exclude light.

Cultures of both pathogens were isolated and maintained in the dark on a clarified V-8 juice agar (PARP) selective for the culture of Oomycete organisms (Jeffers and Martin, 1986).

3.2.5 Resistance assessment

Lesion size, stem water potential (oak only), and biomass were assessed at harvest which took place 108 days after inoculation in the maple experiment and 65 days after inoculation in the oak experiment.

Vertical and horizontal advance of the pathogen, as evidenced by expansion of the necrotic lesion, were measured at the conclusion of the experiment. Lesion measurements were made at the edge of discolored, intact bark tissue. Vertical expansion was recorded as total length of the necrotic region, and horizontal expansion was recorded as the percentage of stem circumference girdled by the necroses. Removal of bark and examination of necrotic phloem tissue confirmed that the margin of discolored bark was indicative of the margin of the underlying necrotic lesion originating from the original inoculation point. Following harvest, 5 plants from each treatment group were assessed to confirm pathogen colonization. Tissue from the margin of the necrosis was placed on PARP-V8 agar (Jeffers and Martin, 1986) and colony identity was verified morphologically.

At the conclusion of the trial following lesion measurements and re-isolation, all seedlings were dried at 65° C for at least 72 hr. Root and stem portions were separated and weighed. Leaf weight was not included because senescence and dropping of leaves during the course of the experiment occurred but was not recorded.

In the oak experiment, mid-day stem water potential was determined at the conclusion of the experiment (18Aug, 2011, 1300-1430) to assess the effects on plant water relations caused by colonization of xylem by the pathogen. A Scholander-type pressure chamber was used for this purpose (PMS Instrument Company, Albany, OR). The terminal 10-15 cm of each seedling was cut with a sharp razor blade and water potential measurements were made immediately. Following measurements, the plant portion used in this analysis was included in the biomass measurements.

3.2.6 **Statistical analysis**

All statistical analyses were conducted using SigmaPlot 12.0 for windows statistical software (Systat Software Inc., San Jose, CA). The type I error rate (α) was set at 0.05 for all treatment comparisons. Lesion size, biomass, and stem water potential were analyzed by one way analysis of variance (ANOVA) with soil treatment (biochar amendment or chemical) as the main factor. When data failed the Shapiro-Wilk test for normality, treatment group means were compared using the Kruskal-Wallis analysis of variance (ANOVA) on ranks with soil treatment as the main factor. Means of treatment groups were compared using the Student-Newman-Keuls method in the case of equal sample sizes (maple) and using Dunn's method for unequal sample sizes (oak). Each treatment group was comprised of 20 seedlings in the maple experiment and 14 or 15 seedlings in the oak experiment.

3.3 Results

Attempts to re-isolate *Phytophthora cactorum* and *P. cinnamomi* from margins of necrotic lesions were successful in all cases where necrosis was evident. *P. cactorum* was not isolated from any non-inoculated plants. *P. cactorum* was isolated from chemically treated and inoculated plants when necrosis was evident (9 out of 20 plants), but was not isolated from plants where no necrosis had developed. In the following sections, treatment groups are identified by the level of biochar amendment or as a chemical treatment (Chem). All biochar-amended plants and all chemically treated plants were inoculated.

3.3.1 **Maple Experiment**

Data for vertical and horizontal expansion of necrotic lesions in the maple experiment failed the Shapiro-Wilk test for normality; therefore treatment group means were compared using the Kruskal-Wallis one way analysis of variance on ranks. Treatment had a significant effect on lesion expansion; differences in means among treatment groups for both vertical and horizontal expansion were greater than would be expected by chance ($p < 0.001$). The Student-Newman-Keuls Method was used for multiple comparisons in order to isolate differences between treatment groups ($p \leq 0.05$). No necrotic lesions developed on non-inoculated plants (0% -) and callus tissue had overgrown all wound sites (data not shown). Compared to the inoculated plants with no biochar amendment (0% +), incorporation of 5% biochar significantly reduced vertical lesion expansion and the percentage of the stem circumference that was girdled by necrosis (Fig. 3.1). Amendment with 10% biochar significantly reduced horizontal expansion of lesions compared to inoculated control plants, while amendment with 20% biochar increased horizontal expansion of lesions compared to inoculated control (Fig. 3.1 A). Amendment of potting mix with 10% or 20% biochar did not significantly affect lesion vertical expansion compared to 0%+ (Fig. 3.1 B). Mean lesion expansion in chemically treated plants was significantly reduced compared to all other inoculated treatment, both vertically and horizontally, though some necrosis did develop on

9 of 20 plants, causing significant differences in mean lesion expansion compared to the non-inoculated control where no lesions developed (not shown).

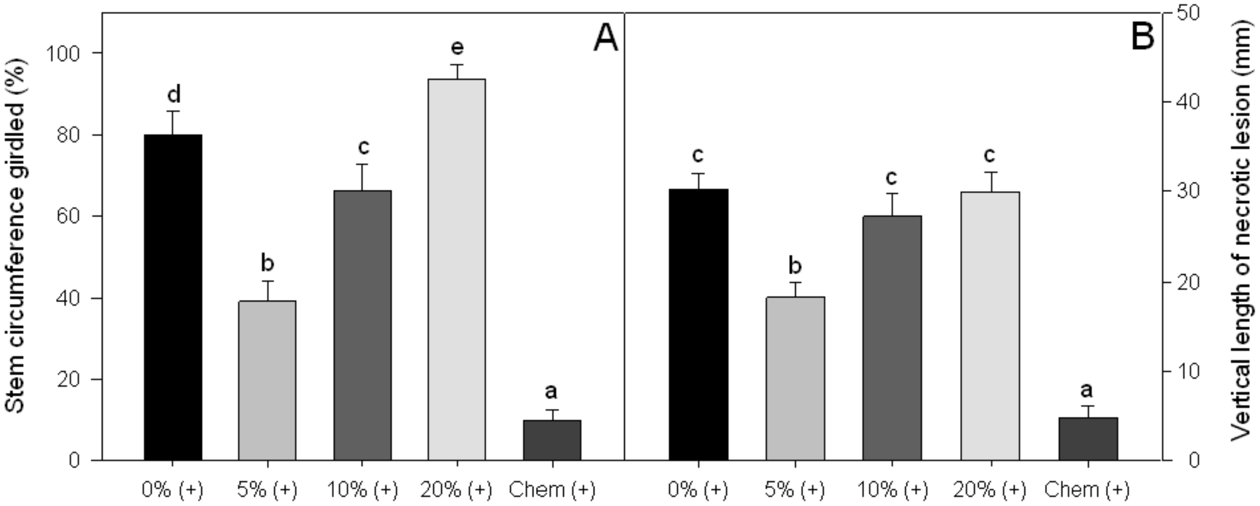

Figure 3.1. Mean horizontal (A, % circumference girdled) and vertical (B, mm) expansion of necrotic lesion caused by *P. cactorum* on *A. rubrum* seedlings (n=20). Treatment names include percent biochar incorporation and inoculation status. No lesions formed on non-inoculated, non-amended control seedlings (data not shown). Data were analyzed by one-way ANOVA with biochar amendment as the main factor and there was a significant treatment effect ($p < 0.001$). Means without a letter in common are significantly different for that measurement (Student-Newman-Keuls Method for multiple comparisons, $p \leq 0.05$). Bars are one standard error.

Final dry biomass data in the maple experiment were normally distributed (Shapiro-Wilk, p = 0.538) and treatment groups were equal in variance (p = 0.328). Analysis of variance indicated a significant effect of treatment on stem, root, and total dry biomass ($p < 0.001$). Non-inoculated plants (0%-) produced significantly more total biomass than any inoculated treatment. Inoculated plants that were chemically treated (Chem) produced significantly more total mean biomass than 0%+, 10%, and 20% plants, but total mean biomass of chemically treated plants was not significantly different than the inoculated plants growing in 5% biochar amendment (Fig. 3.2). Amendment with 5% biochar significantly increased total mean biomass production compared to 10% and 20% treatments, but not compared to 0%+.

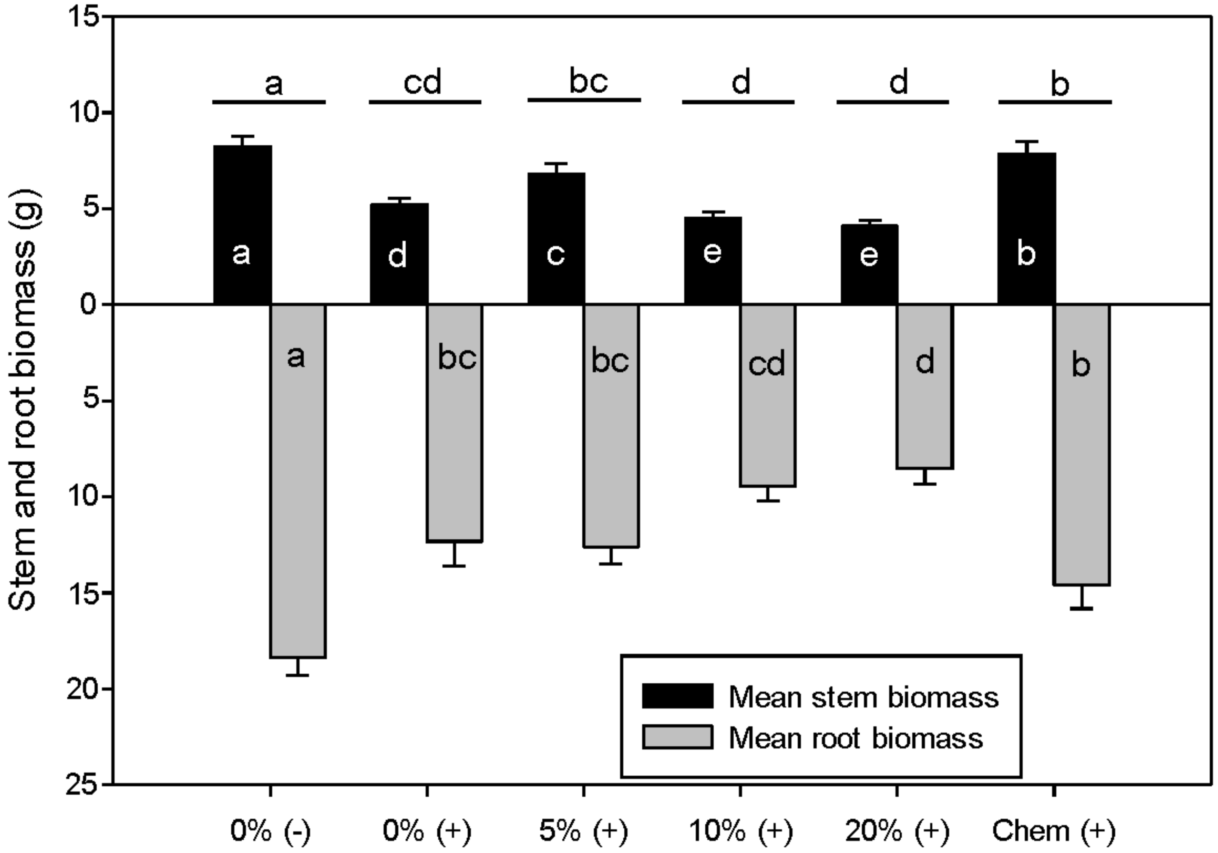

Figure 3.2. Mean stem (above axis) and root (below axis) biomass of *A. rubrum* seedlings following soil treatment (control, % biochar, chemical) and 108 days post-inoculation (+ or -) with *P. cactorum.* Means that do not share a common letter within the plot are significantly different for that parameter, and letters above plots indicate differences in total biomass (Student-Newman-Keuls Method for multiple comparisons, $p \leq 0.05$). Bars are one standard error.

When taken alone, biomass of stems revealed greater differences among treatments. Variability in root weights was greater than in stem weights, and the growth of all root systems had been altered by contact with the pot edges by the end of the experiment. For stem biomass, non-inoculated plants (0%-) produced significantly more biomass than any inoculated treatment group. Inoculated plants that were chemically treated produced less stem biomass than the 0%- treatment, but produced significantly more than any other inoculated treatment. Compared to the inoculated control (0%+), amendment of potting mix with 5% biochar significantly increased stem biomass. Mean stem biomass of plants receiving the 10% or 20% treatments were not significantly different from each other, but both treatment groups produced significantly less mean stem biomass than the other treatments (Fig. 3.2).

3.3.2 Oak Experiment

In the oak experiment, data for both vertical and horizontal lesion expansion failed the Shapiro-Wilk test for normality; therefore means were compared using the Kruskal-Wallis analysis of variance on ranks with soil treatment as the main factor. No significant difference was found between treatments group means for vertical lesion expansion (*p* = 0.311, Table 3.1), however mean values for this parameter in oak followed the same general trends as in the maple experiment. Also, when compared individually to the control, amendment with 5% biochar showed a strong trend toward reduction of lesion vertical expansion (t-test, *p* = 0.094). Significant differences did exist between treatments for horizontal lesion expansion (*p* = 0.002) in the oak experiment, and multiple comparisons were made using Dunn's method due to the unequal sample sizes resulting from disruption by wildlife, which involved birds removing labels or whole plants from pots. Incorporation of 5% biochar in the oak experiment significantly reduced horizontal expansion of lesions compared to all other treatments ($p \leq 0.05$). No significant differences existed in horizontal lesion expansion between the 0%+, 10%, or 20% treatments.

Table 3.1. Expansion of necrotic lesion, mid-day stem water potential, and stem biomass of *Quercus rubra* seedlings at 65 days post inoculation with *Phytophthora cinnamomi*. Values in parentheses are one standard error. When 1-way ANOVA showed significant differences between treatments, Dunn's method was used for multiple comparisons.

	Expansion of necroses		Mid-day stem water potential		
Treatment	% Girdled	Vertical length (mm)	Mpa	t-test vs. control	Biomass (g)
0%	82.0% (5.34) b^z	49.8 (7.82)	-0.528 (0.03)	na	13.93 (0.81)
5%	57.4% (4.55) a	33.0 (5.84)	-0.457 (0.02)	*p* = 0.110	12.72 (0.66)
10%	85.9% (5.14) b	46.5 (8.92)	-0.527 (0.03)	*P* = 0.942	12.95 (0.88)
20%	85.3% (5.69) b	51.1 (7.71)	-0.561 (0.03)	*P* = 0.468	12.71 (0.69)
F-test	*p* = 0.002	*p* = 0.311	*p* = 0.066		*p* = 0.611

^{z}Girdling percentage means without a common letter are significantly different ($p \leq 0.05$)

In the oak experiment, neither total biomass nor above-ground biomass data were normally distributed, and were therefore compared using the Kruskal-Wallis analysis of variance on ranks. No significant differences existed between treatment group means for total biomass ($p = 0.611$) or for mean stem biomass ($p = 0.602$).

In the oak experiment, data for mid-day stem water potential were not normally distributed ($p < 0.05$, Shapiro-Wilk test); means from each treatment group were compared using the Kruskal-Wallis analysis of variance on ranks. Differences between treatment groups were marginally significant (p = 0.066) based on this test. 5% biochar amendment resulted in the least negative mean value for stem water potential (-0.457 MPa), followed by the 10% treatment and the 0%+ treatment which were nearly the same (-0.527 MPa and -0.528 MPa, respectively). The mean stem water potential in the 20% treatment (-0.561 MPa) was the most negative (Table 1).

3.4 Discussion

Overall, amendment of potting mix with 5% biochar resulted in reduced lesion expansion and greater stem biomass when compared to plants growing in media that was not amended or amended with 10% or 20% biochar (v/v). Biologically, the horizontal expansion of lesions, leading to the complete girdling of phloem in stems, probably has a greater effect on overall plant health than vertical expansion, which would not completely girdle the stem, and this parameter showed some of the greatest differences between treatments. While not significant at $p \leq 0.05$, the trends seen in stem water potential data from the oak experiment are also of interest. Visual inspection of discolored tissue indicated that the pathogen had colonized xylem tissue in addition to causing phloem necrosis, and the water potential data support this observation as the smallest lesion sizes and least negative water potentials both occurred in the 5% treatment, indicating a potential physical blockage hindering the movement of water. The degree of potential xylem vessel blockage in this study appears to be similar to or less than the xylem blockage due to other physical damages such as stem bending (Kim et al., 2004). Previous studies have also shown xylem colonization and blockage in woody stems caused by various *Phytophthora* pathogens (Brown and Brasier, 2007; Parke and Lewis, 2007).

Amendment of potting media with 5% biochar did not prevent lesion development or increase biomass as well as treatment with salts of phosphorous acid, a material known to induce resistance against *Phytophthora* spp. (Daniel and Guest, 2006; Daniel et al., 2005; Garbelotto et al., 2009; Jackson et al., 2000; Weiland et al., 2009). However, based on this study and previous studies involving other pathogen/host combinations (Elad et al., 2010; Harel et al., 2012), it appears that amendment of rooting media with 5% biochar has the potential to induce some level of resistance against a wide range of pathogens. The experiment using red oak (*Quercus rubra*) and *Phytophthora cinnamomi* resulted in comparable, though often non-significant trends compared to the experiment using red maple (*Acer rubrum*). In the oak experiment, disruptions of the plots by wildlife lead to a loss of 20-25% of treatment replicates. While many of the parameters under investigation followed similar trends between the maple and oak experiments, the reduction of statistical power in the oak experiment due to loss of plants reduced the potential significance of differences in means among treatment groups in that experiment. Despite the lack of significance for some parameters, the similar results from each experiment suggest promising potential for this soil amendment to benefit other patho-systems involving woody plants and pathogens in the genus *Phytophthora*.

There are many mechanisms by which biochar amendment may lead to increased resistance to plant disease, including increase in overall plant vigor, increased nutrient availability, alteration of soil pH, altered soil moisture, increase in mycorrhizal associations or other beneficial rhizosphere microorganisms, and changes in soil physical properties (Glaser and Zech, 2002; Ishii and Kadoya, 1994; Kolton et al., 2011; Lehmann et al., 2011; Novak et al., 2009; Warnock et al., 2007). Effects of biochar amendment on soil pH, soil drainage, soil physical properties, and microbial communities may directly impact *Phytophthora* spp. in soil. However, the experiments described here were designed to eliminate any direct effects on the pathogen caused by soil physical, chemical, or microbial properties through inoculation of above-soil stem tissue. Effects of soil physical or chemical properties on resistance were controlled for through provision of plants with ample moisture and nutrients in horticultural growth media. Based on this design, the results here are indicative of a systemic response, and are in agreement with previous research that showed a reduction in damage caused by foliar plant diseases in multiple host plants following incorporation of biochar into various media (Elad et al., 2010; Harel et al., 2012). In those pioneering studies, the authors concluded that the induced resistance may have been the result of an increase in beneficial soil microorganisms, elicitation of plant defense responses following stress to the plant caused by low-level phyto-toxic compounds in the biochar material, or chemical elicitors.

While the present study does not explicitly support one potential mechanism or another, based on the apparent dose-dependency of the results, induction of defense through low-level stress appears to be a likely possibility. While amendment of media with 5% biochar did reduce lesion size and increase biomass compared to the untreated control group, higher rates were not beneficial and in some cases appeared to become detrimental. This would appear to support the concept of hormesis, *i.e.*, that low levels of phytotoxic compounds can stimulate growth or defense in plants (Graber et al., 2010; Prithiviraj et al., 2007). Further studies that investigate other key physiological aspects including carbon assimilation, production of defense compounds, photosynthetic efficiency, and foliar starch content will be needed to unveil the mechanisms responsible for the reduction of lesion development and increase in biomass production. A decrease in stomatal conductance, carbon assimilation, and photochemical efficiency following infection with canker-causing *Phytophthora* species has been attributed to several factors in previous studies. Impaired water movement in xylem and subsequent drought stress (Luque et al., 1999), pathogen derived chemical elicitors (Fleischmann et al., 2005;

Manter et al., 2007), or a feedback inhibition caused by starch accumulation in foliage as a result of dead or non-functional phloem (Clemenz et al., 2008) have all been implicated as the main cause for the effects of inoculation on carbon assimilation. While we have shown a reduction in disease progression with biochar amendment, our data do not explicitly support or negate any of these potential mechanisms.

Here I have shown that amendment of potting media with 5% biochar can reduce the expansion of necrotic lesions caused by *Phytophthora* spp. in seedlings of two common landscape tree species. In addition, we have shown an apparent dose-dependency of the benefits of biochar amendment, as amendment of potting mix with 5% biochar limited disease development, but increased levels (10 % and 20 %) caused no change or had deleterious effects on the parameters under investigation.

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Chapter 4

Physiological effects of biochar amendment on *Acer rubrum* seedlings inoculated with *Phytophthora cactorum*

4.1 Introduction

In the previously described studies, I have taken a detailed look at various physiological aspects of red maple (*Acer rubrum*) as they are affected by *P. cactorum*. Seedlings that were inoculated using a stem-wound technique showed a rapid and sustained reduction in carbon assimilation rates and stomatal conductance, in agreement with previous studies regarding stem infection in other combinations of host and *Phytophthora* species (Clemenz et al., 2008; Fleischmann et al., 2005; Luque et al., 1999; Manter et al., 2007). Seedlings that were wounded only (mock inoculated control) showed a more gradual reduction in assimilation rates and stomatal conductance, followed by complete recovery of these parameters to pre-treatment levels. Maximum efficiency of photosystem-II, as evidenced by dark adapted chlorophyll fluorescence, showed a temporary decline followed by recovery in both treatment groups, and the decline was slightly more pronounced in the inoculated treatment. Instantaneous water use efficiency, a measure of carbon assimilation in relation to stomatal conductance, was affected by treatment, gradually rising over time in the inoculated group, indicating that infection was affecting the moisture status of the plant in some way. Taken together, these data indicate that the early declines in photosynthesis were likely due to wounding in both groups, and the larger early reduction in the inoculated group may have been caused by plant recognition of a pathogen derived signal. Later, the sustained reduction of assimilation and conductance in the inoculated group compared to recovery in the control group was not caused by phloem death or dysfunction, leading to a carbohydrate feedback inhibition of photosynthesis. Rather, it appears the reduction in assimilation was related to pathogen derived signals or pathogen-induced hormonal responses in the plant that lead to a reduction in stomatal conductance and subsequent reduction in assimilation due to limitations in the supply of carbon dioxide at the site of carboxylation, in combination with some moisture stress.

In addition to illustrating some of the physiological effects of infection on *A. rubrum* seedlings, I have shown that amendment of potting mix with biochar at optimal rates can reduce the expansion of the necrotic lesion caused by *P. cactorum* on *A. rubrum*, as well as reducing expansion of lesions caused by *P. cinnamomi* on red oak (*Quercus rubra*) (Zwart and Kim, 2012). These findings are similar to several studies that have shown reductions in various foliar diseases as a result of biochar amendment of soil (Elad et al., 2011; Elad et al., 2010; Harel et al., 2012). This supports the conclusion that biochar amendment of soil or potting media has the potential to induce systemic resistance against a wide range of pathogens.

In the first year of experiments, physiological data regarding the effects of inoculation on carbon assimilation rates, stomatal conductance, and photosynthetic efficiency were generally intermediate in the 5 % biochar amended treatment group when compared to the non-inoculated control group and the non-amended inoculated group. However, these differences in physiological parameters were often unclear or not statistically significant. The experiment described here was designed to replicate much of the previous experiment in order to supplement data, illuminate trends, and strengthen conclusions, as well as to compare the effects of phloem death due to pathogen ingress with the effects of physical removal of bark and phloem tissue (manual girdling).

The purpose of this experiment was three-fold: 1) to strengthen the findings that 5 % biochar amendment of potting media can reduce the expansion of necrotic stem lesions in *A. rubrum* caused by *P. cactorum*; 2) to determine what physiological factors are being impacted differently in the biochar-amended inoculated plants compared to the non-amended, inoculated plants, and compared to manually girdled plants; and 3) to attempt to reveal the physiological mechanism leading to the decreased disease progression and impact in biochar amended seedlings.

4.2 Materials and Methods

4.2.1 Treatments

There were four treatment groups of *Acer rubrum* seedlings in this experiment, which are referred to as 'control', 'inoculated', 'biochar', or 'girdled'. There were eight seedlings in the 'control' group, and these seedlings were not amended, wounded, or mock-inoculated. There were eight seedlings in both the 'inoculated' and 'biochar' groups which were stem-wound inoculated with *P. cactorum* on 31 July, 2012 as described below. Seedlings in the 'biochar' group had been amended at planting with 5 % biochar by volume, thoroughly mixed with the potting media. There were five seedlings in the 'girdled' group, which were treated in the same manner as control plants until the girdling treatment was applied on 21 Sept, 2012. Girdling was achieved by carefully removing the bark and phloem in a vertical strip 5-cm long, and encircling 66 % (visual estimate) of the stem using a sterile razor and tweezers.

4.2.2 Plant material

Acer rubrum seedlings used in this experiment were seed-grown 2-year-old plants (15-30 cm bare-root seedlings) obtained from Lawyer Nursery, Olympia, WA, and planted on 10 May, 2012 at the University of Washington Center for Urban Horticulture, Seattle, WA. Root systems were washed, cut to a uniform length of 15-cm, and planted in 2.54-L tree pots containing Sunshine Professional Growing Mix #2 (75-85% Canadian sphagnum peat moss, horticultural grade perlite, dolomitic limestone; Sun Gro Horticulture, Canada Ltd.). In the 'biochar' treatment group, the potting mix was uniformly amended with 5 % biochar by volume prior to planting. All seedlings were grown under greenhouse conditions with average temperature and average relative humidity maintained at 22.8° C and 54 %, respectively, until harvest 66 days post-inoculation. All plants were monitored daily, watered by hand as needed at 1-2 day intervals to maintain near-constant field capacity conditions, and fertilized weekly with Hoagland's #2 nutrient solution mixed at half strength as described in (Kinmonth-Schultz and Kim, 2011).

4.2.3 Pathogens

The isolate of *P. cactorum* used in this experiment was isolated through direct plating of phloem tissue from the margin of necrotic lesions that were associated with symptoms of 'bleeding cankers' typical of *Phytophthora* spp. infections on a red maple (*Acer rubrum,* Indian Land, SC). The isolate was identified by morphology by D. Zwart (Erwin and Ribeiro, 1996; Stamps et al., 1990). The isolate produced distinctly papillate, variable shaped sporangia in culture, terminally borne singly or in clusters. Hyphal-tip subcultures of the isolate readily produced oospores (homothallic) which were pluerotic and smooth-walled. Antheridia were single celled and paragynous.

4.2.4 Inoculation

A stem-wound technique was employed to introduce the pathogen into seedlings in the inoculated treatments. A 5-mm diameter disc of bark and phloem was removed from the lower stem using a sterile cork borer and razor blade. Immediately following wound creation, a 5-mm diameter agar plug was taken from the margin of an actively growing colony of *P. cactorum* and placed on the wound with the upper surface facing inwards. The control treatment was not wounded or mock-inoculated. All inoculation sites were immediately wrapped with moist sterile gauze followed by a layer of parafilm in order to maintain adequate moisture for infection and then covered with a layer of aluminum foil to exclude light.

Cultures of the pathogen were isolated and maintained in the dark on a clarified V-8 juice agar (PARP) selective for the culture of Oomycete organisms (Jeffers and Martin, 1986).

4.2.5 Biochar

The biochar used in this experiment was produced from pine parent material (*Pinus taeda, P. palustris, P. echinata, P. elliottii*), which was pyrolyzed for 1 hour between temperatures of 550° C and 600° C in a pyro-torrefaction style kiln. The biochar was ground to create particle sizes between 4 and 6 mesh. The biochar contained (% dry weight): 1.0 % mobile Carbon (C), 63.1% resident C, 0.1 % mobile

Nitrogen (N), 0.3 % resident N, 17 % mobile Hydrogen-Oxygen (H-O), 6.8 % resident H-O, 8.6% soluble ash, 3.7% non-soluble ash (analyzed July 2011 by Control Laboratories Inc., Soil Control Lab, Watsonville, CA). The biochar was obtained from New Earth Renewable Energy (Quebec, Canada), a commercial producer that is no longer in operation.

4.2.6 Physiological responses

Gas exchange parameters were assessed using a LiCor LI-6400 portable photosynthesis system (LiCor, Lincoln NE) at weekly intervals. At each sampling date, a 6 cm^2 portion excluding the mid-vein of the uppermost fully expanded green leaf was assessed for maximum net carbon assimilation rates (A_{max}) and stomatal conductance (g_s). Gas exchange measures were made under set conditions of 400 ppm CO_2, 25 °C (block temperature), 45-65 % relative humidity, flow rate of 300 $\mu mol\ s^{-1}$, and photon flux density of 1700 μmol photon $m^{-2}\ s^{-1}$, as regulated by the LI-6400 unit. Measurements were made between 700-1300 PDT, and all plants were allowed to stabilize for at least 10 minutes prior to data recording.

Measures of chlorophyll fluorescence (dark adapted Fv/Fm) were taken to monitor the quantum yield and photochemical efficiency of photosystem II (PS-II), and as an indicator of overall plant stress as described in Percival, (2005). Briefly, at each sampling date, leaves were adapted to darkness for at least 40 minutes using light exclusion clips placed on the uppermost fully expanded green leaf, in most cases the same leaf used in gas exchange measures. Chlorophyll fluorescence was measured with a HandyPEA portable fluorescence spectrometer (Hansatech Instruments Ltd., King's Lynn, U.K.), and data were collected up to 1 s with a data acquisition rate of 10 µs for the first 2 ms and 1 ms for the remainder. The fluorescence responses were induced by a red (peak at 660 nm) light with photon flux density of 1500 μmol photon $m^{-2}\ s^{-1}$ provided by an array of six light-emitting diodes.

Measures of leaf chlorophyll content were assessed throughout the experiment using a Konica-Minolta SPAD-502 chlorophyll meter (Spectrum Technologies, Inc. Plainfield, IL). Measurements were made on five leaves per plant, and two measurements were taken per leaf. Reported values are the mean of these 10 measures.

The water soluble phenolic content, as tannic acid equivalents, was assessed on foliar tissue from the 2011 experiment (see chapter 3 for description of plant material and treatments), as plant condition and early senescence precluded this analysis at the conclusion of the 2012 experiment. Fully expanded, upper-most leaves were collected at the end of the 2011 experiment and frozen (-80° C) until the analysis was conducted. Four leaf discs (1.27-cm diameter) were punched from leaves excluding mid-veins using a cork borer, weighed for fresh mass, and placed in 1.5-ml deionized sterile water for 72 hours at 60° C. One mL of the resulting extract solution was moved to a sterile 2.0-ml Eppendorf tube, and centrifuged at 2,000 RPM for 10 minutes. The Folin-Ciocalteu method was used to quantify soluble phenolic compounds in the resultant supernatant solution (Swain and Hillis, 1959). Briefly, 600-µl of sterile deionized water was added to a new Eppendorf tube, followed by 10-µl of sample solution, 50-µl of Folin-Ciocalteau reagent, 150-µl sodium carbonate solution, and 190-µl sterile deionized water. Tubes were thoroughly mixed after each addition. After two hours, absorbance at wavelength 725-nm was assessed using a PharmaSpec UV-1700 series spectrophotometer (Shimadzu Corp., Kyoto, Japan). Absorbance was compared to a standard curve created with tannic acid solutions of various concentrations, and the tannic acid concentration equivalent was divided by leaf disc fresh weight to arrive at phenolic concentration for samples, expressed in mg / g fresh weight.

Lesion size and biomass were assessed at harvest which took place 66 days after inoculation. Vertical and horizontal advance of the pathogen, as evidenced by expansion of the necrotic lesion, were measured at the conclusion of the experiment. Lesion measurements were made at the edge of discolored, intact bark tissue. Vertical expansion was recorded as total length of the necrotic region, and horizontal expansion was recorded as the percentage of stem circumference girdled by the necroses. Removal of bark and examination of necrotic phloem tissue confirmed that the margin of discolored bark was indicative of the margin of the underlying necrotic lesion originating from the original inoculation point. Following harvest, 4 plants from each inoculated treatment group were assessed to confirm pathogen colonization. Tissue from the margin of the necrosis was placed on PARP-V8 agar (Jeffers and Martin, 1986) and isolate identity was verified morphologically by D. Zwart (Stamps et al., 1990).

At the conclusion of the trial following lesion measurements and re-isolation, all seedlings were dried at 65° C for at least 72 hr. Root and stem portions were separated and weighed. Leaf weight was not included because senescence and dropping of leaves occurred during the course of the experiment but was not recorded.

4.2.7 **Statistical analysis**

All statistical analyses were conducted using SigmaPlot 12.0 for windows statistical software (Systat Software Inc., San Jose, CA). The type I error rate (α) was set at 0.05 for all treatment comparisons. Lesion size, biomass, gas exchange parameters, SPAD measures, and chlorophyll fluorescence data were analyzed by one-way analysis of variance with treatment group as the main factor, and the Student-Newman-Keuls or Holm-Sidak method was employed for pairwise multiple comparisons when sample sizes were equal, and Dunn's method was employed for unequal sample sizes. In cases when the inoculated treatment groups ('biochar' and 'inoculated') were compared directly, the Student's t-test was employed, with amendment status as the main factor. Lesion sizes were expected to be decreased in the 'biochar' group compared with the 'inoculated' group based on results from 2011, and were therefore compared via one-tailed t-tests. When data failed the Shapiro-Wilk test for normality, treatment group means were compared using the Mann-Whitney rank sum test (Zar, 1999). 'Control', 'inoculated', and 'biochar' treatment groups were comprised of eight seedlings, and the 'girdled' treatment group contained five seedlings. Weekly gas exchange measures included all seedlings from each treatment group in the 2012 experiment. Because the plant species, pathogen, and treatments were the same for the ‘inoculated’ treatment and the ‘biochar’ treatment groups in the 2011 and 2012 experiments, data for these two treatments were combined and analyzed via two-way analysis of variance, with year and treatment (biochar or no biochar amendment) as the two main factors, and least-square means were compared.

4.3 Results

Phytophthora cactorum was successfully isolated from the margins of necrotic lesions of four plants in each of the inoculated treatment groups (‘inoculated’ and ‘biochar’). Attempts were not made to isolate the pathogen from non-inoculated treatments (‘girdled’ and ‘control’).

4.3.1 **Biomass and stem water potential**

There were no significant differences among treatment groups in mean dry biomass for whole plant mass (p = 0.771), stem mass (0.223), or root mass (0.648), though the largest relative differences occurred in stem mass. The ratio of stem mass to root mass was highest in the inoculated treatment without biochar. Mean stem mass, root mass and stem to root ratio in the biochar amended seedlings was intermediate between control and non-amended, inoculated seedlings for all measures (Table 4.1). There were no significant differences in mean stem water potential measurements among treatment groups (0.802) at the conclusion of the experiment (Table 4.1). In addition, there was no visual evidence of xylem colonization by the pathogen in inoculated treatments, nor did the girdling treatment appear to have impacted xylem function, as evidenced in stem water potential data.

4.3.2 **Expansion of lesions**

Expansion of lesions was significantly reduced in the biochar amended seedlings compared to the non-amended seedlings in 2012. Horizontal expansion, measured as the percentage of the trunk girdled by the necrotic lesion, was reduced from a mean of 55.9 % in ‘inoculated’ seedlings to 44.6 % in the ‘biochar’ seedlings (p = 0.049). Vertical expansion of the necrotic lesion was also reduced from a mean of 31.5-mm in inoculated seedlings to 24.8-mm in biochar amended, inoculated seedlings (p = 0.016) (Table 4.1). When data from the 2011 and 2012 experiment were combined, the reduction in lesion size is clear after allowing for differences in experiment year. Least squares mean of vertical lesion length in the non-amended group was 30.9-mm (± se 1.5-mm), while incorporation of biochar significantly reduced ($p < 0.001$) vertical lesion expansion, resulting in least squares mean lesion length of 21.5-mm (± se 1.5-mm). Horizontal expansion (% stem girdled) was also reduced in the biochar amended group ($p < 0.001$), with least squares mean of stem girdling percentage in the non-amended group of 67.9 % (± se 4.4 %) and 41.9 % (± se 4.4 %) in the biochar amended group.

Table 1. Stem, root, and total biomass, stem to root ratio, lesion expansion, and mid-day stem water potential of *Acer rubrum* seedlings at the conclusion of the experiment. 'Inoculated' and 'Biochar' groups were stem-inoculated with *P. cactorum* 66-days prior to data collection; the 'girdled' group was manually stem girdled through removal of a 15-mm strip of bark and phloem encircling 66% of the stem.

	Dry Biomass (g)				Lesion Progression		
	Stem (se)	Root	Total	S:R ratio^z	Girdle (%)	Length (mm)	SWP (Mpa)
Control	11.29 (0.89)	35.89 (2.57)	47.18 (3.21)	0.318 (0.02)	na	na	-0.105
Inoculated	14.36 (1.89)	31.12 (2.09)	45.48 (2.15)	0.494 (0.10)	55.88 (4.30)	31.50 (2.17)	-0.11
Biochar (5 % v/v)	12.55 (0.77)	34.95 (3.81)	47.50 (4.21)	0.382 (0.04)	44.63 (4.68)	24.75 (1.81)	-0.156
Girdled	10.624 (1.08)	32.24 (3.81)	42.34 (4.19)	0.342 (0.04)	na	na	-0.097
P - value	p = 0.223^y	p = 0.648	p = 0.771	p = 0.17	**p = 0.049^x**	**p = 0.016**	p = 0.802

4.3.3 Gas exchange

Net assimilation of carbon dioxide in the 2012 experiment was marginally lower in the inoculated treatment compared to the biochar treatment and control, though these differences were not significant in any week (Fig. 4.1 A). Mean assimilation rates in the control group were marginally higher than in the biochar treatment group in most, but not all weeks. In the girdled treatment, mean assimilation rates dropped dramatically and mean assimilation in the girdled group was significantly lower than all other treatments just two weeks after girdling was applied ($p < 0.05$).

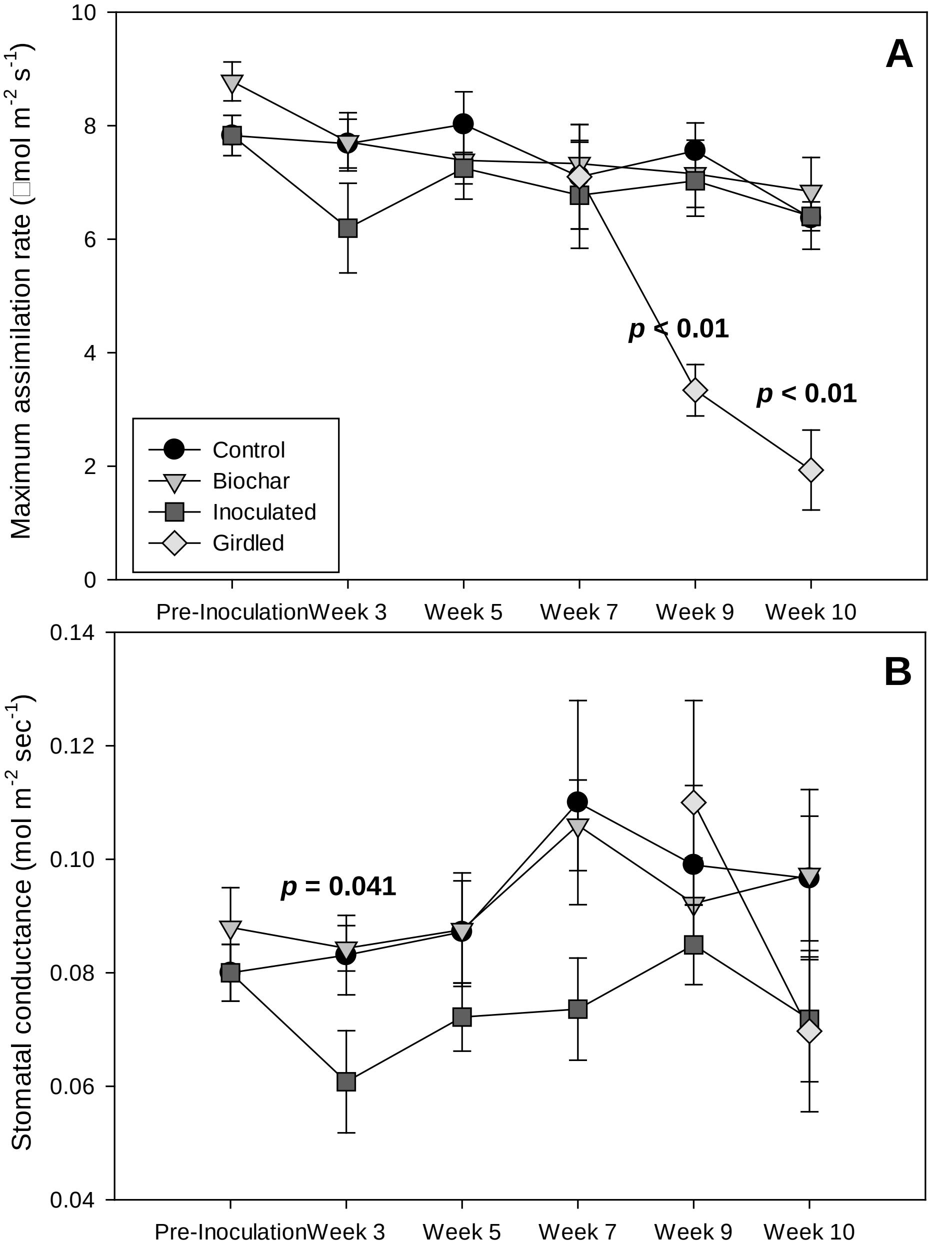

Figure 4.1. Mean maximum assimilation rates (μmol m^{-2} s^{-1}; A) and stomatal conductance (mol m^{-2} s^{-1}; B) over time in *Acer rubrum* seedlings following various treatments ('control'- no amendment, no inoculation; 'biochar'- 5% biochar amendment, stem inoculated with *P. cactorum*; 'inoculated'- no amendment, stem inoculated with *P. cactorum*; 'girdled'- no amendment, 5-cm strip of phloem and bark removed from 66% of the stem circumference prior to measures on week 7.) *P* – values are the result of one-way analysis of variance among treatment groups for that week, bars are one standard error.

Stomatal conductance to carbon dioxide followed the same trends as assimilation rates in the 2012 experiment (Figure 4.1 B). Conductance was always marginally higher in both control and biochar amended groups compared to the inoculated group, and this difference was significant three weeks after inoculation. No differences existed in stomatal conductance between biochar amended and control groups. Mean stomatal conductance in the girdled group dropped rapidly and was significantly lower than all other treatments three weeks after the girdling was applied.

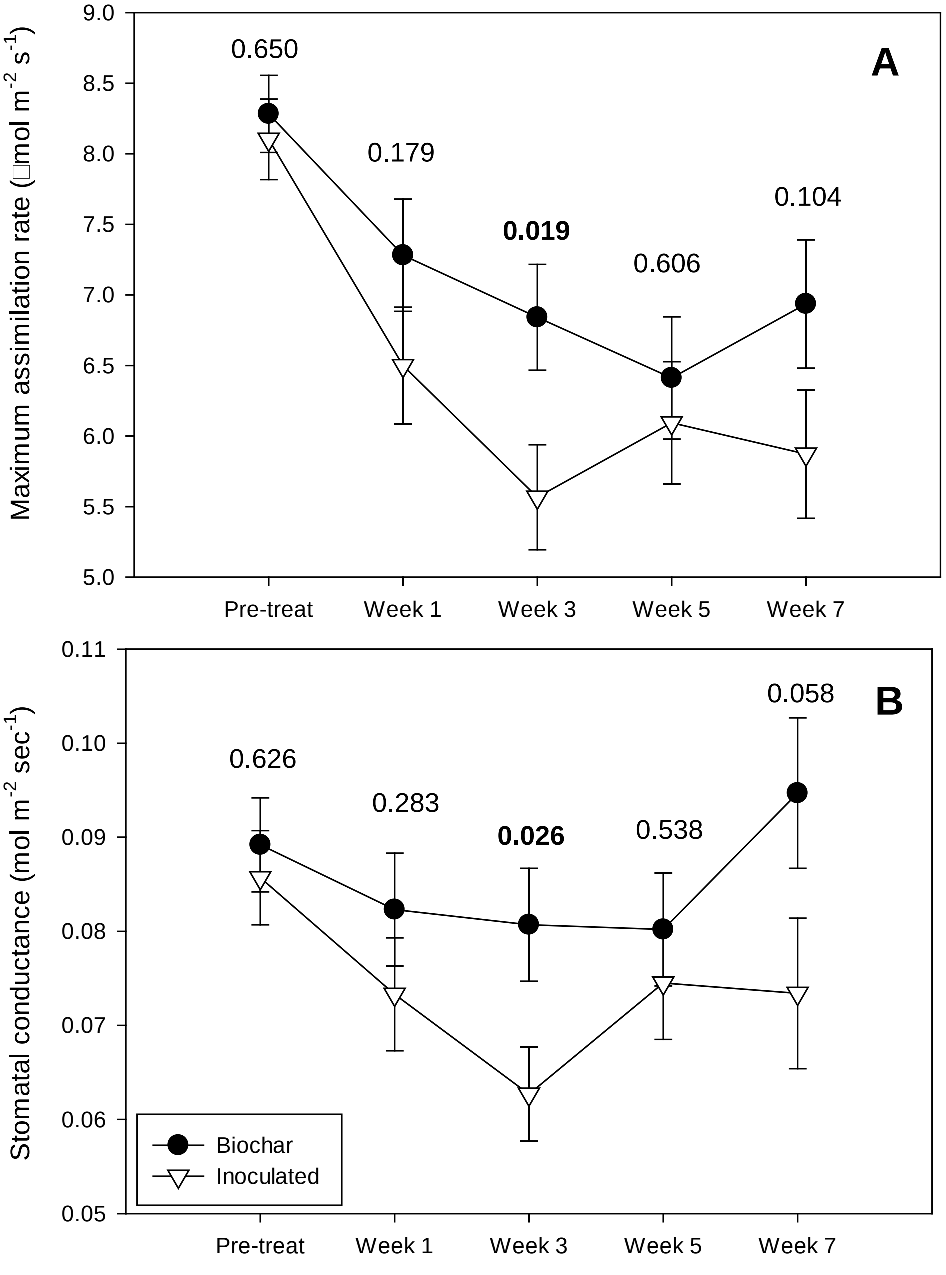

Figure 4.2. Mean maximum assimilation rates (µmol m^{-2} s^{-1}; A) and stomatal conductance (mol m^{-2} s^{-1}; B) over time in *Acer rubrum* seedlings that were amended with 5 % biochar prior to planting ('biochar') or non-amended ('inoculated'), and stem inoculated with *Phytophthora cactorum*. Data represents combined results from 2011 and 2012 experiments. *P* – values represent the effect of treatment after allowing for the effect of experimental year (two-way ANOVA with Year and Treatment as variables). Bars are one standard error.

Over two years, taking differences between years into account, there were clear differences in carbon assimilation and stomatal conductance among treatments (Figure 4.2 A,B). Prior to inoculation, no differences existed between groups in assimilation rates due to year (*p* = 0.575) or due to biochar amendment (*p* = 0.650). Similarly, for stomatal conductance no differences existed due to year (*p* = 0.345) or biochar amendment (*p* = 0.626) prior to treatment. One week after inoculation, the assimilation rate and stomatal conductance were differentially affected based on experiment year (*p* = 0.001; *p* = 0.009, respectively). However, after accounting for effects of year, there remained a strong trend toward reduced assimilation rate in the non-amended group (*p* = 0.18) compared to the biochar amended group, and stomatal conductance in the biochar amended group was also marginally higher than the non-amended group (*p* = 0.283). Three weeks after treatment, the differences between years was significant for assimilation rates (*p* = 0.006), but not for stomatal conductance (*p* = 0.828). After allowing for these differences in year, both assimilation and conductance were significantly higher (*p* = 0.019; *p* = 0.026, respectively) in the biochar amended group compared to the non-amended group. At seven weeks following treatment, after allowing for differences due to year of experiment (*p* = 0.049 for assimilation, *p* = 0.308 for conductance), assimilation rate was still marginally reduced in the non-amended group (5.872 ± se 0.454 µmol m^{-2} sec^{-1}) in relation to the biochar amended group (6.936 ± se 0.454 µmol m^{-2} sec^{-1}; *p* = 0.104), and conductance was also reduced in the non-amended group (0.0734 ± se 0.008 mol m^{-2} sec^{-1}) compared to the biochar amended group (0.0947 ± se 0.008 mol m^{-2} sec^{-1}; *p* = 0.058).

4.3.4 Efficiency of PS-II

One week prior to treatment (inoculation), the efficiency of photosystem-II, as evidenced by the dark-adapted Fv/Fm ratio, was slightly higher (*p* = 0.052 at time = 1 week pre) in 'biochar' treatment group (0.787 ± s.e. 0.005) compared to non-amended soils (0.770 ± s.e. 0.006). However, immediately prior to inoculation this difference had disappeared (*p* = 0.953 at time = 0). While mean Fv/Fm in the ‘inoculated’ treatment group remained marginally lower than the ‘control’ and ‘biochar’ treatment groups throughout the experiment, these differences were never significant (Fig 4.3).

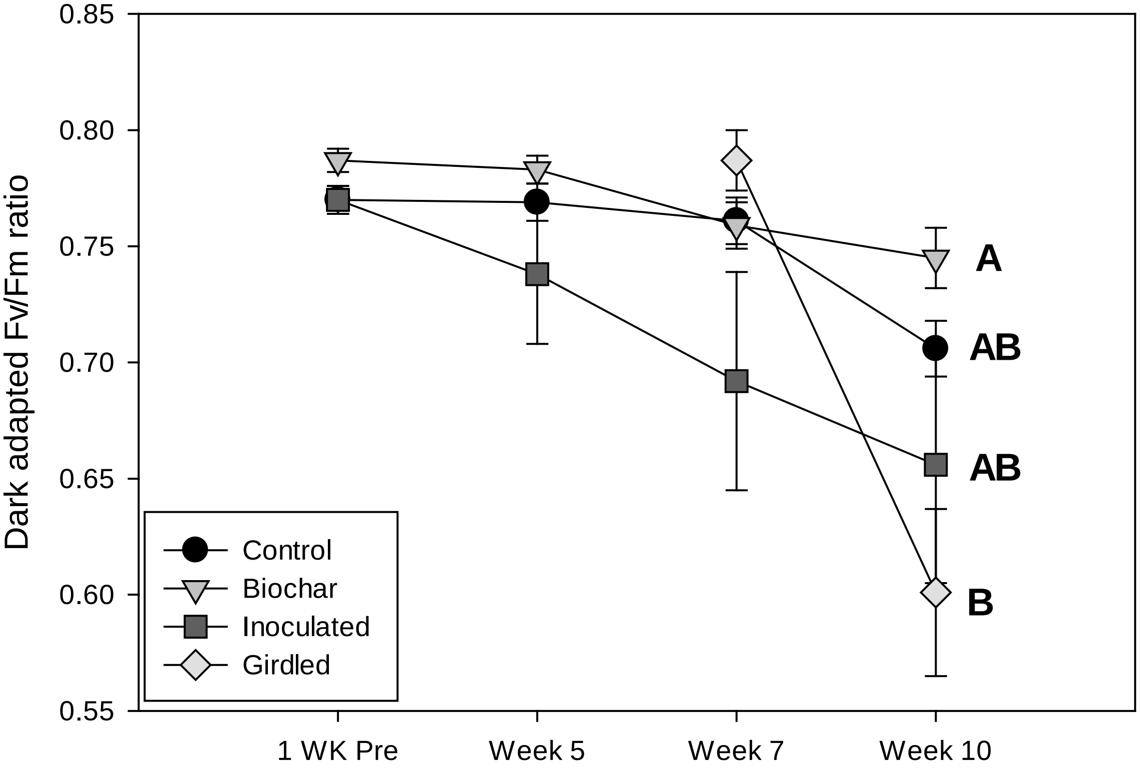

Figure 4.3. Maximum efficiency of PS-II over time (dark adapted Fv/Fm) in seedlings of Acer rubrum which were inoculated with *Phytopthora cactorum* (‘inoculated’), amended with 5 % biochar and inoculated (‘biochar’), manually girdled (‘girdled’; 66 % stem circumference, 5-cm length), or not amended or inoculated (‘control’). Bars are one standard error. At week 10, treatments that do not share a common letter were significantly different at *p* = 0.05 (one-way analysis of variance and S-N-K multiple comparisons). No significant differences existed among treatment groups prior to week 10.

Compared to pre-treatment means for each treatment group, differences in Fv/Fm did not exist in any group until week 7 and week 10. For the ‘girdled’ treatment group, the girdling treatment was applied prior to data collection at week 7, and mean Fv/Fm values in that group declined rapidly, showing significant differences compared to pre-treatment at week 10, just 3 weeks post-girdling (p = 0.0011; t = -4.949, 8 d.f.). At week 10, the final time of data collection, significant differences existed among treatment groups (p = 0.040). Dunn’s method for multiple comparisons was used to separate treatment group means, and this analysis indicated that mean Fv/Fm in the ‘biochar’ treatment group was significantly higher than in the 'girdled' treatment group, while means for the 'control' and 'inoculated' treatment groups were intermediate between the two and not significantly different from either. At the end of the experiment, mean Fv/Fm values had declined from pre-treatment values for that group by 10.3 % (± s.e. 1.6 %) in the 'control' group, 5.88 % (± s.e. 1.6 %) in the 'biochar' group, 16.8 % (± s.e. 6.5 %) in the 'inoculated' group, and 23.6 % (± s.e. 5.1 %) in the girdled group, though these reductions were not significantly different among groups.

4.3.5 **SPAD analysis**

Mean SPAD values did not vary among treatments prior to inoculation (*p* = 0.472), at week 5 (*p* = 0.148), at week 7 (*p* = 0.198), or at the conclusion of the experiment 10 weeks after treatment (*p* = 0.398) (Figure 4.4).

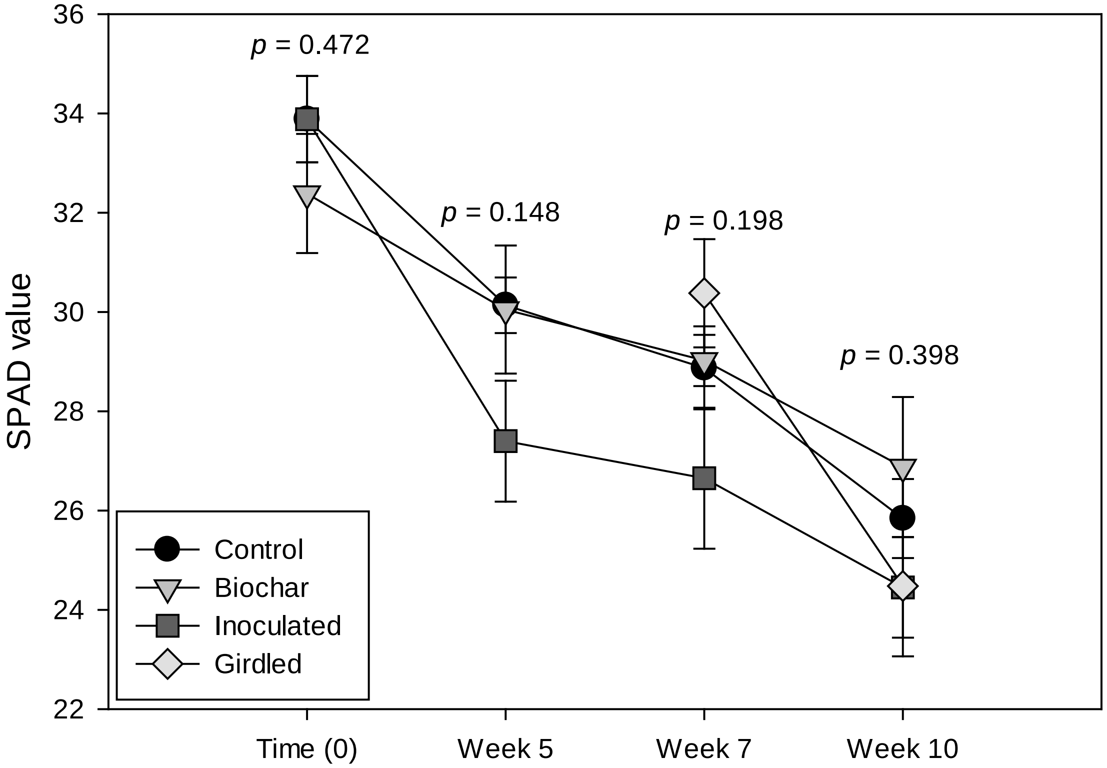

Figure 4.4. Mean SPAD values over time of 'control' (non-inoculated, non-amended), 'biochar' (inoculated with *Phytophthora cactorum*, 5 % biochar amendment), 'inoculated' (inoculated, non-amended), and 'girdled' (manually girdled, non-amended) treatments in *Acer rubrum* seedlings. *P* – values are the result of one-way analysis of variance among treatments for that week. Bars are on standard error.

By the end of the experiment, mean SPAD values for all treatment groups were significantly reduced compared to pre-treatment values for that group ('control' and 'inoculated', $p < 0.001$; 'biochar', $p = 0.010$; 'girdled', $p = 0.011$). However, the 'control' and 'inoculated' treatment groups were reduced compared to pre-treatment levels for that group by week 5 ($p = 0.009$, $p < 0.001$, respectively), while mean SPAD values in the 'biochar' treatment group were not different from pre-treatment values at week 5 ($p = 0.238$) or week 7 ($p = 0.118$). Mean SPAD values for the 'girdled' treatment group were significantly reduced just three weeks after treatment ($p = 0.011$).

4.3.6 **Phenolic content**

One-way analysis of variance of leaf soluble phenolic content on foliage from the 2011 experiment indicated that there were significant differences among treatment groups that were greater than would be expected by chance ($p = 0.016$). Phenolic data were normal and equal in variance, and pairwise multiple comparisons were made using the Holm-Sidak method. The chemically treated group had the lowest mean concentration of soluble phenolics (113.0 mg/g fresh weight ± s.e. 7.72), followed by the biochar amended group (143.60 mg/g fresh weight ± s.e. 6.54), then the control group (144.91 mg/g fresh weight ± s.e. 10.67), and the inoculated group had the highest mean concentration (151.39 mg/g fresh weight ± s.e. 10.0), though not all differences were significant (Figure 4.5). The only difference in group means that was significant was between the chemically treated group and the inoculated group ($p = 0.021$; t = 3.053), and differences were marginal between the chemically treated group and the biochar group ($p = 0.071$) or the control group ($p = 0.068$). No differences existed between the inoculated group and the control group ($p = 0.847$), between the inoculated and biochar groups ($p = 0.902$), or between the control and biochar groups (p = 0.917).

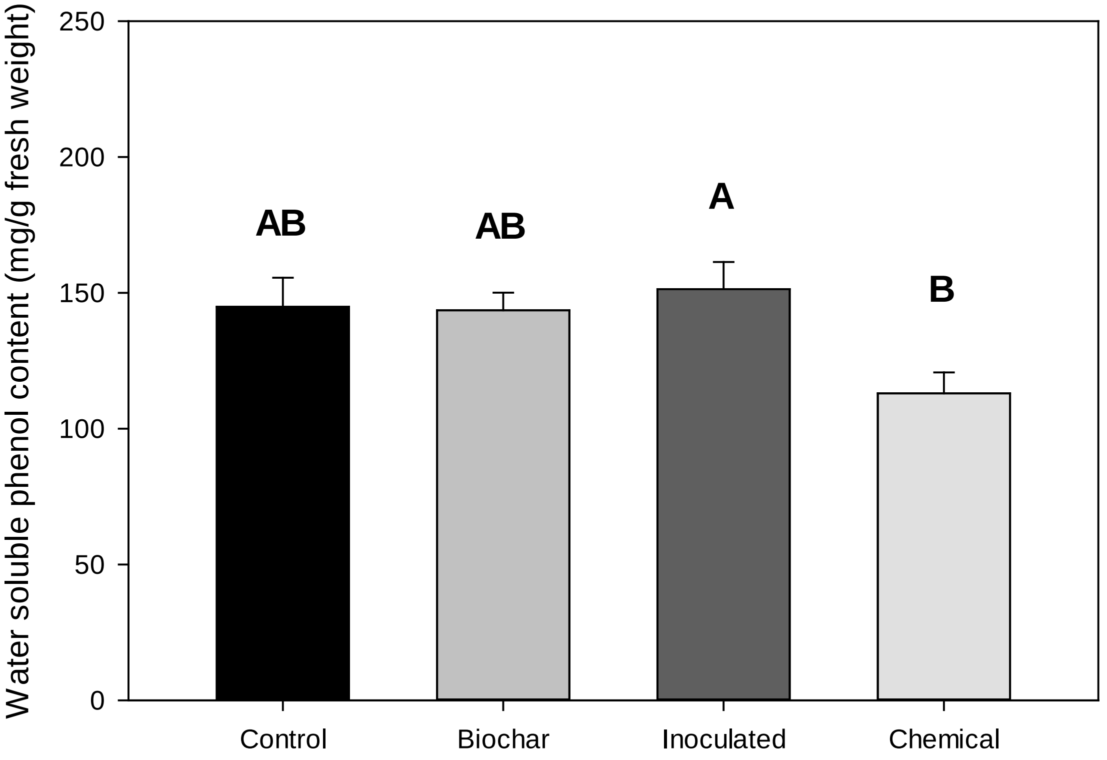

Figure 4.5. Mean water soluble phenolic content (foliar) determined by tannic acid equivalents in seedlings of Acer rubrum which were inoculated with *Phytopthora cactorum* ('inoculated'), amended with 5 % biochar and inoculated ('biochar'), chemically treated ('Chemical'; Agrifos® applied at labeled rates), or not amended or inoculated ('control'). Bars are one standard error; treatments that do not share a common letter were significantly different from each other (*p* = 0.05, Holm-Sidak method).

4.4 Discussion

After obtaining similar results in separate experiments over two growing seasons, it is clear that amendment of potting media with 5 % biochar resulted in a reduction in the expansion of stem lesions caused by *Phytophthora cactorum* in *Acer rubrum* seedlings. Both vertical extension and stem-girdling percentage of 'above-ground' lesions were reduced by incorporation of biochar in the root zone, indicating a systemic effect of biochar on relative disease resistance, in agreement with previous research in other host-pathogen combinations (Elad et al., 2010; Harel et al., 2012). Lesion size was reduced in biochar treated seedlings; however, the pathogen was not eliminated, as evidenced by the presence of lesions beyond the site of inoculation and the successful recovery of the pathogen from lesion margins at the conclusion of both experiments.

While there were clear and consistent results in regard to lesion expansion, differences in plant condition and growth between years made comparisons of physiological parameters somewhat difficult. In 2011, there was a clear reduction followed by recovery in the control plants and biochar amended plants, while in 2012, all plants, including controls, showed a decline in most parameters as the experiment progressed, and while there was no apparent recovery phase in the control or biochar amended groups in 2012, most parameters declined more slowly in these groups compared to the inoculated group that was not amended with biochar. Nonetheless, much of the physiological data reflect that the damage caused by the pathogen was reduced but not completely prevented by biochar amendment; in almost all parameters, the biochar amended, inoculated plants performed at an intermediate level between the inoculated plants that were not amended, and the non-inoculated control plants.

The biomass of ‘biochar’ treatment group in the 2011 experiment was intermediate between the ‘control’ and the ‘inoculated’ group, but in 2012 there were no differences in biomass. In 2011, the differences might have been directly related to net carbon assimilation, as mean assimilation in most weeks followed the same trends (control > biochar > inoculated) as biomass at the conclusion. In 2012, while there were no differences in overall biomass, there was an interesting trend in the ratio of stem to root mass. The proportion of stem mass in relation to root mass (S:R ratio) was highest in the inoculated group, followed by the biochar amended and inoculated group, the girdled group, and lastly the control group had the smallest stems in relation to root mass. This pattern coincides with stem lesion size; potentially indicating that disruption of phloem transport caused the deposition of carbohydrates above the lesion site (stem) rather than contributing to root mass. While the girdled treatment resulted in removal of more phloem than was killed in the inoculated treatment, this treatment was applied later in the season and would not have affected the transport of photosynthetic products for much of the growing season.

Carbon assimilation and stomatal conductance varied based on the treatment and the year of experiment, but over the course of the two experiments, strong trends emerged indicating that in addition to, or perhaps due to the reduction of lesion expansion, amendment of inoculated plants with 5 % biochar improved photosynthetic performance. Patterns of carbon assimilation over time indicate that the biochar amended seedlings showed a trend toward recovery by the end of the experiment, similar to control plants in 2011, while assimilation in inoculated seedlings remained steadily depressed or in decline. Measures of photosynthetic efficiency of PS-II indicated significant differences between 'inoculated' and 'biochar' treatment groups in just one week in 2011, indicating that damage to the photosynthetic apparatus was probably not caused directly by the pathogen, and differences in assimilation rates are not due to the photochemical efficiency of PS-II. Dark-adapted Fv/Fm values trended toward being less impacted in biochar amended plants in both years, and the decline in Fv/Fm values over the course of the experiment in 2012 was less pronounced in 'biochar' treatment group compared to the 'inoculated' treatment group. This trend in 2012 may be explained partially by leaf chlorophyll content. In 2012, SPAD values in all treatments decline through the course of the experiment, and differences between groups were never significant, but mean SPAD readings in the 'biochar' group were always higher than in the 'inoculated' group. The higher levels of chlorophyll and the delayed reduction in this factor are likely to contribute to the differences in chlorophyll fluorescence because reduction in chlorophyll content will increase non-photochemical quenching of light energy (Baker, 2008).

The effects of girdling on physiology of red maple seedlings were interesting in their own right. This treatment was applied in order to compare physical removal of phloem with the slower onset of phloem necrosis caused by pathogen ingress, with expectations of similarity between the two treatments. As expected, physiological parameters were similarly depressed in the 'inoculated' and 'girdled' treatments by the end of the experiment, but the reductions in the 'girdled' group was much more rapid than anticipated. One likely explanation for the rapid crash in assimilation, conductance, and photosynthetic efficiency is that the girdling treatment was not limited to phloem, and functional xylem was severed in the process, resulting in water shortage and the subsequent reduction in assimilation common to that stress. However, stem water potential measures taken at mid-day at the conclusion of the experiment did not show any differences in water status among any of the treatment groups. In fact, in the 'girdled' treatment group, the mean stem water potential was the least negative of the four groups. The most likely explanation for this rapid decrease in the absence of effects on water relations is that the girdling wound was significantly larger than the necroses caused by the pathogen, and the instant removal of phloem tissue does not directly mimic the slow advance of the pathogen as it grows in phloem tissue.

Unfortunately, the data regarding concentrations of soluble phenolic compounds do not clarify the potential mechanism leading to increased disease resistance in biochar amended plants. There was no difference in phenolic concentrations caused by inoculation or by biochar amendment followed by inoculation. One theory regarding the mechanism behind increased resistance in biochar amended plants is hormesis (Graber et al., 2010), which is based on the known phenomenon that compounds that may be toxic at high concentrations can be beneficial at lower levels (Calabrese and Blain, 2009; Prithiviraj et al., 2007). If this were occurring, systemic phenolic concentrations would be expected to be highest in the biochar amended group due to low-level phytotoxic compounds originating from the biochar material 'priming' the defense response, but this was not seen. Interestingly, the lowest concentration of phenolic compounds was found in the group that was treated with a disease management compound known to induce resistance in plants against *Phytophthora* pathogens, salts of phosphorous acids (Daniel and Guest, 2006; Jackson et al., 2000). This may be explained by the fact that the increased defense reactions in treated plants is only apparent after pathogen challenge, and then localized to the site of pathogen ingress (Daniel et al., 2005). In this experiment, it seems that the resistance afforded by biochar does not stem from the same underlying mechanism as resistance induced by the chemical treatment.

In combination with the experiment described in previous chapters, here I have shown that amendment of potting media with 5 % biochar (v/v) can help to reduce the expansion of necrotic lesions caused by *P. cactorum* on *A. rubrum* seedlings, and mitigate some of the negative physiological impacts of infection on the photosynthetic processes. As with the previous experiment, the mechanism underlying the reduction in assimilation and other physiological parameters appears to be linked to the reduction in supply of carbon dioxide to the site of carboxylation, caused by reduced stomatal conductance. This reduction in assimilation may have been caused by direct recognition by the plant of pathogen derived signals, or due to hormonal signaling in the plant upon wounding and recognition of the pathogen. By the end of the experiment in 2011, moisture stress might have also played a role in the reduced photosynthesis of the inoculated plants as evidenced by increasing water use efficiency and decreasing stomatal conductance. While the exact mechanism underlying the biochar-induced mitigating effect is unknown, it appears to be of a systemic nature as improvements were seen despite a physical separation between the pathogen and the biochar as the biochar was incorporated in the rooting media and the inoculation site was on the seedling stems well above the soil-line.

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CHAPTER 5

Conclusions and Future Directions

5.1 Conclusions

The experiments described in the preceding chapters were designed to determine the specific physiological cause of reduced photosynthetic performance in tree seedlings inoculated with *Phytophthora* stem canker pathogens, using *P. cactorum* infection of *Acer rubrum* as a model system. Further, the potential for biochar as a soil amendment to induce resistance in tree seedlings against the negative impacts of stem infection by *Phytophthora* pathogens was tested. Finally, an additional experiment was conducted in an attempt to understand the physiological mechanisms underlying the impact of biochar soil amendment on the progression and effects of *P. cactorum* stem infection in *Acer rubrum*.

Similar to many studies on *Phytophthora* stem-canker pathogens in tree seedlings, *P. cactorum* inoculation of *A. rubrum* seedlings resulted in a decrease in carbon assimilation and stomatal conductance, as well as a decrease in photosynthetic efficiency, and some moisture stress as indicated by an increase in water use efficiency. Three general mechanisms have been speculated to be the cause of the reduction in assimilation rates in infected seedlings: 1) xylem blockage and water stress, 2) phloem damage limiting transport of carbohydrates, resulting in feedback inhibition, and 3) effects of a pathogen derived signal or plant resistance response that shifts metabolism from assimilation to respiration and production of defense related compounds.

In the first study, designed to determine the cause of reduced assimilation in infected plants, foliar starch levels were highest in the control plants, which also showed the highest assimilation rates throughout both seasons, indicating that feedback inhibition was not the cause of reduced assimilation in the infected plants. Xylem did not appear to be colonized visually, but water use efficiency did increase in the infected plants over time, indicating a slight effect of the pathogen or wounding process on water relations. However, this indication of moisture stress arose slowly throughout the course of the experiment, while the reduction in assimilation and stomatal conductance in infected plants was rapid and dramatic, and remained steadily depressed over time, as opposed to slowly decreasing. This indicates that the effects of infection on moisture relation and stomatal conductance may have played a longer term role in reduced photosynthesis, but some other mechanism likely caused the initial rapid reduction in assimilation. Additionally, the recovery in efficiency of photosystem-II seen in fluorescence data shows that long-term damage to or down-regulation of the photosynthetic apparatus did not occur, which would be expected under extreme drought stress. While not tested explicitly, the evidence suggests that a signal derived directly from the pathogen acted as an elicitin, or a hormonal response by the plant in response to wounding and infection resulted in reduced stomatal aperture and a subsequent reduction in assimilation due to supply limitations of carbon dioxide.

In the biochar amendment experiment, 5 % biochar incorporation in rooting media successfully reduced the size of lesions caused by *P. cactorum* in *A. rubrum*, and caused by *P. cinnamomi* in *Quercus rubra* seedlings. In *A. rubrum*, 5 % biochar amendment of inoculated seedlings also resulted in improved photosynthetic performance and delayed senescence when compared to inoculated seedlings that had not been amended. In *Q. rubra* seedlings, amendment with 5 % biochar reduced the size of lesions and also the negative impact of infection on plant water relations as evidenced by stem water potential. While biochar amendment at 5 % resulted in several measurable benefits compared to infected plants that had not been amended, incorporation of 10 or 20 % biochar did not result in similar benefits, and in some cases appeared detrimental. Also, seedlings treated with Agri-fos®, a systemic fungicide known to induce resistance to *Phytophthora* pathogens, performed better than any seedlings in any biochar treatment.

In the final experiment, reduction of lesion sizes and improvement in photosynthetic performance in seedlings amended with 5 % biochar compared to non-amended inoculated seedlings was demonstrated once again. Biochar amended plants showed higher assimilation and conductance rates, higher photosynthetic efficiency, and greater leaf chlorophyll content, with a delayed reduction in these factors over time. However, analysis of leaf defense chemistry did not provide a clear mechanism underlying this improvement. Stimulation of plant defenses through low level toxicity of biochar (hormesis) or through alterations to the soil microbial community resulting in a shift toward more plant-growth and defense promoting microbes have both been suggested as possible mechanisms. In either case, I expected to see an increase in phenolic defense compounds in both the biochar amended and the chemically treated plants. In fact, there were no significant differences in leaf phenolic content between the biochar amended and control, or inoculated plants that had not been amended. Additionally, the chemically treated plants showed the lowest levels of foliar phenolic compounds, despite this treatment resulting in the smallest lesions of all inoculated treatments. This may be explained by research that has shown the induced response to pathogen ingress resulting from chemical treatment, or by microbial instigated defense, is often highly localized to the area of infection, while the analysis here was performed on foliage that was not directly adjacent to the infection site. Also, while care was taken to provide plants with ample nutrients and water in order to control for the beneficial aspects of biochar in relation to those factors, it is still possible that the presence of biochar improved overall plant vigor in some way, resulting in an improved capacity to defend against pathogens.

While the exact mechanisms underlying the improvement in disease resistance and photosynthetic performance afforded by biochar amendment in inoculated plants was not uncovered entirely, these results do show a reduction in progression and impact of *Phytophthora* pathogens in two common landscape tree species. Further, the results of this research refute the hypothesis that reduced assimilation in infected plants is caused by feedback inhibition as a result of phloem damage and reduced transport of carbohydrates. Rather, the reduction in photosynthesis caused by *Phytophthora* stem lesions in tree seedlings appears to be related to the impacts on the supply of carbon dioxide, with either pathogen derived or induced signals, wound-related signals, or a combination thereof leading to a reduction in stomatal conductance and subsequent decrease in carbon dioxide supply at the site of carboxylation.

5.2 Improvements and further research

In retrospect, there were several elements of these experiments that may be altered or expanded upon in order to improve and strengthen results and conclusions. First, as with any experiment, increasing the size of treatment groups, and obtaining a more homogeneous plant population will result in decreased variability and increased statistical power. While 20 replicates of each treatment in the 2011 experiments proved ample to show treatment effects and statistical differences, the reduction to 8 replicates in 2012 due to the poor condition of plant material resulted in high variability and decreased ability to test for the differences among treatments. Also, while the seedlings used here were all *Acer rubrum*, using a cloned line of seedlings would decrease genetically based differences in disease resistance. It may also have been of interest to delay harvest until the season following treatment and inoculation. Multiple-year studies are difficult, but may be more accurate in reflecting the disease progression in woody plants. Recovery or mortality might have occurred in various treatments after a second season, and more dramatic physiological effects may have been apparent. An additional treatment group with 5 % biochar amendment and no inoculation could also have been useful to determine if there was any improvement in plant performance resulting from biochar compared to non-amended seedlings despite attempts to control for differences in soil nutrient retention, moisture, and physical properties.

Regarding the effects of infection on foliar starch levels, a series of samples through time, corresponding to assimilation rate data, might have provided more information on the relation between infection, assimilation, phloem disruption, and assimilate partitioning. In this study the highest level of starch corresponded with the highest assimilation rates, suggesting that there was never a demand-based limitation to carbon assimilation caused by feedback, but previous studies have shown that starch accumulation precedes reduced assimilation, which, if it occurred, would have been more easily observed from samples taken earlier in the experiment.

Similarly, in the analysis of defense chemistry, both the timing of sampling and the plant portion sampled probably affected my ability to draw solid conclusions from phenolic data. First, both chemical and microbial induced resistance to pathogen attack, including to *Phytophthora* infection specifically, have been shown to occur in the area immediately surrounding the infection site. Analysis of the stem tissue immediately surrounding the lesions might have resulted in more definite results, however, my intention was to detect a systemic, low-level induction in foliage resulting from biochar incorporation, albeit unsuccessfully. Any short-term effects on foliar phenolic levels following inoculation may also have subsided by the end of the experiment when foliage was harvested for analysis.

A final aspect that could have altered or improved the results would have been to use a more natural infection process in the inoculation of seedlings rather than a physically damaging stem-wound inoculation technique. There are several methods of inoculation that do not directly damage phloem or xylem tissue, including placing agar plugs directly on bark, or creating a zoospore suspension and wetting the stem with spores. One drawback to these methods is the need for many more replicates in order to assure a useful number of plants that actually develop infection. Artificial wounding of the stem probably resulted in some unintended wound responses from the plant which must be separated from the response to infection, but the method also ensures a very high infection rate.

5.3 Future directions

As an application-minded plant pathologist and physiologist, my future research regarding biochar and *Phytophthora* disease will be focused predominantly on “real life” field applications. While many important physiological aspects of plant growth and disease development can be assessed using seedlings in a controlled greenhouse setting, the true test for developing plant health care practices comes from mature plants in the field that are subject to multiple biotic and abiotic stress factors, in addition to normal symbiotic relationships. Experiments concerning the incorporation of biochar into the root zones of mature trees and the effect on plant disease are needed to determine if the disease reduction seen in these experiments holds under field conditions. Additionally, and in specific reference to the epidemiology of Sudden Oak Death caused by *Phytophthora ramorum*, the spore production of *P. ramorum* on foliar hosts (particularly *Umbellularia californica*, California bay-laurel) is widely considered to be the driving factor in disease spread and infection of oaks. Both greenhouse and field trials examining the effects of biochar on foliar host spore production is another interesting aspect that should be explored. Reduction of lesion size, number, or spore production by *P. ramorum* in foliar hosts could have a significant impact on local inoculum pressure and disease transmission. The effects of biochar incorporation on soil-survival and spore production of any *Phytophthora* species that predominantly cause root diseases are also of great interest.

Longer term studies on biochar and disease resistance are also needed in order to determine the length of time that biochar amendment may impact plant disease resistance. All studies published to date examine the effects on disease in the first year after biochar incorporation. While this approach is logical in annual crops, in long-lived tree species, this approach does not provide all of the necessary information for plant health managers. For example, if the improvements in plant resistance are a result of improved vigor resulting from nutrient, soil, moisture, or microbial impacts of biochar incorporation, then the reduction in disease could be expected to be a long-term phenomenon. However, if the reduction in disease, or increase in beneficial microbes, is related to the soluble or mobile fraction of biochar composition, the effects may be transitory and re-application of biochar may be necessary to maintain disease reductions. Additional studies on the relation between biochar and soil microbial communities, and the potential to ‘seed’ soil with biochar that has been colonized by beneficial microorganisms are also areas of considerable interest that may impact the management of trees in the landscape.