CONTROL OF FIRE BLIGHT THROUGH SYSTEMICALLY ACQUIRED RESISTANCE AND INTRASPECIES CHARACTERIZATION OF ERWINIA AMYLOVORA

by

Lorraine May Fleck

A thesis submitted in conformity with the requirements for the degree of Master of Science in Forestry (M.Sc.F.) Graduate Department of Faculty of Forestry University of Toronto

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Abstract

*Ophiostoma ulmi* and *Verticillium dahliae* elicitors were tested for efficacy against *Erwinia amylovora*, causal agent of fire blight, in apple and pear cultivars. Despite inconsistent disease control in apple seedlings with *O. ulmi* elicitors, both elicitors provided significant protection against *E. amylovora* in 4 year-old apple and pear trees (*P* < 0.05). *E. amylovora* intraspecies differences in extracellular protease production and PCR products of the 16S/23S rDNA intergenic transcribed spacer (ITS) region were also assessed for correlation with host species-specificity. Both *Maloideae* and *Rubus* isolates secreted an extracellular protease of similar size and activity. The ITS PCR products of *Maloideae* and *Rubus* *E. amylovora* isolates encoded for tRNA^Ala^ and tRNA^Glu^, and shared significant homology. The smallest ITS PCR product of *E. amylovora Rubus* isolate 1-97 was homologous with the ITS region of *E. pyrifoliae*, contradicting previous conclusions that *E. amylovora* and *E. pyrifoliae* were not homologous in the ITS region.
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# Table of Contents

**Abstract**

**Acknowledgements**

**Table of Contents**

**List of Tables**

**List of Figures**

<table>
<thead>
<tr>
<th>1</th>
<th>Introduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Host plants and geographic distribution</td>
</tr>
<tr>
<td>1.2</td>
<td>Economic impact</td>
</tr>
<tr>
<td>1.3</td>
<td>The pathogen: <em>E. amylovora</em></td>
</tr>
<tr>
<td>1.3.1</td>
<td>Life cycle and symptoms</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Pathogenicity</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Intraspecies differences in the 16S-23S rDNA spacer region</td>
</tr>
<tr>
<td>1.4</td>
<td>Fire blight management</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Orchard maintenance</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Predictive models</td>
</tr>
<tr>
<td>1.4.3</td>
<td>Chemical control</td>
</tr>
<tr>
<td>1.4.4</td>
<td>Biological control</td>
</tr>
<tr>
<td>1.4.5</td>
<td>Control through systemic acquired resistance and growth regulation</td>
</tr>
<tr>
<td>1.5</td>
<td>Plant defence mechanisms in plant-pathogen interactions</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Compatibility in plant-pathogen interactions: the elicitor-receptor model</td>
</tr>
<tr>
<td>1.5.2</td>
<td>Elicitors and the host response</td>
</tr>
<tr>
<td>1.5.3</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>1.6</td>
<td><em>Ophiostroma ulmi</em> elicitor</td>
</tr>
<tr>
<td>1.7</td>
<td><em>Verticillium dahliae</em> elicitor</td>
</tr>
<tr>
<td>1.8</td>
<td>Objectives</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2</th>
<th>Materials and Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td><em>E. amylovora</em> isolates, media and culture conditions</td>
</tr>
<tr>
<td>2.2</td>
<td><em>O. ulmi</em> elicitor preparation</td>
</tr>
<tr>
<td>2.3</td>
<td><em>V. dahliae</em> elicitor preparation</td>
</tr>
<tr>
<td>2.4</td>
<td>Elicitor efficacy testing</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Seedling experiments</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Inoculum dose experiment</td>
</tr>
<tr>
<td>2.4.3</td>
<td>Field experiments</td>
</tr>
</tbody>
</table>
# Table of Contents

<table>
<thead>
<tr>
<th>2</th>
<th>Materials and Methods continued</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td><em>E. amylovora</em> host species-specificity: <em>E. amylovora</em> intraspecies characterization by secreted protease bioassay</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Crude protease fraction preparation</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Protein concentration assay</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Protease electrophoresis and activity</td>
</tr>
<tr>
<td>2.6</td>
<td><em>E. amylovora</em> host species-specificity: <em>E. amylovora</em> intraspecies characterization by intergenic transcribed spacer (ITS) regions</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Genomic DNA isolation</td>
</tr>
<tr>
<td>2.6.2</td>
<td>PCR amplification</td>
</tr>
<tr>
<td>2.6.3</td>
<td>Cloning of PCR products</td>
</tr>
<tr>
<td>2.6.4</td>
<td>Plasmid DNA isolation</td>
</tr>
<tr>
<td>2.6.5</td>
<td>DNA sequencing and analysis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Efficacy of <em>O. ulmi</em> and <em>V. dahliae</em> elicitors against <em>E. amylovora</em></td>
</tr>
<tr>
<td>3.2</td>
<td>Correlation between <em>E. amylovora</em> host species-specificity and extracellular protease activity</td>
</tr>
<tr>
<td>3.3</td>
<td>Correlation between PCR products of the ITS region and <em>E. amylovora</em> host species-specificity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4</th>
<th>Discussion</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Efficacy of <em>O. ulmi</em> and <em>V. dahliae</em> elicitors against <em>E. amylovora</em></td>
</tr>
<tr>
<td>4.2</td>
<td>Host species-specificity and extracellular proteolytic activity of <em>E. amylovora</em></td>
</tr>
<tr>
<td>4.3</td>
<td>PCR products of the <em>E. amylovora</em> ITS region and host species-specificity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>References</td>
</tr>
<tr>
<td>Table</td>
<td>Microbial isolates, hosts, sources, and their role in experimentation</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------------------------------------------</td>
</tr>
<tr>
<td>Table 1</td>
<td>Microbial isolates, hosts, sources, and their role in experimentation</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Photomicrograph of <em>E. amylovora</em> (x 18,000)</td>
<td>5</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Life cycle of <em>E. amylovora</em></td>
<td>6</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Fire blight symptoms on pome fruit trees</td>
<td>8</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Schematic representation of systemic acquired resistance (SAR)</td>
<td>25</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Disease severity index used for evaluating fire blight symptoms in <em>E. amylovora</em> inoculated apple seedlings</td>
<td>37</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Efficacy of <em>O. ulmi</em> Q412 elicitor against <em>E. amylovora</em> in Golden delicious apple seedlings</td>
<td>49</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Efficacy of <em>E. amylovora</em> Ea 6-4 in fire blight symptom development in Golden delicious apple seedlings</td>
<td>50</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Efficacy of <em>O. ulmi</em> elicitors against <em>E. amylovora</em> in Golden delicious apple seedlings</td>
<td>51</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Efficacy of <em>O. ulmi</em> and <em>V. dahliae</em> elicitors (1 mg/tree) in 4-year old Bartlett pear trees</td>
<td>52</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Efficacy of <em>O. ulmi</em> (40 mg/tree) and <em>V. dahliae</em> (20 mg/tree) elicitors against fire bight in 4 year-old Ida Red apple trees.</td>
<td>53</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Efficacy of <em>O. ulmi</em> Q412 elicitor against fire blight in 4 year-old Bartlett pear trees</td>
<td>54</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Efficacy of <em>V. dahliae</em> elicitor against fire blight in 4 year-old pome fruit trees</td>
<td>55</td>
</tr>
<tr>
<td>Figure 13</td>
<td>SDS-gelatin-PAGE assay of secreted proteases produced by <em>Maloideae</em> and <em>Rubus E. amylovora</em> isolates</td>
<td>58</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Agarose gel electrophoresis of PCR-amplified ITS region of <em>E. amylovora</em> isolates</td>
<td>60</td>
</tr>
<tr>
<td>Figure 15</td>
<td>DNA sequence of <em>E. amylovora</em> pear isolate D-7 739 bp ITS PCR product, encoding for tRNA&lt;sup&gt;Glu&lt;/sup&gt;</td>
<td>61</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 16</td>
<td>DNA sequence of <em>E. amylovora</em> pear isolate D-7 870 bp ITS PCR product, encoding for tRNA$^{\text{Ala}}$</td>
<td>62</td>
</tr>
<tr>
<td>Figure 17</td>
<td>DNA sequence of <em>E. amylovora</em> pear isolate D-7 1088 bp ITS PCR product, encoding for tRNA$^{\text{Ala}}$</td>
<td>63</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Comparison of the two DNA sequences of <em>E. amylovora</em> isolate D-7 which encode for tRNA$^{\text{Ala}}$</td>
<td>64-65</td>
</tr>
<tr>
<td>Figure 19</td>
<td>DNA sequence of <em>E. amylovora</em> raspberry isolate 7-96b 737 bp ITS PCR product, encoding for tRNA$^{\text{Glu}}$</td>
<td>66</td>
</tr>
<tr>
<td>Figure 20</td>
<td>DNA sequence of <em>E. amylovora</em> raspberry isolate 7-96b 942 bp ITS PCR product, encoding for tRNA$^{\text{Ala}}$</td>
<td>67</td>
</tr>
<tr>
<td>Figure 21</td>
<td>DNA sequence of <em>E. amylovora</em> raspberry isolate 1-97 730 bp ITS PCR product, encoding for tRNA$^{\text{Glu}}$</td>
<td>68</td>
</tr>
<tr>
<td>Figure 22</td>
<td>DNA sequence of <em>E. amylovora</em> raspberry isolate 1-97 945 bp ITS PCR product, encoding for tRNA$^{\text{Ala}}$</td>
<td>69</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Comparison of the DNA sequences of the smallest 16S/23S ITS PCR product (tRNA$^{\text{Glu}}$) in <em>Maloideae</em> and <em>Rubus</em> <em>E. amylovora</em> isolates</td>
<td>70-71</td>
</tr>
</tbody>
</table>
1 Introduction

Fire blight, caused by the bacterium *Erwinia amylovora* (Burill) Winslow *et al* is a serious necrotic wilt disease that affects the Rosaceae plant family. The disease is termed fire blight (Coxe, 1817), as affected tissue appears persistently blackened and the plant, tree or shrub looks as through scorched by fire. Fire blight was initially observed in the Hudson River Highlands of New York state in 1780 (Stapp, 1961). *E. amylovora*, which is native to North America, was the first bacteria shown to cause a plant disease (Arthur, 1885; Burrill, 1880).

1.1 Host plants and geographic distribution

Despite initial reports of fire blight in North America during the late eighteenth century, the disease was not a frequent problem for another 25 years (van der Zwet and Beer, 1991). The first incidence of fire blight in Canada occurred in 1865 (Bonn and van der Zwet, 2000). In recent years, severe infestations have destroyed entire orchard blocks in southern Ontario and British Columbia (Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA), 2000). *E. amylovora* is established in the major apple producing regions of the United States, including California, the Northwest, Midwest and Appalachian regions (van der Zwet and Beer, 1991). The disease’s ubiquity has severely restricted pear cultivation in the eastern United States (Thomson, 1992).

Fire blight has spread beyond North America and is now a disease of international importance. Novel fire blight occurrences are common given the wide distribution of *E. amylovora* infections (Thomson, 1992). Fire blight is established in Mexico,
Guatemala, Bermuda, the United Kingdom, most of Europe, the Middle East and New Zealand (Plant Quarantine Policy Branch, Australian Quarantine and Inspection Service, 2000).

*E. amylovora*’s host range includes susceptible plants in at least 174 species and 40 genera, all in the family *Rosaceae* (van der Zwet and Beer, 1991). The disease is especially destructive to *Maloideae* (*Pomoideae*) subfamily, which includes the pome fruits pear (*Pyrus*) and apple (*Malus*) (Momol and Aldwinkle, 2000). Other economically important hosts include *Cotoneaster* (cotoneaster), *Crataegus* (hawthorn), *Cydonia* (quince), *Pyracantha* (firethorn), and *Sorbus* (mountain ash) (van der Zwet and Beer, 1991). Fire blight infections can also occur on *Rubus* plant species (e.g. raspberries). *E. amylovora* strains isolated from *Rubus* hosts display host species-specificity, as *Rubus* isolates are not pathogenic on other fire blight hosts (Heimann and Worf, 1985; Ries, and Otterbacher, 1977; Starr *et al*., 1951; Vanneste, 1995). Further, non-*Rubus* *E. amylovora* isolates are non-pathogenic on *Rubus* hosts (Vanneste, 1995). In general, *E. amylovora* isolates are not host-species specific (Momol and Aldwinkle, 2000).

1.2 Economic impact
Cultivated apples and pears are the most seriously affected *E. amylovora* host species. Fire blight losses can be substantial, given both the agricultural importance of these host species and the fact that superior horticultural traits (e.g. taste, storage, and marketing qualities) have been difficult to combine with high levels of disease resistance (Johnson and Stockwell, 1998). Due to consumer demand for
specific cultivars, the majority of commercial apple cultivars currently planted have shifted towards those with greater fire blight susceptibility (Stockwell et al., 1998). Further, the most popular rootstocks, “dwarfing” varieties whose compact growth allows more trees to be planted per unit area (e.g. M9), are also the most susceptible to fire blight (Steiner, 2000).

Estimates for orchard losses due to fire blight are scarce; the damage caused by the disease is erratic, and in many cases an actual loss cannot be assessed (Thomson, 1992). The current season’s crop production can be eliminated with blighting of virtually every blossom as well as the death of spurs and subsequent loss of future fruit production. Severe outbreaks regularly result in damage totalling several million U.S. dollars (Bonn and van der Zwet, 2000). A recent fire blight outbreak in southwest Michigan, which occurred in the year 2000, is estimated to have a total loss of approximately $42 million U.S. (Longstroth, 2000). This figure is based on the loss of 350,000 to 450,000 apple trees over 1,550 to 2,300 acres, and includes orchard development costs (approximately $9 million U.S.), immediate apple yield reductions of approximately $10 million U.S. (2.7 million bushels), and an additional 4 years for apple production to recover from the outbreak (a cumulative loss of $23.2 million U.S.) (Longstroth, 2000).

The mere report of fire blight in a country can be an extremely important economic and trade factor. Many countries will not accept susceptible fruit or nursery stock from countries where fire blight occurs. Further, quarantine measures are estimated
to cost several million U.S. dollars each year (Thomson, 1992). Given fire blight’s impact on agriculture, and the fact that the disease has never been eradicated from a country with an established fire blight history (Thomson, 1992), a substantial body of research has focused on disease control and prevention.

1.3 The pathogen: *E. amylovora*

*E. amylovora* (figure 1) is a Gram-negative, rod-shaped bacterium that is a member of the *Enterobacteriaceae* family (Holt, 1994). The bacterium, motile via peritrichous flagella, is 0.5 - 1.0 by 1.0 - 3.0 μm in size and can occur singly, in pairs, and in short chains of bacterial cells. Optimal growth of *E. amylovora* occurs between 24°C and 29°C; however, the pathogen can grow over a much broader temperature range of 4°C to 32°C.

1.3.1 Life cycle and symptoms

The life cycle (figure 2) begins in spring with the production of inoculum and blossom infection, continues through summer with progressive infection into the tree, and ends in late summer or early autumn with canker development. The pathogen is quiescent during host dormancy. Infection is either primary or secondary, depending on the time of year. Fire blight can be classified as blossom, fruit, leaf, shoot, twig, limb, trunk, collar or root blight, depending on infection location (van der Zwet and Beer, 1991). Bacterial invasion through wounds created by extreme weather events (rain, hail and wind storms), insect feeding, or mechanical damage results in trauma blight (Thomson, 1992).
Figure 1. Photomicrograph of *E. amylovora* (x 18,000).

Spring

Extension of cankers in the spring

Flower infections through nectarrhodes and other flower parts

Endophytic populations in bud wood or trees

Summer

Honeybees and other flower visiting insects spread bacteria to flowers & leaves

Ooze spread by rain, insects, aerosols, birds, humans.

Direct infection of flowers

Rain dissemination

Low populations of bacteria are deposited on stigmas, where they multiply and are spread by rain to other flowers

Shoot infections

Infection of immature fruit

Strands, aerosols and ooze provide inoculum for insects, rain and hail to spread to other shoots

Progression of flower infections to produce cankers

Cankers develop on branches and provide a site for overwinter survival of the pathogen

Late summer or autumn, shoot infections often result in indeterminate cankers

Winter

Inside the plant

Outside the plant

Autumn

Figure 2. Life cycle of E. amylovora.

Adapted from Thomson (2000).
Primary infection is the first infection of the season, and usually occurs in spring when *E. amylovora* invades the blossom or shoots of a susceptible host plant. The main inoculum originates from ooze-producing cankers (figure 31) that become active in early spring, when *E. amylovora* multiplies in adjacent healthy bark tissue (van der Zwet and Beer, 1991). These cankers (necrotic tissue in the cortex or bark) developed in previous years and are typically located on larger limbs or the tree trunk. Cankers with indeterminant margins (lacking a defined border between healthy and diseased tissue) are more likely to harbour the bacteria than those with determinate margins (Beer and Norelli, 1977; Thomson, 2000). Ooze is a combination of the exopolysaccharide (EPS) amylovoran produced by *E. amylovora* (section 1.3.2) and the bacterium itself; the consistency can vary with atmospheric humidity from discrete drops to thin strands. Ooze constitutes the primary inoculum source and facilitates the dissemination of the bacterium. Disease control becomes difficult, since ooze is vectored by insects and birds. Ooze also spreads via water, orchard equipment, and wind.

Blossom infection may occur when insects (e.g. flies) that have contacted ooze vector the pathogen, or when rain or heavy dews wash the ooze into the flower base (OMAFRA, 2000). Honeybees do not visit the ooze and are not responsible for primary dispersal of inoculum (Thomson, 2000). Once a blossom is colonized, *E. amylovora* multiplies on the stimagatic surfaces of the flower. The pathogen enters blossom tissue through nectarthodes (specialized nectar-secreting stomata) (Vanneste and Eden-Green, 2000). Bacterial growth and migration results in
Figure 3. Fire blight symptoms on pome fruit trees.


blossoms with small ooze droplets on the flower stem and darkened sepals. Blossom infection is important in *E. amylovora*'s distribution, since the bacterium is easily vectored to healthy blossoms by pollinating bees. This disperses fire blight throughout an orchard.

In susceptible cultivars, continued spread of *E. amylovora* in the host, or infection through stomatal openings and wounds, causes leaf and shoot blight. Leaf blight in susceptible cultivars is characterized by blackening of the petiole and leaf midrib (figure 3B); ooze may be present. Sometimes infection is limited primarily to the central leaf vein; complete leaf necrosis (figure 3C) is common. Shoot blight may result from internal bacterial multiplication in the cortical parenchyma (Vanneste, 1995). When succulent shoots are affected, they bend characteristically to form a shape known as the shepherd's crook (figure 3E). Fire blight progresses very rapidly in the shoots of susceptible cultivars and may advance 10 to 30 cm in one day (Thomson, 1992). Humid weather is also favourable for shoot blight (van der Zwet and Beer, 1991). Ooze frequently appears on blighted shoots (figure 3F).

Advancing disease in infected shoots leads to twig (figure 3D), limb or trunk blight (figure 3G) (van der Zwet, and Beer, 1991). Susceptible cultivars with extensive fire blight can develop collar or root blight, both usually lethal. Cankers (figure 3I) often accompany limb, trunk or collar blight. Because cankers develop when *E. amylovora*'s multiplication slows, they may appear in late spring, summer or autumn (van der Zwet and Beer, 1991). The bacterium overwinters in the cankers when the
host is dormant, providing inoculum for primary fire blight infections in the spring. The cankers may expand and cause an obstruction of the vascular bundles termed girdling (Tainter and Baker, 1996). Girdling disrupts nutrient and water transport, killing all tissue distal to the canker. Girdling on limbs can lead to a substantial loss of tree structure; girdling around the trunk's circumference will kill the entire tree.

Abundant ooze from cankers often flows along the bark and ensures secondary infections (infections that are vectored from primary infection inoculum during the current life cycle). The inoculum for secondary infections can be also vectored by rain, wind, insects and pruning equipment (Thomson, 1992). These infections often occur through late-emerging secondary blossoms or wounds created on shoots and fruit created by storm damage and insects. Immature fruit often display copious ooze (figure 3H), and are an important inoculum source for secondary infections. Fruit can alternatively become infected through internal invasion of the fruit spur. Secondary infections are usually more numerous and serious than primary ones (van der Zwet and Beer, 1991).

1.3.2 Pathogenicity

*E. amylovora* has been studied intensively in order to understand its pathogenicity and control fire blight. While considerable research has been conducted (see Vanneste (1995) and Vanneste (2000) for reviews), the precise mechanism of *E. amylovora*’s virulence remains unclear. Initial investigations attempted to identify virulence factors common to many plant pathogens, including plant tissue degrading enzymes, toxins, and plasmids (Keen, 1999; Vanneste, 1995). Despite extensive
research, no cell wall degrading enzymes (Seemüller and Beer, 1977) or toxins (Vanneste, 1995) have been identified. Mutants devoid of the pEA29 plasmid, unique to E. amylovora, are still pathogenic (Falkenstein et al, 1989; Laurent et al, 1989; Vanneste, 1995). It is now established that fire blight’s evolutive necrosis is a direct consequence of E. amylovora’s migration in host tissues and multiplication in the intercellular parenchyma (Vanneste and Eden-Green, 2000). Further, two pathogenicity factors produced by E. amylovora, amylovoran and harpin, have been identified.

Amylovoran, a constituent of exopolysaccharide (EPS) or ooze (section 1.3.1), is an acidic heteropolysaccharide that encapsulates the bacterial cell of most E. amylovora strains (Vanneste, 1995). Mutational analysis of the amylovoran synthesis (ams) gene cluster demonstrated mutations in any ams gene resulted in both an absence of EPS production and bacterial pathogenicity (Bernhard et al, 1993). ams mutations that alter or disrupt amylovoran synthesis are correlated with altered pathogenicity and poor (or non-existent) growth in planta (Menggad and Laurent, 1998; Tharaud et al, 1994). These observations confirm prior reports linking E. amylovora encapsulation with virulence (Ayers et al, 1979; Bennett and Billing, 1978). However, since some non-pathogenic E. amylovora strains are encapsulated (Bennett and Billing, 1978), amylovoran is not the sole pathogenicity determinant.

Another pathogenicity factor is harpin, a protein produced by E. amylovora (Wei et al, 1992) that is an elicitor of the hypersensitive response (section 1.5.2). Harpin,
which has demonstrated efficacy as preventative treatment for fire blight (section 1.4.5), is encoded by \textit{hrpN}, located in the \textit{hrp} gene cluster. Genetic studies of several necrogenic bacteria species have determined that the \textit{hrp} (hypersensitive response and pathogenicity) gene cluster is essential for pathogenicity, as the gene cluster controls the production of elicitors (section 1.5.2) and pathogenicity factors (Lindgren, 1997). \textit{E. amylovora} \textit{hrpN} mutants which do not produce harpin are non-pathogenic on pear and do not elicit the hypersensitive response (Wei \textit{et al}, 1992), while many \textit{hrpN} mutants still produce amylovoran (Vanneste, 1995). These observations demonstrate that harpin is required for \textit{E. amylovora}'s pathogenicity and fire blight development.

Research by Zhang \textit{et al} (1999) identified PrtA, a 48 kDa metalloprotease secreted by apple \textit{E. amylovora} isolates that assists host tissue colonization. PrtA was produced by five wild type isolates and three amylovoran-deficient mutants, indicating it is highly conserved in \textit{E. amylovora}. Creation of a protease mutant interrupted protease secretion by the bacterium (Zhang \textit{et al}, 1999). While the mutant’s virulence was unaffected by the mutation, the ability to colonize apple leaf parenchyma was impaired relative to the wild type. Zhang \textit{et al} (1999) concluded the protease mutant possessed a reduced ability to degrade barriers for \textit{E. amylovora} in parenchyma tissue, inhibiting the bacterium’s ability to efficiently colonize host tissue. This indicates PrtA assists in plant tissue colonization, and challenges the predominant opinion that \textit{E. amylovora} does not produce enzymes that degrade host tissues (Vanneste and Eden-Green, 2000). As Zhang \textit{et al} (1999) did not use \textit{E.}}
*amylovora Rubus* isolates in their work, whether PrtA production was present in *Rubus* isolates or correlated with host species-specificity was undetermined.

1.3.3 Intraspecies differences in the 16S-23S rDNA spacer region

Previously, the only intraspecies variation known within *E. amylovora* was the host species-specificity of *Rubus* isolates. The origin of this host-specificity was unknown. However, there is a correlation between the 16S-23S rDNA spacer region (intergenic transcribed spacer or ITS) and *E. amylovora* host species-specificity (Jeng *et al.*, 2001). The 16S-23S ITS region has been previously exploited for bacterial species identification (Jensen *et al.*, 1993; McManus and Jones, 1995; Tyler *et al.*, 1995).

Polymerase chain reaction (PCR) amplification of the ITS region demonstrated that the PCR profile could differentiate *E. amylovora* at the intraspecies level (Jeng *et al.*, 2001). *E. amylovora* isolates obtained from *Maloideae* hosts (apple and pear) exhibited three distinct amplified PCR products, while isolates obtained from *Rubus* hosts (raspberries) exhibited two PCR products. The latter were similar in size to the two smallest fragments obtained from apple and pear isolates (~ 800 kb and ~ 1000 kb). DNA sequencing of the smallest and largest (~ 1200 kb) PCR products from a pear isolate showed these products contained tRNA<sup>Glu</sup> and tRNA<sup>Ala</sup> genes, respectively (Jeng *et al.*, 2001). The ITS PCR products of *Rubus* isolates were not sequenced and compared with PCR products of similar size from *Maloideae* isolates. Therefore, it is unknown if any genes contained within *Rubus* isolates' ITS PCR products correlate with host-species specificity or differ from *E. amylovora Maloideae* isolates.
1.4 Fire blight management
Fire blight prevention and control is achieved through integrated orchard management (IOM) (van der Zwet and Beer, 1991). IOM recognizes that cultural and seasonal conditions (e.g. physiological changes in the host during dormancy cessation) and increased ambient temperature and humidity (Vanneste and Eden-Green, 2000) contribute to disease development. Therefore, IOM utilizes a number of practices to prevent and control fire blight in pome fruit orchards. These include nutrition and soil management; orchard maintenance; and control measures using bactericides and predictive models. Since infection is most likely initiated at bloom, control programs are usually directed towards preventing blossom blight. While there are some fire blight resistant apple cultivars, the majority of commercial apple cultivars planted have shifted towards those with greater fire blight susceptibility due to consumer demand (Stockwell et al, 1998). A similar situation exists with commercial pear cultivars (Lespinasse and Aldwinkle, 2000). Therefore, the use of fire blight resistant trees is not an important control method.

1.4.1 Orchard Maintenance
E. amylovora can be readily vectored by hands, clothing, shoes, pruning tools and the wheels of orchard equipment that have contacted the bacterium. It is vital that pruning tools are completely sterilized between cuts with an appropriate disinfectant (e.g. household bleach or alcohol), as contaminated tools readily spread bacteria from blighted to healthy branches. Insect control is important, as a number of undesirable insect species readily transfer the bacteria to host plants (Hildebrand et al, 2000; van der Zwet and Beer, 1991). Rapid E. amylovora multiplication in
succulent shoot tissue prohibits the use of high dose nitrogen fertilizers and nitrogen-fixing cover crops, as surplus nitrogen stimulates excessive succulent tissue growth. Overhead irrigation should be avoided, since the resulting elevated humidity increases the probability of both fire blight infection and severity (van der Zwet and Beer, 1991). This watering method also disperses bacteria within an infected tree and to uninfected trees, disseminating fire blight throughout an orchard.

1.4.2 Predictive Models
The observation that fire blight epidemics are associated with warm temperatures during bloom has resulted in predictive models that assess the probability of blossom infection, based on observed climatic conditions. These models identify the periods conducive for epiphytic E. amylovora proliferation and dispersion among blossom populations before infection occurs. Such models have been widely adopted by growers to aid decisions on the need for and timing of chemical applications (Johnson and Stockwell, 1998). Mills (1955) was the first to establish a predictive model for fire blight control. This model correlated the seasonal severity of blossom blight with total degree days above a base temperature of 18.3°C (Mills, 1955). Subsequent research reported a positive correlation between the detection of epiphytic E. amylovora in pear blossoms and the accumulated degree hours above 18.3°C (Zoller and Sisevich, 1979).

The use of accumulated thermal units to track epiphytic growth of E. amylovora has been expanded in two recent computerized predictive models: MARYBLYT (Steiner, 1990a; Steiner, 1990b) and COUGARBLIGHT (Smith, 1993; Smith, 1996).
MARYBLYT, which calculates the number of degree hours above 18.3°C, uses a threshold value of 110 cumulative degree hours from the blossom pink stage to determine if the epiphytic inoculum potential is of sufficient size to cause blossom blight. Similarly, COUGARBLIGHT identifies a high-risk period for blossom blight when the total number of degree hours exceeds a certain threshold value. COUGARBLIGHT sums degree hours above 15.5°C on a 4 day moving total. Both models consider rain or blossom wetness during warm weather to determine more precisely the timing or likelihood of blossom infection. This is dependent upon local climate characteristics and previous fire blight outbreaks.

1.4.3 Chemical control
Chemical control of fire blight is obtained using bactericides. Bactericides, which are either antibiotics or copper compounds, reduce inoculum survival in early spring and inhibit the multiplication of *E. amylovora*. This prevents the development of new infections in blossoms or shoots. As bactericides have negligible systemic action (van der Zwet and Beer, 1991), these chemicals must be applied before infection occurs, with several regular applications during the bloom period. Thorough application on the tree surface is essential, especially in the interior of blossoms, where *E. amylovora* frequently enters. Bactericides should be applied immediately after, if possible, or within 24 hours following severe rain, wind or hail storms, since wind and rain favour the development of blossom blight (van der Zwet and Beer, 1991).
Antibiotics used for fire blight control include streptomycin (a.k.a. plantomycin, sold as Agri-Mycin-17° and Agri-strep®), tetracycline, and oxytetracycline (a.k.a. terramycin, sold as Mycoshield®). Streptomycin is the only antibiotic licensed for fire blight control in Canada. Unfortunately, bacterial resistance to some of these antibiotics has developed in some locations, particularly to streptomycin (OMAFRA, 2000). Many countries, especially in Europe, forbid the use of medically important antibiotics in agriculture in order to reduce the development of antibiotic resistance in human pathogens (Epton et al, 1994). Generally, copper compounds are less effective in controlling fire blight and are more phytotoxic than antibiotics. Many copper compounds cause leaf chlorosis or necrosis and fruit russetting (fruit epidermal necrosis). This damage on the fruit finish prevents copper compounds from being used in high quality fruit (Paulin, 1996).

In Ontario, copper-spray oil formulations are advised at the pre-bloom stage; streptomycin use is confined to bloom periods and after traumatic events (i.e. hail and wind storms (OMAFRA, 2000). Limiting streptomycin applications to three or four per season will decrease the selection pressure and slow down resistance development to streptomycin. Pome fruit growers control the number of antibiotic applications by using predictive models (section 1.4.2) in the spring.

1.4.4 Biological Control
Biological control of fire blight has focussed the use of antagonistic bacteria to suppress E. amylovora populations. BlightBan A506° (Plant Health Technologies, Boise, Idaho) is the only commercial preparation available for biological control of
fire blight in the United States. This product is currently not licensed for use in Canada. The active ingredient is *Pseudomonas fluorescens* A506, a bacterium which competes with *E. amylovora* for nutrients on blossoms (Wilson and Lindow, 1993). This competition reduces the *E. amylovora* population to a level that hinders infection. BlightBan A506® must be applied early so *P. fluorescens* A506 can become established before *E. amylovora* colonization. When sprayed, BlightBan A506® establishes well on apple and pear blossoms; however, sometimes the bacteria die rapidly and do not persist long enough to suppress fire blight. *P. fluorescens* low osmotolerance of the blossom nectar's high sugar content (Pusey, 1999) may explain this observation. BlightBan A506® can be used safely with antibiotics (Stelljes and Senft, 1998).

*Erwinia herbicola* (Löhnis) Dye, synonym *Pantoea agglomerans* (Ewing & Fife) Gavini *et al.*, is another bacterium that is a potential biological control against fire blight. While a number of *E. herbicola* strains have been studied (Epton *et al.*, 1994; Johnson and Stockwell, 1998), the most efficacious produce antibiotic compounds. Promising strains include Eh252, which constitutively produces an antibiotic compound (Wodzinski *et al.*, 1987), and Eh318, which produces two antibiotics, pantocin A and pantocin B (Wright *et al.*, 2001). Another strain, C9-1, produces two antibiotics, herbicolin O and I (Epton *et al.*, 1994). Competition for colonization sites and growth-limiting nutrients may contribute to *E. herbicola*’s inhibition of *E. amylovora*. The population dynamics of strain C9-1 are not adversely affected by streptomycin or oxytetracycline application (Stockwell *et al.*, 1996; Johnson *et al.*, 1998).
2000); no published studies indicate copper tolerance. Pusey (1999) demonstrated that *E. herbicola* C9-1 is more osmotolerant and effective against *E. amylovora* in high sugar conditions than *P. fluorescens* A506, an advantage for blossom colonization. A preparation containing C9-1 is being registered in the United States by Plant Health Technologies (Boise, Idaho) under the name BlightBan C9-1® (Stelljes and Senft, 1998).

1.4.5 Control through systemic acquired resistance and growth regulation
A number of novel fire blight control methods have been recently developed. Some stimulate the host plant's defence mechanisms against *E. amylovora*, resulting in long-term protection of all subsequently developed tissue. Such stimulation is termed systemic acquired resistance (SAR) (see section 1.5.3 for a detailed discussion). The advantage of such control methods is that SAR, unlike antibiotics, does not cause intense selection pressures that can induce pathogen resistance to the control measure (Grisham, 2000).

A compound that activates SAR is Messenger® (Eden BioScience, Bothell, Washington), the harpin protein of *E. amylovora* (section 1.3.2). While the complete harpin-stimulated defence mechanism has not been characterized, it is known that Messenger® activates physiological and molecular signalling and induces the activity of pathogenicity-related genes and proteins (Grisham, 2000). Messenger® is approved for use in the United States and Puerto Rico, with the exception of California, Colorado, and New York; it is currently unavailable in Canada.
Another SAR-inducer that demonstrates fire blight control is the synthetic compound acibenzolar-S-methyl (ASM, marketed as Actigard® or Bion® by Novartis Crop Protection, Basel, Switzerland) (Brisset et al, 2000). ASM induces two families of plant-defence enzymes, peroxidases and β-1,3-glucanases (section 1.5.2) in apples (Brisset et al, 2000). ASM is currently in the initial testing stages as a fire blight control.

Prohexadione calcium (Apogee®, BASF, Mount Olive, New Jersey) is another novel fire blight treatment. Apogee®, which is used on apples, inhibits gibberellin biosynthesis, resulting in a reduction of cell elongation and less vegetative shoot growth (BASF U.S.A., 2000). By controlling new shoot growth, and consequently reducing the amount of tissue most susceptible to infection, Apogee® assists in fire blight control by preventing secondary infections. Currently, Apogee® is licensed for agricultural use only in the United States.

1.5 Plant defence mechanisms in plant-pathogen interactions
Plants, including trees, are continuously exposed to a multitude of pathogenic microorganisms, yet the majority of plants are healthy. This is because plants possess highly effective mechanisms for preventing, or at least limiting, disease. In a plant-pathogen interaction, a plant may recognize a specific pathogen and activate defence responses (Lucas, 1998). Alternatively, a plant unable to recognize a pathogen could become susceptible to disease. Thus, plants possess a sophisticated sensory system that can detect signals from pathogens and translate these signals into appropriate defence responses (Ebel and Mithöfer, 1998).
1.5.1 Compatibility in plant-pathogen interactions: the elicitor-receptor model

Whether disease results from a plant-pathogen interaction depends on the combined compatibility of the plant and pathogen to produce disease symptoms. This is dependant on both host susceptibility and pathogen virulence, and is explained by the gene-for-gene hypothesis (Flor, 1942). In this model, if a plant carries a resistance (R) gene corresponding to an avirulence (Avr) gene in the pathogen, the plant will be resistant to the pathogen. This is an incompatible interaction, as the host and pathogen genotypes are incompatible for disease development (Lucas, 1998). If the plant’s R gene does not correspond to the Avr gene of the pathogen, the plant is susceptible to the pathogen and disease occurs (Flor, 1942). This is a compatible interaction, since the host and pathogen genotypes are compatible for disease development (Lucas, 1998).

Keen (1990) proposed that under the gene-for-gene hypothesis, the R gene product is an intra- or extra-cellular receptor, while the Avr gene product encodes a secreted or surface-located signal molecule that binds to the receptor. In an incompatible interaction, the signal molecule elicits a defence response, triggering the complex cascade of responses involved in active plant defence (Hutcheson, 1998). The Avr gene product that elicits the defence response is an elicitor. This term denotes molecules that induce any plant defence response (Lucas, 1998).

1.5.2 Elicitors and the host response

Evaluating the role of elicitors in host-pathogen interactions has been difficult. Many early experiments used crude microbial extracts containing complex chemical
mixtures in which biologically active fractions were not clearly defined. Purification attempts have since shown that microbial-derived elicitors are chemically diverse, and include polysaccharides, glycoproteins, and proteins (Lucas, 1998). There is also functional diversity within these chemical classes, as different elicitors stimulate different defence mechanisms (Ebel and Mithöfer, 1998).

Elicitor host specificity varies widely. In the gene-for-gene system, an elicitor must be a specific product, derived from a pathogen’s Avr gene, which is recognized by the product of the appropriate host resistance gene to activate defence (Flor, 1942; Keen, 1990). However, elicitors may be involved in cultivar-specific, species-specific, or non-host resistance responses of a given plant pathogen system (Ebel and Mithöfer, 1998). Thus, some elicitor molecules may be important as a part of a non-specific surveillance system enabling plant cells to recognize foreign organisms, and most appear unlikely to be Avr gene products (Lucas, 1998). In light of this, it is not surprising that inorganic elicitors, such as acibenzolar-S-methyl (ASM, section 1.4.5) have been identified (Oostendorp et al, 2001).

Incompatible plant-pathogen interactions, associated with disease resistance, are characterized by active defence mechanisms that constitute the host response (Keen, 1992). The host response is stimulated by the pathogen-derived elicitor (Lucas, 1998) and may be localized to the infection site. Active defence mechanisms may also be stimulated systemically (Hutcheson, 1998). Systemically acquired responses lead to systemically acquired resistance (SAR), discussed in section 1.5.3.
The localized host response is restricted to cells in contact with or in close proximity to the pathogen, and involves the recognition of elicitor molecules whose presentation and display by the pathogen are critical to active defence. The frequent outcome of a localized host response is a rapid, localized cellular necrosis at the infection site called the hypersensitive response (HR) (Kombrink and Schmelzer, 2001). The HR occurs in incompatible plant-pathogen interactions (Lucas, 1998), and is often associated with SAR in distal plant tissues (Ryals et al, 1996). *hrp* (*hypersensitive reaction and pathogenicity*) genes, including the *hrp* gene cluster of *E. amylovora* (Beer et al, 1993) which secretes harpin (sections 1.3.2 and 1.4.5), modulate the ability of phytopathogenic bacteria to cause disease and elicit the HR in resistant plants (Lindgren, 1997).

The HR initiates with membrane aberrations and continues with an oxidative burst, changes in cell morphology, metabolism, and gene expression (Hutcheson, 1998). Enhanced expression of phenylpropanoid metabolism genes leads to ligninification and phytoalexin synthesis (Lucas, 1998). Ligninification serves to physically isolate the pathogen, while the antimicrobial activity of phytoalexins restricts pathogen growth and replication. Pathogenesis-related (PR) proteins are often observed in tissues involved in the HR (Hutcheson, 1998). PR proteins are host-encoded proteins that accumulate predominantly in the intercellular spaces of plant tissues in response to infection (Lucas, 1998). Some are involved in phenylpropanoid metabolism; others, such as β-1,3-glucanase and chitinase, delay fungal growth and release molecules which may act as endogenous elicitors (Lucas, 1998).
1.5.3 Systemic acquired resistance

The concept that plants may develop a form of systemic resistance to disease was first comprehensively reviewed by Chester (Chester, 1933a; Chester, 1933b). Early researchers likened systemic resistance following pathogen exposure to the mammalian immune system and vaccination (Lucas, 1998). This phenomenon is now termed systemic acquired resistance (SAR), alternately known as induced systemic resistance (ISR) (Conrath et al, 2001).

In SAR, disease resistance is expressed both locally at the site of induction and systemically in tissues remote from the initial induction site (figure 4). This resistance extends to the roots as well (Sticher et al, 1997). The inducer or elicitor, which often stimulates the HR, may be a pathogen, pathogen-derived compound, or chemical agent (Kessmann et al, 1994). Like elicitors of HR, SAR inducers are chemically diverse. It is clear that SAR inducing agents are active because of what they do, rather than what they are (Kúc, 2001). While the majority of detailed genetic and molecular analyses have been confined to model systems such as tobacco, cucumber, and Arabidopsis thaliana, over 20 plant species from six different plant families have demonstrated SAR. A recent study in conifers (Christiansen et al, 1999) and work with elm tree species (Hubbes, 2000a) indicate that at least two forest tree species exhibit SAR.
Figure 4. Schematic representation of systemic acquired resistance (SAR).

1. The SAR inducer or elicitor is applied to plant tissue.

2. Localized induced resistance develops, usually accompanied by lesions characteristic of the hypersensitive response (HR).

3. Signal transduction sensitizes distal tissues to pathogen infection.

4. SAR stimulates plant defense mechanisms when sensitized tissues are subject to pathogen challenge.
When SAR is activated, a normally compatible plant-pathogen interaction can become an incompatible one (Ryals et al, 1996). The tissues of plants displaying SAR react more rapidly and efficiently to a challenge infection than plants without systemic resistance, a phenomenon termed conditioning or sensitizing (Conrath et al, 2001; Sticher et al, 1997). The biochemical changes in a sensitized plant usually become apparent only at a challenge infection. There is often a delay of several days between induction and full SAR expression. Disease resistance is expressed as a reduction in lesion number and/or size, in addition to pathogen multiplication and/or growth. Protection is long-lasting, often for weeks or even months (Lucas, 1999). Unlike mammalian immunity, the protection conferred by SAR is non-specific i.e. it is effective against pathogens unrelated to the inducing agent. SAR protection is not transmitted to seed progeny.

While the underlying mechanism of SAR is not understood, some details have emerged. It appears changes in cellular activity and various plant defences are stimulated by SAR. Associated with SAR is the expression of genes termed SAR genes (Ward et al, 1991), whose induction correlates with the onset of SAR and defence responses in uninfected tissue. SAR genes encode SAR proteins, whose activity also correlates with disease resistance (Ryals et al, 1996). Many SAR proteins are PR proteins (section 1.5.1) e.g. β-1,3-glucanase and chitinase. SAR gene expression also stimulates lignification and the formation of structural barriers (Sticher et al, 1937), both of which are active defence mechanisms.
Much research has focussed on how SAR activates resistance in tissues remote from the induction site. Since most elicitors have restricted mobility and are not involved in long distance signalling (Ebel and Mithöfer, 1998), it appears SAR is activated throughout the plant by signal transduction. Signal transduction describes the process by which a receptor interacts with a binding molecule (e.g. elicitor), transmitting a signal that triggers a pathway within a cell (Lewin, 1994). It is postulated translocation of a systemic signal, produced at the SAR induction site, stimulates SAR in distant tissues (Sticher et al, 1997). This signal sensitizes the plant against further pathogen attacks, probably stimulating a complex array of defence responses through signal transduction. This would include SAR gene activation, SAR protein expression and structural barrier formation. Thus, the SAR signalling pathway appears to function as a potentiator or modulator of other disease resistance mechanisms (Ryals et al, 1996).

A substantial body of evidence suggests that salicylic acid (SA) plays a pivotal role in SAR signalling and disease resistance. This includes observations that endogenous SA concentrations increase locally and systemically in tobacco plants inoculated with tobacco mosaic virus (TMV) and exhibiting SAR (Malamy et al, 1990). The same PR genes are also activated after both the elevation of endogenous SA (Malamy et al, 1990) and exogenous SA application (Ward et al, 1991). SAR was displayed in the latter study. The importance of SA in SAR was reinforced using transgenic tobacco and Arabidopsis plants that overexpress a bacterial salicylate hydroxylase (the NahG gene), an enzyme that converts SA to the
SAR-inactive catechol. In these plants, SA levels are low and SAR is absent, indicating that SA is crucial for SAR induction (Bi et al, 1995; Delaney et al, 1994; Friedrich et al, 1995; Gaffney et al, 1993; Lawton et al, 1995). Additionally, SA-insensitive Arabidopsis mutants do not display SAR while wild type Arabidopsis plants do (Summermatter et al, 1996), establishing that the ability to perceive SA is essential for SAR. Despite SA’s importance in SAR, whether it is the translocated systemic signal is unproven (Métraux, 2001).

1.6 Ophiostoma ulmi elicitor
A crude glycoprotein elicitor preparation, isolated from Ophiostoma ulmi (Buisman) Nannfeldt, the Dutch Elm Disease (DED) fungus, has been patented as a preventative treatment for wilt diseases (Hubbes, 2000b). This elicitor preparation, hereafter termed O. ulmi elicitor, has demonstrated efficacy as a preventative treatment for DED in uninfected elm trees (Hubbes, 2000a). SAR has been observed in the former; elm tree tissues remote from the elicitor treated site have resisted aggressive O. ulmi isolates administered as a challenge inoculation (Hubbes, 2000a).

The O. ulmi elicitor is obtained from the culture filtrate of two non-aggressive O. ulmi isolates, either Q412 (Yang et al, 1989) or WRLS (Hubbes, 2000b). The only known difference between the strains is that Q412 produces spores, while WRLS does not sporulate (M. Hubbes, personal communication). Non-aggressive O. ulmi strains are preferred, as elicitors obtained from these strains cause elm tissues to accumulate phytoalexins termed mansonones faster and in larger quantities than elicitors
isolated from aggressive O. ulmi strains (Yang et al, 1989; Yang, 1991). An aspartic protease obtained from the O. ulmi elicitor preparation (Yang, 1991; Yang et al, 1994) has been sequenced (Hubbes, 2000b); however, the full compliment of SAR-inducing molecules within the elicitor preparation has not been determined.

Since the O. ulmi elicitor induced SAR against a tree vascular wilt disease, specifically against DED in elms, it was hypothesized that the elicitor may also induce SAR against other vascular wilt pathogens in woody perennial plants. An initial experiment in apple seedlings, using the elicitor as preventative treatment against fire blight, was promising (Svircv, 1999). However, additional research was required to confirm the elicitor’s efficacy as preventative treatment for fire blight.

1.7 Verticillium dahliae elicitor
A crude elicitor preparation has also been isolated from the culture filtrate of Verticillium dahliae Klebahn. V. dahliae is destructive vascular wilt fungus that infects many plant species, including agricultural crops (cotton, pepper, potato and tomato), forest trees, and woody and herbaceous ornamentals (Bhat and Subbarao, 1999). Like O. ulmi, V. dahliae a vascular wilt fungus that is non-pathogenic on apples or pears (Chatfield et al, 2000). Given this similarity to O. ulmi, the V. dahliae elicitor was used as a control treatment in field experiments to determine if the O. ulmi elicitor was the only vascular wilt fungus elicitor to induce resistance to E. amylovora.
Culture filtrate elicitors obtained from *V. dahliae* have stimulated plant defense responses in cotton tissue culture. One crude *V. dahliae* culture filtrate elicitor stimulated cell wall lignification in cotton hypocotyls (Smit and Dubery, 1997). Another crude *V. dahliae* culture filtrate elicitor stimulated both phytoalexin formation and the oxidative burst in cotton cell suspension cultures (Davis *et al*, 1998), while a glycoprotein purified from the same crude elicitor only elicited phytoalexin synthesis (Davis *et al*, 1998).

### 1.8 Objectives

The objectives of this research thesis were as follows:

1. To evaluate the efficacy of the *O. ulmi* elicitor against fire blight in susceptible apple and pear cultivars;
2. To determine if secreted protease production by *E. amylovora* correlates with host species-specificity; and
3. To determine whether the 16S/23S intergenic spacer region (ITS) DNA sequences of *E. amylovora* isolates correlates with host species-specificity.
2 Material and Methods
The list of bacterial and fungal isolates used in all experimentation is contained in table 1.

2.1 E. amylovora isolates, media and culture conditions
Erwina amylovora isolates were maintained for long-term storage at -20°C in a storage media composed of Luria Broth (LB):glycerol (85:15 v/v). LB contained 10 g/L tryptone, 5 g/L yeast extract and 5 g/L sodium chloride, pH 7.5. All E. amylovora isolates were initially cultured in 15 cm sterilized plastic Petri plates containing LB agar (10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride and 15 g/L bacteriological agar, pH 7.5) and incubated at 28°C for 20 to 24 hours prior to use.

2.2 O. ulmi elicitor preparation
O. ulmi elicitor was prepared as described in Yang et al (1989), with adaptations by Hubbes (2000a). O. ulmi cultures were initiated from individual mycelial plugs previously maintained in 10% glycerol at -70°C. Both Q412 and WRLS elicitors were isolated from 10 day old O. ulmi cultures of the appropriate non-aggressive strain grown in Takai-Richard (TR) media (1 g/L KH2PO4, 0.48 mg/L FeCl3 6H2O, 0.36 mg/L MnCl2 4H2O, 0.1 g/L MgSO4 7H2O, 0.44 mg/L ZnSO4 7H2O, 2 g/L L-asperagine, 2 g/L yeast extract, 10 g/L sucrose) at room temperature. Culture media were prepared in 12 L quantities in 15 L Nalgene® culture vessels (Fisher Scientific, Nepean, ON) and autoclaved at 121°C for 30 minutes. The culture vessels were inoculated with 25 mL of the appropriate fungal culture grown in TR media, and incubated at room temperature with agitation on a Barnstead Thermolyne orbital shaker (Type M71735, Fisher Scientific, Nepean, ON) at 125 rpm for 10 days. To obtain WRLS culture filtrate, culture solids were removed.
Table 1. Microbial isolates, hosts, sources, and their role in experimentation.

<table>
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<tr>
<th>Species</th>
<th>Use</th>
<th>Host</th>
<th>Donator</th>
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<td>ITS PCR</td>
<td>Raspberry</td>
<td>A.M. Svírcev¹</td>
</tr>
<tr>
<td>4-96</td>
<td>Extracellular protease extraction and bioassay</td>
<td>Raspberry</td>
<td>A.M. Svírcev¹</td>
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<tr>
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<td>Elicitor efficacy testing, extracellular protease extraction and bioassay</td>
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<td>A.M. Svírcev¹</td>
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<td>ITS PCR</td>
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<td>A.M. Svírcev¹</td>
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<tr>
<td><em>Ophiostoma ulmi</em></td>
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<tr>
<td>Q412</td>
<td>Elicitor preparation and efficacy testing</td>
<td>Elm</td>
<td>M. Dumas²</td>
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<tr>
<td>WRLS</td>
<td>Elicitor preparation and efficacy testing</td>
<td>Elm</td>
<td>M. Dumas²</td>
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<td><em>Verticillium dahliae</em></td>
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<td>122 (race 1)</td>
<td>Elicitor preparation and efficacy testing</td>
<td>Tomato</td>
<td>V.J. Higgins³</td>
</tr>
</tbody>
</table>

¹: Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, Vineland Station, Ontario, Canada.

²: Natural Resources Canada, Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada.

³: Department of Botany, University of Toronto, Toronto, Ontario, Canada.
by centrifugation. Q412 culture filtrate was initially prepared by centrifugation to remove spores and mycelia, and then subsequently purified via a wine press filter system to remove remaining spores.

Once cell-free *O. ulmi* culture filtrate was obtained, polysaccharides were precipitated with an equal volume of 95% ethanol at 4°C. The precipitated culture filtrate was filtered through Whatman® #42 filter paper (Fisher Scientific Limited, Nepean, ON) via vacuum filtration, and the ethanol distilled on a Brinkmann Buchi® Rotovapor-R (Fisher Scientific Limited, Nepean, ON) under reduced pressure. The water fraction was filtered through an Amicon® Bioseparations YM10 regenerated cellulose ultrafiltration membrane (Millipore Corp., Bedford, MA) to concentrate and retain molecules ≥ 10 kDa. The concentrated retentate, which constituted the elicitor fraction of the culture filtrate, was then lyophilized.

2.3 *V. dahliae* elicitor preparation
*V. dahliae* culture filtrate elicitor was prepared using the method of Smit and Dubery (1997) with modifications. One mycelial plug of *V. dahliae*, grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) for 7 days, was cultured in 2 L of potato dextrose broth medium (Difco Laboratories, Detroit, MI) for 7 days. The liquid culture was incubated at room temperature with agitation on a Barnstead Thermolyne orbital shaker (Type M71735, Fisher Scientific Limited, Nepean, ON) at 125 rpm. Culture filtrate was obtained by centrifugation to separate mycelia and spores from the supernatent. Once cell-free culture filtrate was obtained, it was filtered through an Amicon® Bioseparations YM10 regenerated cellulose
ultrafiltration membrane (Millipore Corp., Bedford, MA) to concentrate and retain molecules $\geq 10$ kDa. The concentrated retentate, which constituted the elicitor fraction of the culture filtrate, was then lyophilized.

2.4 Elicitor efficacy testing
In both seedling and tree experiments, elicitor suspensions were prepared immediately before use. The elicitor suspension consisted of the appropriate elicitor dissolved in sterilized distilled water.

2.4.1 Seedling experiments
_Malus x domestica_ cv. Golden delicious, a fire blight susceptible apple cultivar, was used for all seedling experiments. Apple seed dormancy was broken either through stratification (cold-chilling) in peat at 4°C for three months, or by using the cold water treatment method described by Bogs et al (1998). In the later method, apple seeds were stratified for 5 days in ice water, germinated for 2 to 4 weeks at 10°C, and then transferred into soil. The plantlets were illuminated in a growth chamber for 16 h at 22°C and allowed to grow further for at least 3 weeks. Regardless of stratification method, germinated seeds were planted in a soil-peat moss-sand-fertilizer mixture containing 40 cu. ft. five year old compost soil, 20 cu. ft. peat moss, 5 cu. ft. sand, 4 lbs. magnesium sulphate, 1.5 lbs. calcium nitrate, 12 lbs. 10-10-10 fertilizer, and 1 lb. green house grade 0-20-0 (super phosphate) fertilizer. Once planted in sterilized pots, the seedlings were grown in greenhouse conditions at approximately 27°C, with natural light supplemented by 400 W high pressure sodium lighting.
To evaluate the *O. ulmi* elicitor’s efficacy against fire blight, a completely randomized design was employed to assign elicitor and control treatments. A sample size of 10 seedlings/treatment was used. Twenty µL of elicitor suspension (10 mg/mL) was injected into the seedling, in the junction between the petiole and the stem, at the seventh leaf from the apical meristem using a repeating dispenser (Cat. # PB600-1, Hamilton Company, Reno, NV). Sterilized distilled water was used for control treatments. Seedlings were maintained in a greenhouse at 27°C for 1 week before challenge.

In challenge inoculations, overnight cultures of *E. amylovora* isolate Ea 6-4 were suspended in sterile 0.01 M sodium phosphate buffer (PB) pH 6.8. The turbidity of the suspension was adjusted to $A_{600} = 0.1$ (equivalent to $10^8$ CFU/mL) with PB. This inoculum dose is routinely used in evaluating the efficacy of compounds to control fire blight (Tharaud *et al.*, 1997). Twenty µL of the bacterial suspension was injected, using the aforementioned repeating dispenser, at the junction between the petiole and the stem of the first open leaf from the apical meristem. Later seedling experiments (00Ea2 through 00Ea4) used $10^6$ CFU/mL; the $10^8$ CFU/mL suspension was diluted 100 times with the appropriate amount of PB. The lower inoculum concentration was employed in light of concerns a $10^8$ CFU/mL was too high (section 2.4.2). Experiments using $10^8$ CFU/mL inoculum were replicated four times with Q412 elicitor; experiments using $10^6$ CFU/mL inoculum were replicated three times with both Q412 and WRLS elicitor.
The challenged seedlings were maintained in a 27°C greenhouse, and fire blight symptoms were evaluated at 7, 14, 21, and 28 days post-inoculation, using the disease index in figure 5. Observations recorded 28 days post-inoculation were used to determine the presence of statistically significant treatment differences in SAS® statistical software (version 7, Microsoft Windows 95® platform). Data was analyzed using Duncan's Multiple Range Test at \( P < 0.05 \).

2.4.2 Inoculum dose experiment
In response to concerns that the \( 10^8 \) CFU/mL Ea 6-4 inoculum dose was too high, an inoculum dose experiment was performed in 'Golden delicious' apple seedlings. The objective was to find, if possible, a lower inoculum concentration that produced fire blight symptoms in all injected seedlings. Ten-fold serial dilutions of the \( 10^8 \) CFU/mL dose were made, producing inoculum concentrations ranging from \( 10^3 \) CFU/mL to \( 10^8 \) CFU/mL. This resulted in 20 CFU/plant to \( 2 \times 10^6 \) CFU/plant when the inoculum was administered via the 20 \( \mu \)L repeating dispenser. A completely randomized design was used to assign the different inoculum concentrations, with a sample size of 10 seedlings/concentration. The inoculum was administered as in the seedling elicitor efficacy experiments (section 2.4.1). The seedlings were maintained in a greenhouse at 27°C for 4 weeks. Symptoms were evaluated using the disease severity index in figure 5. Percent disease was expressed as the percent of seedlings in each treatment expressing disease symptoms.
Figure 5. Disease severity index used for evaluating fire blight symptoms in *E. amylovora* inoculated apple seedlings. Ratings: 0=no disease; 1=wilting of shoot tip and leaves; 2=wilting and blackening of apical tip and young leaves; 3=blackening from shoot tip into main veins, leaves and stem of upper leaves; 4=blackening from shoot tip extending into leaves and down seedling stem; and 5=complete blackening of upper leaves and upper stem.

2.4.3 Field experiments
Four year old potted *Malus x domestica* cv. Ida red, grafted onto M9 rootstocks, and *Pyrus communis* cv. Bartlett were used for tree efficacy experiments. The experiments were conducted in a closed structure (or screen house) between May and September 2000 at Agriculture and Agri-Food Canada, Vineland Station, Ontario.

Both Q412 and WRLS *O. ulmi* elicitors were evaluated for efficacy against fire blight. An additional culture filtrate elicitor, obtained from *V. dahliae* 122 (race 1), was also employed as a control treatment. The *V. dahliae* elicitor was used to assess whether the two *O. ulmi* elicitors were the only non-host fungal wilt disease elicitors which possessed efficacy against fire blight. Negative (i.e. unchallenged) *O. ulmi* and *V. dahliae* elicitor controls were used to evaluate phytotoxicity. Positive water controls were used to assess the elicitors’ efficacy and to ensure the viability of the *E. amylovora* inoculum.

A completely randomized block design was used, with each treatment and control assigned 10 trees (1 tree/treatment/block, 10 blocks total). Five actively growing shoots per tree were treated, just below the apical meristem, with 20 µL elicitor injections administered via repeating dispenser. The treated shoots were labelled with flagging tape for evaluation purposes. The trees were challenged two weeks later with $10^8$ CFU/mL *E. amylovora* 6-4 ($A_{600} = 0.1$) suspended in 0.01 M PB pH 6.8, via apical leaf incision. The inoculum was administered via scissors dipped in the bacterial suspension.
For experiment 00Ea128 (1 mg/tree), each elicitor suspension was prepared at a concentration of 10 mg/mL. This elicitor dose was based on a previous field study in 4 year old apple trees using O. ulmi elicitors (Svircev, 1999). Each of the five shoots was injected once at the apical meristem, resulting in an elicitor dose of 1 mg/tree. For experiments 00Ea130 and 00Ea131, both O. ulmi elicitor suspensions were prepared at a concentration of 80 mg/mL. Each shoot was injected five times (once at the apex, and then at the petiole base of the four subsequent leaves), resulting in 40 mg O. ulmi elicitor/tree. Due to elicitor scarcity arising from contamination of V. dahliae liquid cultures, only 20 mg of V. dahliae elicitor was applied to each tree. The V. dahliae elicitor was applied in the same manner as the O. ulmi elicitors, except at a concentration of 40 mg/mL. The water controls for all three experiments were administered in a manner identical to the treatments.

The water controls of experiment 00Ea128 were not challenged until two weeks after the remaining treatments, due to human error. The 10 water control trees were challenged at this time, along with three additional trees, assigned to individual blocks (11, 12 and 13), to ensure adequate data for comparison with the elicitor treatments.

In all experiments, the fire blight lesion length and total shoot length were measured for each treated shoot. The percent of diseased tissue for each shoot was calculated; expressing disease as a percent value accommodated for the different growth rates among shoots. The percent diseased tissue was averaged among all
five shoots for each tree. The resulting average (expressed as percent disease) was used to determine the presence of statistically significant treatment differences in SAS® statistical software (version 7, Microsoft Windows 95® platform). Data was analyzed using Duncan’s Multiple Range Test at $P < 0.05$.

2.5 *E. amylovora* host species-specificity: *E. amylovora* intraspecies characterization by secreted protease bioassay

The following protocol was modified from previously published research on secreted proteases obtained from *E. amylovora* apple isolates (Zhang *et al*, 1999). *E. amylovora* isolates and sources are listed in table 1.

2.5.1 Crude protease fraction preparation

P2 broth, 500 mL (5 g sorbitol, 4 g casein hydrolysate, 5 mg ZnSO$_4$·7H$_2$O, 20 mg Ca(Ac)$_2$, 2.5 mg MgSO$_4$·7H$_2$O, 2 g yeast extract, and 25 mM Tris-HCl, pH 7.0) was inoculated with the appropriate overnight bacterial culture and incubated at room temperature for 48 hours. The supernatant was collected by centrifugation in a Sorvall® RC-5B centrifuge (Mandel Scientific, Guelph, ON) using a SS-34 rotor rotating at 6000 rpm for 20 minutes ($4^\circ$C). The crude extracellular protease fraction in the supernatant was isolated through ammonium sulphate precipitation. Ammonium sulphate was added to 50% using the formula of Scopes (1982). The resulting precipitate was centrifuged at in a Sorvall® RC-5B centrifuge using a SS-34 rotor rotating at 8000 rpm for 20 minutes ($4^\circ$C) and resuspended in Tris-EDTA (TE) buffer ($pH 8.0$). The supernatant was reserved, and the precipitation process repeated with ammonium sulphate at 70% and 80% saturation. After each precipitation, the centrifugation process was repeated. Once the precipitates from
the 80% saturated ammonium sulphate solution were collected, all of the precipitated fractions were combined, dialyzed for 48 hours against distilled water at 4°C, and then lyophilized. The resulting product was the crude protease fraction obtained from the *E. amylovora* culture filtrate.

2.5.2 Protein concentration assay
After lyophilization, the protease fraction’s protein concentration was determined using Bio-Rad Laboratories (Richmond, CA) protein assay dye reagent concentrate. The protein concentration was measured to determine a suitable sample concentration for subsequent protein electrophoresis. Ten milligrams of the protease fraction was dissolved in 1 mL distilled water and centrifuged to remove any insoluble particles. The resulting supernatant (10 μL) was added to a test tube containing a pre-mixed solution of Bio-Rad Laboratories protein assay dye concentrate (1 mL) and distilled water (4 mL). The reaction was vortexed and allowed to incubate at room temperature for 5 minutes. Samples were measured in a Shimadsu® UV-1601 benchtop spectrophotometer (Mandel Scientific Co. Ltd., Guelph, ON) at *A*$_{595}$ and standardized using the diluted Bio-Rad Laboratories dye concentrate.

2.5.3 Protease electrophoresis and activity
A SDS-gelatin-PAGE (sodium dodecyl sulphate–gelatin–polyacrylamide gel electrophoresis) assay, also known as a zymogram, was used to determine protease activity and molecular mass in the crude protease fraction. Ten, 15, and 17.5 μL of the previously prepared 10 mg/mL culture filtrate were mixed with an equal amount
of NOVEX™ Tris-Glycine SDS Sample Buffer (Cat. # LC2676, Invitrogen, San Diego, CA) and incubated at room temperature for 10 minutes. The entire volume of each sample (10, 15 and 20 µg of protease fraction, respectively) was applied onto a pre-cast NOVEX™ Zymogram gel (0.1% gelatin, 10% Tris-Glycine, pH 8.6; Cat. # EC6175) assembled in a NOVEX™ XCell Surelock™ Mini-Cell apparatus (Cat. # EI0001) with 1X Tris-Glycine SDS running buffer (Cat. # LC2675). The gel was run at 125 V for approximately 90 minutes.

Once electrophoresis was complete, the gel was incubated in 1X NOVEX™ Zymogram Renaturing Buffer (Cat. # LC2670) with gentle agitation for 30 minutes at room temperature. The renaturing buffer was decanted and replaced with 1X NOVEX™ Developing Buffer (Cat. # LC2671) and incubated at 37°C overnight with gentle agitation. Protease activity was visualized by incubating the gel with 0.5% Coomassie blue strain for 30 minutes, and destaining with an aqueous solution containing 7% acetic acid and 5% of 95% ethanol.

2.6 E. amylovora host species-specificity: E. amylovora intraspecies characterization by intergenic transcribed spacer (ITS) regions

The following protocol was modified from Jeng et al (2001).

2.6.1 Genomic DNA Isolation

A single colony from overnight cultures of E. amylovora were transferred to 1 mL of LB medium and incubated overnight in a shake culture at room temperature. Genomic DNA was extracted using QIAamp® Tissue Kit (Cat # 29304, QIAGEN®, Mississauga, ON, Canada) with modifications to the manufacturer’s instructions.
Bacterial culture (1 mL) was placed in a 1.5 mL microfuge tube, and centrifuged in a benchtop microcentrifuge for 5 minutes at 7500 rpm (room temperature). The bacterial pellet was resuspended in 180 μL Buffer ATL. Proteinase K stock solution (20 μL) was mixed with the resuspended pellet, and the sample was incubated at 55°C in a shaking water bath until the sample was clear and bacteria completely lysed.

Next, 200 μL of Buffer AL was added to the sample, which was then vortexed thoroughly and incubated at 70°C for 10 minutes. After incubation, 210 μL of 95% ethanol was added to the sample and then mixed. The sample was applied to a QIAamp® spin column in a 2 mL collection tube, and spun in a microfuge for 1 minute. The QIAamp® column was then placed in a clean collection tube. 500 μL of Buffer AW was added, and the sample was centrifuged in a microfuge for 1 minute. The QIAamp® column was transferred to a clean collection tube, and another 500 μL of Buffer AW was added. The sample was centrifuged for 3 minutes, and the resulting filtrate was discarded. The QIAamp® spin column was placed in a clean 2 mL microfuge tube. DNA in the column was incubated for 5 minutes with 200 μL of buffer AE preheated to 70°C, and then microfuged for 1 minute at 6000 x g (8000 rpm). This process was repeated, and the two eluted DNA samples combined before storage at -20°C.
2.6.2 PCR Amplification
PCR amplification was performed using the method of Jeng et al (2001) with adaptations. The primer pair used for the intergenic spacer (ITS) was ITS16 (5'-TTGTACACACCGCCCGTC) and ITS23 (5'GGTACCTTAGATGTTTCAG) (Jeng et al, 2001; McManus and Jones, 1995). Prior to amplification, undigested genomic DNA was run on a 0.8% agarose gel (BioShop Canada, Burlington, ON) to test for degradation. Each PCR reaction contained 2.5 μL of each primer, 2.5 μL of 1X PCR buffer with MgCl₂ (Cat. # 27-2094-01, Amersham Pharmacia Biotech, Piscataway, NJ), 1 unit of Taq DNA polymerase (Cat. # 27-0799-01, Amersham Pharmacia Biotech), 2.5 μL of 100 mM dNTP (Cat. # 27-2035-01, Amersham Pharmacia Biotech) and 50–100 ng of genomic DNA in a final volume of 25 μL. Amplification was performed under mineral oil in a thermal cycler (Hybaid, Ashford, UK) using the following PCR program: (1) denaturation at 95°C for 2 min; (2) a cycle of 45°C, 50 s; 72°C, 1 min; 95°C, 1 min; 50°C, 50 s; and 72°C, 1 min, repeated 35 times; and (3) extension at 72°C for 10 minutes. For molecular size analysis of the 16S/23S spacer regions, PCR products were directly separated on a 1.8% agarose gel (BioShop Canada, Burlington, ON) with a 100 base-pair DNA molecular weight marker (Cat. # 27-4001-01, Amersham Pharmacia Biotech).

2.6.3 Cloning of PCR products
PCR products were cloned into a pCR®2.1-TOPO® plasmid vector using a TOPO TA Cloning® kit (Cat. # K4500-40, Invitrogen) containing TOP10 One Shot® chemically competent cells, following the manufacturers instructions. Fresh PCR product (3 μL), salt solution, 1 μL (1.2 M NaCl and 0.06 M MgCl₂) and plasmid vector (1 μL)
were combined, mixed gently, and incubated for 5 minutes at room temperature. After incubation, the cloning reaction was placed on ice. Two μL of the cloning reaction was added into a vial of TOP10 One Shot® chemically competent cells. After gentle mixing, the transformation reaction was incubated on ice for 10 minutes.

Following incubation, the competent cells were heat-shocked for 30 seconds in a 42°C water bath without shaking. The vial was immediately transferred to ice, and 250 μL of room temperature SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) was added aseptically. The vial was agitated horizontally (200 rpm) at 37°C for 1 hour. After incubation, 150 μL of transformed cells were plated on LB agar media pre-treated with 30 μL XGAL (Bioshop Canada, Burlington, ON) and 50 μL ampicillin (Cat. # A9393, Sigma-Aldrich Canada Ltd., Oakville, ON). Plates were incubated overnight at 37°C. After incubation, 10 white colonies were selected and individually cultured overnight in LB (3 mL) medium at 37°C.

2.6.4 Plasmid DNA Isolation
Plasmid DNA was isolated using the mini-prep plasmid DNA method of Sambrook et al (1989). To harvest bacteria, 1.5 mL of fresh overnight bacterial culture was centrifuged in a benchtop microcentrifuge for 60 seconds at 4°C. After the supernatent was removed by aspiration, the bacteria were lysed by alkali. The bacterial pellet was re-suspended in 100 μL ice-cold Solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, and 10 mM EDTA pH 8.0) by vigorous mixing. Next, 200 μL of
freshly prepared Solution II (0.2 N NaOH and 1% SDS) was incorporated by rapidly inverting the microfuge tube 15 times. The reaction was stored on ice, and 150 μL of ice-cold Solution III (60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid, and 28.5 mL distilled water) was added. The resulting mixture was vortexed gently in an inverted position for 10 seconds to disperse Solution III through the viscous bacterial lysate.

After a 10 minute incubation on ice, the bacterial lysate was centrifuged for 10 minutes in a benchtop microcentrifuge at 4°C. The supernatent was transferred to a fresh tube. Double-stranded DNA was precipitated with two volumes of 95% ethanol at room temperature. After mixing, the solution was incubated for 60 minutes at -20°C to maximize DNA precipitation. Upon incubation completion, the reaction was centrifuged for 5 minutes in a benchtop microcentrifuge at 4°C. Supernatent was removed by gentle aspiration. The DNA pellet was rinsed with 1 mL of 70% ethanol (4°C), and dried in air for 10 minutes. Once the DNA pellet was dry, it was dissolved in 100 μL Tris-EDTA buffer (pH 8.0) and stored at -20°C.

2.6.5 DNA sequencing and analysis
Prior to sequencing, insertion of PCR product into pCR<sup>®</sup>2.1-TOPO<sup>®</sup> plasmid DNA was verified by EcoRI restriction digestion (Cat. # E10404, Amersham Pharmacia Biotech, Piscataway, NJ), followed by electrophoresis on a 1.8% agarose gel (Bioshop Canada, Burlington, ON). DNA sequencing was performed at the York University Core Molecular Biology and DNA Sequencing Facility (Toronto, ON) on a Perkin-Elmer/ABI 373A DNA Sequencer (Applied Biosystems Group, Foster City,
CA), using M13 reverse (5' CAGGAAACAGCTATGAC) and T7 forward (5' CCCTATAGTGAGTCGTATTA) primers. DNA sequence searches were completed using the National Center for Biotechnology Information (Bethesda, MD) GenBank database nucleotide BLAST© utility (http://www.ncbi.nlm.nih.gov/BLAST/). DNA sequence alignment of both the two Rubus isolates, the two smallest PCR products of the Maloideae isolate, and the smallest PCR products of all three isolates was completed using PC/Gene™ release 6.60 (Intelligenetics Inc., Mountain View, CA). In PC/Gene™, two nucleotide sequence alignment was performed using the method of Myers and Miller (1988) (NALIGN version 1.10), and multiple sequence alignment was generated using CLUSTAL version 1.10.
3 Results
3.1 Efficacy of O. ulmi and V. dahliae elicitors against E. amylovora

The efficacy of O. ulmi elicitor against an E. amylovora Maloideae isolate (6-4) varied between seedling and tree experiments. In apple seedlings, four initial experiments with an Ea 6-4 inoculum concentration of $10^8$ CFU/mL ($2 \times 10^7$ CFU/plant) produced no significant differences between control and Q412 elicitor treatments at $P < 0.05$ (fig. 6). Concerns that the inoculation dose in these experiments was too high led to three new experiments using an inoculation dose of $10^6$ CFU/mL (fig. 8). The new inoculation dose was determined by an apple seedling experiment using 20 µL injections of Ea 6-4 at concentrations of $10^2$, $10^4$, $10^6$ and $10^8$ CFU/mL (fig. 7). The objective was to find the lowest possible inoculum concentration that induced disease symptoms in all challenged apple seedlings. Doses of $10^6$, $10^7$, and $10^8$ CFU/mL produced visible fire blight symptoms in all seedlings tested; the lowest dose of $10^6$ CFU/mL was selected for subsequent seedling experiments. In two of the three experiments using the lower inoculum concentration, at least one of the two O. ulmi elicitors demonstrated efficacy, relative to water controls, against fire blight (fig. 8).

In the screen house experiments, older plant material was used. Apple and pear cultivars highly susceptible to fire blight were selected. A significant statistical difference ($P < 0.05$) was not observed between Q412 elicitor (1 mg/tree) and the water control in the pears (fig. 9 and 11A). No treatment effect ($P < 0.05$) was observed with the WRLS and V. dahliae elicitors at 1 mg/tree (fig. 9). However, a subsequent experiment with pear trees using a Q412 elicitor dose of 40 mg/tree
Figure 6. Efficacy of *O. ulmi* Q412 elicitor (0.2 mg/seedling) against *E. amylovora* in Golden delicious apple seedlings. Seedlings were challenged with *E. amylovora* 6-4 at $10^8$ CFU/mL. Disease symptoms were evaluated as described in Materials and Methods. Different letters denote a statistically significant difference between treatments at $P < 0.05$ for each experiment, as determined by Duncan's Multiple Range Test. The experiment was replicated four times, with no significant differences between treatments.
Figure 7. Efficacy of *E. amylovora* 6-4 in fire blight symptom development. Inoculum was administered to Golden delicious apple seedlings via a 20 μL repeating injector, resulting in a dose of 20 to 2 x 10^6 CFU/seedling. Disease symptoms were evaluated as described in Materials and Methods. Only concentrations of 10^6 to 10^8 CFU/mL generated symptoms in all injected seedlings.
Figure 8. Efficacy of O. ulmi elicitors Q412 and WRLS (0.2 mg/seedling) against E. amylovora in Golden delicious apple seedlings. Experiments were replicated three times (A, experiment 00Ea2; B, experiment 00Ea3; and C, experiment 00Ea4). All experiments were challenged with E. amylovora 6-4 at 10^6 CFU/mL. Disease symptoms were evaluated as described in Materials and Methods. Different letters denote statistically significant difference between treatments at $P < 0.05$ for each experiment, as determined by Duncan's Multiple Range Test.
Figure 9. Efficacy of *O. ulmi* elicitors (Q412 and WRLS) and *V. dahliae* elicitor (VD), applied at 1 mg/tree against fire blight in 4 year-old Bartlett pear trees (experiment 00Ea128). Percent disease was measured as described in Materials and Methods. Different lower case letters denote statistical difference at $P < 0.05$, as determined by Duncan’s Multiple Range Test.
Figure 10. Efficacy of *O. ulmi* Q412 and WRLS elicitors (40 mg/tree) and *V. dahliae* elicitor (VD, 20 mg/tree) against fire blight in 4 year-old Ida red apple trees (experiment 00Ea130). Percent disease was measured as described in Materials and Methods. Different lower case letters denote statistical difference at $P < 0.05$, as determined by Duncan's Multiple Range Test.
Figure 11. Efficacy of O. ulmi Q412 elicitor against fire blight in 4 year-old Bartlett pear trees. Elicitor was administered at 1 mg/tree in experiment 00Ea128 (A) and 40 mg/tree in experiment 00Ea131 (B). Percent disease was measured as described in Material and Methods. Different letters denote statistical difference at $P < 0.05$ respectively, as determined by Duncan's Multiple Range Test.
Figure 12. Efficacy of *V. dahliae* elicitor against fire blight in 4 year-old pome fruit trees. Elicitor was administered at 1 mg/tree in experiment 00Ea128 (A) to Bartlett pear trees, and at 20 mg/tree in experiment 00Ea131 (B) to Ida red apple trees. Percent disease was measured as described in Material and Methods. Different letters denote statistical difference at $P < 0.05$ respectively, as determined by Duncan’s Multiple Range Test.
showed a statistically significant difference between the elicitor treatment and water control (fig. 11B). As the Q412 elicitor used in both pear experiments was from the same batch, and the pear cultivar and *E. amylovora* isolate in both experiments remained constant, the increased elicitor dose was responsible for this dramatic efficacy increase. An experiment in 'Ida Red' apples, using Q412 and WRLS elicitors at 40 mg/tree, also produced a statistically significant difference between the two *O. ulmi* elicitors and control treatments (fig. 10). There was no statistically significant difference between the two elicitors.

A similar dose effect was observed with the *V. dahliae* elicitor. There was no treatment effect (*P* < 0.05) when the *V. dahliae* elicitor was applied at 1 mg/tree to pears (fig. 9 and 12A). However, there was a significant treatment effect, relative to the water control, when the *V. dahliae* elicitor was applied at 20 mg/tree to apples (fig. 10 and 12B). The *V. dahliae* elicitor used in both experiments was from the same batch. This indicates a dose affect, as the apple and pear cultivars used in both experiments have equal fire blight susceptibility. In both instances, there was no statistically significant difference (*P* < 0.05) between the *V. dahliae* and *O. ulmi* elicitors. It should be noted that in the first pear tree experiment, all three elicitors were applied at 1 mg/tree, while in the apple tree experiment, the *V. dahliae* elicitor was applied at half the rate (20 mg/tree) as the *O. ulmi* elicitors (40 mg/tree). This indicates that the *V. dahliae* elicitor is as effective as the *O. ulmi* elicitors at half the *O. ulmi* elicitors' dose.
3.2 Correlation between *E. amylovora* host species-specificity and extracellular protease activity

Proteolytic activity, indicated by the hydrolysis of gelatin substrate, was demonstrated in the SDS-gelatin-PAGE assay of both Maloideae (Ea 6-4) and *Rubus* (Ea 4-96) *E. amylovora* isolates (fig. 13). Both *E. amylovora* isolates possessed significant protease activity in the culture supernatant and are similar in size (approximately 50 kDa). The size of both proteases is similar to that of PrtA, a 48 kDa protease secreted by *E. amylovora* Maloideae isolates (Zhang et al, 1999). Since PrtA is conserved in *E. amylovora* Maloideae isolates (Zhang et al, 1999), the Ea 6-4 secreted protease is likely homologous to PrtA. The *Rubus* strain (Ea 4-96) also secretes a protease of similar molecular mass and activity (fig. 13). This suggests the *Rubus* isolate’s protease may also be homologous to PrtA, indicating that PrtA is neither unique to Maloideae *E. amylovora* isolates nor confers host specificity.

3.3 Correlation between PCR products of the ITS region and *E. amylovora* host species-specificity

The number of ITS PCR products obtained from *E. amylovora* isolates differed with respect to plant host species. *E. amylovora* isolate D-7, pathogenic on Maloideae, had three PCR products (739, 870, and 1088 bp) (fig. 14). *E. amylovora* isolates obtained from raspberries, a *Rubus* host (1-97 and 7-96b) generated two PCR products (fig. 14). The observation that the ITS PCR products can differentiate between *E. amylovora* isolates with respect to host has been previously demonstrated by Jeng *et al* (2001) and McManus and Jones (1995).
Figure 13. SDS-gelatin-PAGE assay of secreted proteases produced by Maloideae (Ea 6-4) and Rubus (Ea 4-96) E. amylovora isolates. The proteases (approximately 50 kDa in size) are visible as clear, discrete bands on the blue background (as indicated by the arrow).

M, molecular migration marker; lane 1, Ea 4-96 at 10 μg; lane 2, Ea 6-4 at 10 μg; lane 3, Ea 4-96 at 15 μg; lane 4, Ea 6-4 at 15 μg; lane 5, Ea 4-96 at 20 μg; and lane 6, Ea 6-4 at 20 μg. All masses are the total amount of crude protease fraction loaded onto the gel.
A search of the NCBI Genbank database, using the DNA sequences of the ITS PCR products, indicated that the ITS region encoded for specific transfer RNAs (tRNAs). For isolate D-7, the 739, 870, and 1088 bp PCR products encoded for tRNA^{Glu}, tRNA^{Ala}, and tRNA^{Ala}, respectively (fig. 15, 16, and 17). The tRNA genes contained within the D-7 PCR amplified ITS region are homologous to the *E. amylovora* G-7 ITS tRNA genes recently described by Jeng *et al* (2001). This is not surprising, since isolate G-7, like isolate D-7, is pathogenic on *Maloideae* hosts. The two tRNA^{Ala} gene sequences of isolate D-7 are 94.2 % homologous (fig. 18). The two ITS PCR products of raspberry isolate 7-96b also encode for tRNA genes homologous to those of isolates D-7 and G-7. The smaller PCR product (737 bp) encodes for tRNA^{Glu} (fig. 19), while the larger PCR product (942 bp) encodes for tRNA^{Ala} (fig. 20).

The larger (945 bp) ITS PCR product of *Rubus* isolate 1-97 was also homologous to the tRNA^{Ala} genes of *Rubus* isolate 7-96b and *Maloideae* isolates D-7 and G-7 (fig. 22). However, a Genbank search revealed that the smallest PCR product of isolate 1-97 (730 bp), which encodes for tRNA^{Glu}, shared greater homology with the tRNA^{Glu} gene of *Erwinia pyrifoliae* than with the previously characterized *E. amylovora* G-7 tRNA^{Glu} gene (fig. 21). *E. pyrifoliae* is a recently described bacterium that causes a fire blight-like disease on Asian pears and is closely related to *E. amylovora* (Kim *et al*, 1999). The smallest ITS PCR products of all three isolates, which encode for tRNA^{Glu} genes, had a significant level of homology (88.3%) and a consensus length of 750 basepairs (fig. 23).
Figure 14. Agarose gel electrophoresis of PCR-amplified ITS region of *E. amylovora* isolate D-7, obtained from pear (lane 1), and of *E. amylovora* isolates obtained from raspberry (isolates 1-97 and 7-96b, lanes 2 and 3 respectively). M, 100 bp DNA molecular weight marker.
Figure 15. DNA sequence of *Erwinia amylovora* pear isolate D-7 739 bp ITS PCR product, encoding for tRNA^{Glu}. The sequence is shown beginning with the 5' ITS23 primer and ending with the 3' ITS16 primer (both in bold). Homology with *E. amylovora* pear isolate G-7 tRNA^{Glu} is identified by underlined text.
Figure 16. DNA sequence of *Erwinia amylovora* pear isolate D-7 870 bp ITS PCR product, encoding for tRNA\textsuperscript{Ala}. The sequence is shown beginning with the 5’ ITS23 primer and ending with the 3’ ITS16 primer (both in bold). Homology with *E. amylovora* pear isolate G-7 tRNA\textsuperscript{Ala} is identified by underlined text.
Figure 17. DNA sequence of *Erwinia amylovora* pear isolate D-7 1088 bp ITS PCR product, encoding for tRNA$^{\text{Ala}}$. The sequence is shown beginning with the 5' ITS23 primer and ending with the 3' ITS16 primer (both in bold). Homology with *E. amylovora* pear isolate G-7 tRNA$^{\text{Ala}}$ is identified by underlined text.
Figure 18. Comparison of the two DNA sequences of *E. amylovora* isolate D-7 which encode for tRNA^A_{11}_. The DNA sequences are identified by size (873 and 1088 bp, respectively). The sequence comparison begins with the 5' ITS23 primer and ends with the 3' ITS16 primer (both in bold). The character to indicate that two aligned residues are identical is '|'. The sequences are 94.2% homologous, with 2 gaps inserted in the larger sequence (1088 bp) and 30 gaps inserted in the smaller sequence (873 bp).
Figure 19. DNA sequence of *Erwinia amylovora* raspberry isolate 7-96b 737 bp ITS PCR product, encoding for tRNA^{Glu}. The sequence is shown beginning with the 5' ITS23 primer and ending with the 3' ITS16 primer (both in bold). Homology with *E. amylovora* pear isolate G-7 tRNA^{Glu} is identified by underlined text.
Figure 20. DNA sequence of *Erwinia amylovora* raspberry isolate 7-96b 942 bp ITS PCR product, encoding for tRNA\textsuperscript{Ala}. The sequence is shown beginning with the 5' ITS23 primer and ending with the 3' ITS16 primer (both in bold). Homology with *E. amylovora* pear isolate G-7 tRNA\textsuperscript{Ala} is identified by underlined text.
Figure 21. DNA sequence of *Erwinia amylovora* raspberry isolate 1-97 730 bp ITS PCR product, encoding for tRNA<sup>Glu</sup>. The sequence is shown beginning with the 5' ITS23 primer and ending with the 3' ITS16 primer (both in bold). Significant alignment with tRNA<sup>Glu</sup> genes of *E. pyrifoliae* 1-96 and *E. amylovora* G-7 indicated in gray and by underlining, respectively.
Figure 22. DNA sequence of *Erwinia amylovora* raspberry isolate 1-97 945 bp ITS PCR product, encoding for tRNA\(^{\text{Ala}}\). The sequence is shown beginning with the 5' ITS23 primer and ending with the 3' ITS16 primer (both in bold). Homology with *E. amylovora* pear isolate G-7 tRNA\(^{\text{Ala}}\) is identified by underlined text.
<p>| Ea D-7    | GGTACCTTAGATGTTTTCAGTTCCCGGTTGTCGCCCTATGCACCTATGATT | 50  |
| Ea 7-96   | GGTACCTTAGATGTTTTCAGTTCCCGGTTGTCGCCCTATGCACCTATGATT | 50  |
| Ea 1-97   | GGTACCTTAGATGTTTTCAGTTCCCGGTTGTCGCCCTATGCACCTATGATT | 50  |
| Ea D-7    | TCATGACATGATGATGCAACGATGGGATTCGACCCGCCTTCATGCTACATGAT | 100 |
| Ea 7-96   | TCATGACATGATGATGCAACGATGGGATTCGACCCGCCTTCATGCTACATGAT | 100 |
| Ea 1-97   | TCATGACATGATGATGCAACGATGGGATTCGACCCGCCTTCATGCTACATGAT | 100 |
| Ea D-7    | CGTCGGTTGTTGCGGTTTCTGATACCTTTACGCTGCTTTATCGCAGATTAG | 150 |
| Ea 7-96   | CGTCGGTTGTTGCGGTTTCTGATACCTTTACGCTGCTTTATCGCAGATTAG | 150 |
| Ea 1-97   | CGTCGGTTGTTGCGGTTTCTGATACCTTTACGCTGCTTTATCGCAGATTAG | 150 |
| Ea D-7    | CACGCTTTTCACGCTTCTGAGCTGCTGCGCTTATCGCAGATTAG | 200 |
| Ea 7-96   | CACGCTTTTCACGCTTCTGAGCTGCTGCGCTTATCGCAGATTAG | 200 |
| Ea 1-97   | CACGCTTTTCACGCTTCTGAGCTGCTGCGCTTATCGCAGATTAG | 200 |
| Ea D-7    | TCGCTTAACCTAACAACCCACAAAGCGTCCCCGCCAGGACGATGGGATCGTTTCGC | 250 |
| Ea 7-96   | TCGCTTAACCTAACAACCCACAAAGCGTCCCCGCCAGGACGATGGGATCGTTTCGC | 250 |
| Ea 1-97   | TCGCTTAACCTAACAACCCACAAAGCGTCCCCGCCAGGACGATGGGATCGTTTCGC | 250 |
| Ea D-7    | AGGCATTGAGAGACTCGAACATATCGTGATTTCATTCT-GATTACGGAGA | 299 |
| Ea 7-96   | AGGCATTGAGAGACTCGAACATATCGTGATTTCATTCT-GATTACGGAGA | 300 |
| Ea 1-97   | AGGCATTGAGAGACTCGAACATATCGTGATTTCATTCT-GATTACGGAGA | 299 |
| Ea D-7    | ATGAACACGACATGTCGTTTCAATTTTCAGCTTGTTCCGGATTGTTAAAG | 349 |
| Ea 7-96   | ATGAACACGACATGTCGTTTCAATTTTCAGCTTGTTCCGGATTGTTAAAG | 350 |
| Ea 1-97   | ATGAACACGACATGTCGTTTCAATTTTCAGCTTGTTCCGGATTGTTAAAG | 349 |
| Ea D-7    | AGCAATATCTTAAAC----CTGACTTT-GCAG--TCAGCTTTAAGATATT | 392 |
| Ea 7-96   | AGCAATATCTTAAAC----CTGACTTT-GCAG--TCAGCTTTAAGATATT | 393 |
| Ea 1-97   | AGCAATATCTTAAAC----CTGACTTT-GCAG--TCAGCTTTAAGATATT | 399 |
| Ea D-7    | TTTAGCGACACCTTTCACCT-TCACCAAAAGCAGTGGCGGCTCCCCCTAAGG | 411 |
| Ea 7-96   | TTTAGCGACACCTTTCACCT-TCACCAAAAGCAGTGGCGGCTCCCCCTAAGG | 440 |
| Ea 1-97   | TTTAGCGACACCTTTCACCT-TCACCAAAAGCAGTGGCGGCTCCCCCTAAGG | 440 |
| Ea D-7    | ATTCGAAACCCTCTGGTGGCTCCTGACCTTAG | 490 |
| Ea 7-96   | ATTCGAAACCCTCTGGTGGCTCCTGACCTTAG | 488 |
| Ea 1-97   | ATTCGAAACCCTCTGGTGGCTCCTGACCTTAG | 496 |
| Ea D-7    | CGAAGGGGACACGGTGCTGCGGCTCCCCTTCATGATTACCTCTGCTACATGAT | 540 |
| Ea 7-96   | CGAAGGGGACACGGTGCTGCGGCTCCCCTTCATGATTACCTCTGCTACATGAT | 538 |
| Ea 1-97   | CGAAGGGGACACGGTGCTGCGGCTCCCCTTCATGATTACCTCTGCTACATGAT | 531 |</p>
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**Figure 23.** Comparison of the DNA sequences of the smallest 16S/23S ITS PCR products (tRNA^Glu^) in *E. amylovora* isolates D-7 (pear), 7-96b (raspberry) and 1-97 (raspberry). The sequence is shown beginning with the 5' ITS23 primer and ending with the 3' ITS16 primer (both in bold). '●' indicates that a nucleotide position in the alignment is perfectly conserved; '●' denotes a nucleotide position in the alignment is well conserved. The three sequences have a consensus length of 750 bp, with homology of 88.3%.
**4 Discussion**

### 4.1 Efficacy of *O. ulmi* and *V. dahliae* elicitors against *E. amylovora*

While the results of apple seedling experiments were inconsistent, experiments using 4 year-old apple and pear trees indicate both Q412 and WRLS *O. ulmi* elicitors are effective in controlling fire blight. It is noteworthy that the *V. dahliae* elicitor is also as effective against fire blight at half the dose (fig. 11). Both *O. ulmi* and *V. dahliae* elicitors appear to induce resistance against fire blight via SAR (section 1.5.3), since challenge inoculations at sites remote from elicitor treatment produce diminished or no fire blight symptoms. While the *O. ulmi* elicitors have induced SAR in elm trees inoculated with aggressive *O. ulmi* isolates distant from the elicitor treatment site (Hubbes, 2000a; Hubbes, 2000b), this is the first published report of a *V. dahliae* elicitor stimulating SAR against *E. amylovora* in Maloideae. The protection conferred by the elicitors is non-specific i.e. it is effective against pathogens unrelated to the inducing agent. This is characteristic of natural or synthetic compounds that induce SAR (Lucas, 1999).

The maturity of the plant tissue used for elicitor experiments may impact treatment efficacy. The most representative plant material for fire blight experiments is probably a mature apple or pear tree (Vanneste and Eden-Green, 2000). While seedlings are the most practical material for laboratory experiments, the development of symptoms in seedlings, where the tissues are in a more susceptible juvenile state (van der Zwet and Beer, 1991), might not allow the extrapolation of these results to mature trees (Vanneste and Eden-Green, 2000). Using plant material whose developmental stage is closer to maturity allows not only for
extrapolation to mature trees, the target for fire blight treatment, but will more accurately assess the elicitor's efficacy against E. amylovora.

Both O. ulmi and V. dahliae elicitors are promising treatments for fire blight. Alternative control methods must be developed since fire blight prevention relies chiefly on streptomycin. Streptomycin is currently the most effective and common fire blight preventative, but its efficacy is decreasing due to resistant E. amylovora strains (OMAFRA, 2000). The elicitors also provide alternative treatments in jurisdictions where agricultural use of medically important antibiotics is prohibited (Epton et al, 1994). Because the elicitors induce the plant's defences and do not work directly on the fire blight bacterium, E. amylovora is unlikely to develop resistance to elicitor treatment. Further, the O. ulmi elicitors are non-toxic (Hubbes, 2000a; Hubbes, 2000b), which is desirable from an environmental perspective.

The two elicitors compare favourably with the novel fire blight treatments described in sections 1.4.4 and 1.4.5. Both BlightBan A506® and BlightBan C9-1® can only be applied when blossoms are present, while either elicitor can be injected whenever the tree is non-dormant. Further, because both O. ulmi and V. dahliae elicitors are not biological control organisms, there is no danger of the control organism population declining or dying before fire blight control is established. This has occurred with BlightBan A506® (Pusey, 1999).
The elicitors also compare favourably with other SAR activators and growth regulators that control fire blight. Messenger® (section 1.4.5) activates the jasmonic acid-ethylene pathways and other plant growth systems that stimulate plant growth (Grisham, 2000). The increased growth stimulated by Messenger® could curtail its efficacy as a fire blight control, since succulent shoot tissue is most vulnerable to and severely affected by *E. amylovora* (van der Zwet and Beer, 1991). In contrast, Apogee® inhibits gibberellin biosynthesis, controlling new shoot growth (BASF U.S.A., 2000). While this growth regulation reduces the quantity of fire blight susceptible tissue (van der Zwet and Beer, 1991), this decrease could also affect fruit yield.

Since both the *O. ulmi* and *V. dahliae* elicitors demonstrate potential as fire blight controls, future research must establish how the elicitors activate the plant defence response. Greater understanding of the elicitation mechanism will assist improvements in the elicitors’ efficacy against fire blight. Because SA is appears to be integral in SAR induction (section 1.5.3), SA isolation must be attempted following initial elicitor treatment and subsequent challenge inoculation to confirm the elicitor’s activation of SAR. The SAR genes and proteins activated by the elicitor must also be identified. Identifying the genetic components of elicitor-mediated defence can aid research focussed on other aspects of the host defence response. These may include the production of active oxygen species (AOS) generated during the oxidative burst, phytoalexins, and ligninification. The generation of AOS in the form of hydrogen peroxide has been observed in *O. ulmi* elicitor-treated elm callus.
tissue (L. Fleck, R. Jeng and M. Hubbes, unpublished data). Further, there have been sporadic observations of antibiotic compounds, isolated from *O. ulmi* elicitor-treated apple seedlings, which inhibit *E. amylovora* growth *in vitro* (L. Fleck, unpublished data). While members of the Rosaceae family are characterized by a notable absence of isolated phytoalexins (Harborne, 1999), future experiments should investigate whether the elicitors stimulate phytoalexin formation, and whether differences in phytoalexin accumulation exist between elicitor treated plant material and controls.

Both elicitors' efficacy against fire blight can be improved in future research by addressing several issues. Isolation and purification of the eliciting compounds that induce resistance is essential. While an asparatic protease obtained from the *O. ulmi* elicitor has been sequenced, whether it is the only eliciting compound is unproven. Once the SAR-inducing compounds have been identified, an assay must be developed to enforce quality control for future experiments, eliminating a source of variation in future experiments.

Establishing an optimal elicitor dose is crucial for enhancing treatment efficacy. Comparisons between experiments 00Ea128 and 00Ea131 (fig. 11), which used different doses of the same elicitor batch, highlight the importance of the *O. ulmi* elicitor dose. A dose effect was also observed for *V. dahliae* elicitor (fig. 12). Elicitor dose may vary due to tree age and size. It is possible a combination of the three
elicitors improves fire blight control relative to single elicitor treatment. If so, the optimum ratio of elicitors must be calculated.

Measuring the duration of SAR activation and the efficacy of multiple elicitor treatments may improve fire blight control; data from such experiments could aid the development of an elicitor application schedule. The ability of the elicitor to alleviate active fire blight in infected trees should be assessed. Elicitor treatment in Dutch Elm Disease-infected elm trees has proven effective in inhibiting further disease development (Hubbes, 2000a). A similar scenario may exist for fire blight.

4.2 Host species-specificity and extracellular proteolytic activity of E. amylovora
Proteolytic activity, indicated by the hydrolysis of gelatin substrate, was demonstrated in the SDS-gelatin-PAGE assay of Maloideae (Ea 6-4) and Rubus (Ea 4-96) E. amylovora isolates (fig. 13). This assay (also termed a zymogram) was practical for comparing the protease activity and size of the two E. amylovora isolates. In a zymogram, proteases are readily identified as clear bands against a stained background where the enzyme has digested the substrate. Zymograms detect various types of proteases, including metalloproteases such as the PrtA protease of E. amylovora apple isolates (Zhang et al, 1999). Gelatin is often incorporated into the matrix of a zymogram to detect protease activity, since it is a good substrate for many proteases (Heussen and Dowdle, 1980). While casein is also used as a protease substrate, gelatin was chosen for this analysis since PrtA utilizes gelatin substrate (Zhang et al, 1999).
The proteases of both *E. amylovora* isolates are similar in size (approximately 50 kDa) and possess significant enzymatic activity (fig. 13). The highly conserved nature of the PrtA protease in *Maloideae E. amylovora* isolates (Zhang et al, 1999), combined with a similar size (48 kDa), suggest that the protease obtained from the *E. amylovora Maloideae* isolate (Ea 6-4) is PrtA. It is noteworthy that the *Rubus* isolate (Ea 4-96) also secretes a protease whose size and activity is similar to that of Ea 6-4 (fig. 13). This indicates that both *Maloideae* and *Rubus E. amylovora* isolates secrete homologous proteases, and that PrtA secretion is not unique to *E. amylovora Maloideae* isolates. This signifies that PrtA does not confer host species-specificity in *E. amylovora*. Further research, including DNA and protein sequencing, is required for a complete homology assessment of the secreted proteases produced by the two *E. amylovora* isolates assayed.

4.3 PCR products of the *E. amylovora* ITS region and host species-specificity

There are some similarities in the ITS regions of *E. amylovora* isolates studied, irrespective of host. The ITS region of each isolate encodes for at least one tRNA<sup>Ala</sup> gene, which are all homologous to the ITS tRNA<sup>Ala</sup> gene of *E. amylovora Maloideae* isolate G-7 (fig. 17, 17, 20 and 22). There is 94.2% homology between the two tRNA<sup>Ala</sup> genes of *Maloideae* isolate D-7 (fig. 18) Further, in both the *Maloideae* and *Rubus* isolates studied, the smallest ITS PCR amplified fragment codes for a tRNA<sup>Glu</sup> gene homologous to that of *E. amylovora* G-7 (fig. 15, 19, and 21) (Jeng et al, 2001). The size of the smallest ITS PCR product is well conserved (~ 730 bp), and a multiple sequence alignment of the three isolates indicates a high level of homology within the tRNA<sup>Glu</sup> gene of *E. amylovora* isolates (88.3%) (fig. 23).
Despite the high level of homology between the three ITS tRNA\textsuperscript{Glu} genes, the nature of the tRNA\textsuperscript{Glu} gene homology differs between Rubus isolates. The tRNA\textsuperscript{Glu} genes of both \textit{E. amylovora Maloideae} isolate D-7 (fig. 15) and \textit{Rubus} isolate 7-96b (fig. 19) are homologous to that of \textit{E. amylovora Maloideae} isolate G-7 (Jeng \textit{et al}, 2001). However, the tRNA\textsuperscript{Glu} gene of \textit{Rubus} isolate 1-97 (fig. 21) is homologous to that of both \textit{E. amylovora} G-7 and \textit{E. pyrifoliae}, a pathogenic bacterium that causes a disease similar to fire blight on Asian pear species (Kim \textit{et al}, 1999). The fact that the raspberry isolate 1-97's tRNA\textsuperscript{Glu} gene is homologous to both \textit{E. amylovora} and \textit{E. pyrifoliae} indicates that genetic recombination has occurred between the two species in the ITS region. This contradicts previous homology comparisons between \textit{E. pyrifoliae} and \textit{E. amylovora Maloideae} isolates (Kim \textit{et al}, 1999). Kim \textit{et al} (1999) concluded the two bacteria were not homologous in the ITS region. It is possible that the host species-specificity of raspberry isolates has developed because of this recombination. To determine whether this is the case, the ITS region of a larger number of raspberry isolates should be examined for homology to \textit{E. pyrifoliae}.

The intraspecies variability within the \textit{E. amylovora} ITS region is not surprising. The spacer region between the 16S and 23S rRNA genes is extremely variable in size and sequence even within closely related taxonomic groups (Gurtler and Stanisich, 1996). This variability makes the ITS region useful in bacterial species identification. However, there is some conservation within the ITS region, especially within discrete species (Anton \textit{et al}, 1998; Garcia-Martinez \textit{et al}, 1999; Perez-Luz \textit{et al}, 1998). A study comparing the ITS region of two \textit{Bacillus subtilus} isolates found that despite
some differences between the two isolates, a high level of conservation existed in the ITS region. Variation in the 16S/23S spacer regions of closely related bacterial strains probably reflects frequent insertion-deletion events (Garcia-Martinez et al, 1996). Despite this variation, the high level of conservation found in tRNA genes of the ITS region is necessary, as the formation of tRNA secondary structures is vital to cellular metabolism. Thus, nucleotide changes affecting tRNA stability are usually tolerated at very low degrees or only allowed when compensatory mutations appear (Garcia-Martinez et al, 1999).
5 Conclusion
Despite the inconsistent efficacy observed in seedling experiments, both Q412 and WRLS O. ulmi elicitors displayed significant efficacy against fire blight in susceptible apple and pear cultivars at a dose of 40 mg/tree ($P < 0.05$). V. dahliae elicitor possessed equivalent efficacy against fire blight at half the dose of both O. ulmi elicitors (20 mg/tree, $P < 0.05$). Both O. ulmi and V. dahliae elicitors, which appear to stimulate plant defense mechanism via SAR, are promising preventative treatments for fire blight. This is especially significant given the appearance of streptomycin-resistant E. amylovora isolates. Future research must focus on elucidating the complete mechanism of host defense activated by the elicitors studied. As these elicitors appear to activate SAR, whether salicylic acid is involved in the signal transduction pathway should be established. Purification and identification of the eliciting compounds contained within the O. ulmi and V. dahliae crude elicitor preparations, along with establishment of an optimal elicitor dose and application schedule, are essential to enhance the both elicitors’ efficacy against fire blight.

Both Maloideae and Rubus E. amylovora isolates secrete extracellular proteases that possess considerable proteolytic activity. The conserved nature of the previously identified E. amylovora protease (Zhang et al, 1999) suggests the proteases of both isolates assayed are identical. This indicates that extracellular protease production does not confer host species-specificity in E. amylovora.
Variation in the ITS region of *E. amylovora* isolates may be linked to host specificity. Other than host species-specificity, variation in the number of ITS PCR products and ITS tRNA$^{\text{Ala}}$ genes, in addition to tRNA$^{\text{Glu}}$ gene homology variation within the smallest ITS PCR products, are the only known intraspecies differences within *E. amylovora*. The homology of the *E. amylovora* Rubus 1-97 isolate's tRNA$^{\text{Glu}}$ gene with both *E. pyrifoliae* and *E. amylovora* tRNA$^{\text{Glu}}$ genes suggests that Rubus host species-specificity has arisen from DNA recombination between *E. amylovora* and *E. pyrifoliae* isolates. Despite the variation in the *E. amylovora* ITS region, both Maloideae and Rubus isolates encode for only two tRNA genes (tRNA$^{\text{Ala}}$ and tRNA$^{\text{Glu}}$). A high level of homology existed in the tRNA$^{\text{Glu}}$ genes of all three *E. amylovora* isolates sequenced.
6 References
diversity in the 16S-23S intergenic spacer region (ISR) of the rRNA operons in
representatives of the Escherichia coli ECOR collection. Journal of Molecular

Arthur, J.C. (1885). Proof that bacteria are the direct cause of the disease in trees
known as pear blight. American Association for the Advancement of Science

Erwinia amylovora: a correlation with virulence. Applied and Environmental
Microbiology 38: 659-666.

www.basf.com/businesses/consumer/agproducts/solutions/usa_products/Apogee.ht
ml.

Beer, S.V., Wei, Z.M., Laby, R.J., He, S.Y., Bauer, D.W., Collmer, A., and Zumoff,
and is a determinant of pathogenicity. Acta Horticulturae 338: 243-244.


synthesis in Erwinia amylovora: Characterization and relationship to cps genes in
Erwinia stewartii. Molecular and General Genetics 239: 158.

Phytopathology 89: 1218-1225.

does not function downstream of salicylic acid in the induction of PR protein

Bonn, W.G. and van der Zwet, T. (2000). Distribution and Economic Importance of

by the fire blight pathogen Erwinia amylovora marked with genes for

Burrill, T.J. (1880). Anthrax of fruit trees; or the so-called fire blight of pear, and twig blight of apple trees. *Proceedings of the American Association for the Advancement of Science* **29**: 583-597.


