ABSTRACT

Bell, Andrew Claude. Host Plant Resistance to Fire Blight (*Erwinia amylovora*) in the Rosaceae subfamily Maloideae (Under the direction of Dr. Thomas G. Ranney.)

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is a devastating disease of apple (*Malus* Mill. spp.), pear (*Pyrus* L. spp.), and other taxa in the Rosaceae subfamily Maloideae. Currently, the antibiotic, streptomycin, is the only effective chemical to control this disease among susceptible taxa; however, development of streptomycin-resistant strains of *E. amylovora* threatens the future use of this antibiotic. Moreover, environmental and health concerns associated with the use of antibiotics may ultimately result in a widespread ban that already exists in some countries. Significant variation in resistance to fire blight among some host plants has been reported. Selecting and developing new cultivars with enhanced fire blight resistance is an environmentally safe, cost effective, and practical means of control for this disease. Therefore, three studies were conducted to document resistance to fire blight and investigate the role of secondary metabolites in disease resistance.

In the first study, artificial inoculations, using a virulent strain of *E. amylovora* were conducted to determine levels of resistance to fire blight among taxa of flowering pear and quince (*Chaenomeles* Lindl. spp.). Significant variation in resistance to fire blight was observed among many widely cultivated taxa of flowering pear and quince and highly resistant taxa were identified. *Pyrus calleryana* ‘Chanticleer’ was significantly more resistant than ‘Bradford’. Prior to this study,

only limited information was available regarding fire blight resistance among taxa of flowering quince.

In a second study, resistance to fire blight among 49 taxa of flowering crabapples was evaluated based on observations of natural infection and results from artificial inoculations using three virulent strains of *E. amylovora.* Considerable variation in resistance was observed with cultivars ranging from highly susceptible to highly resistant. Artificial inoculations provided a consistent method for evaluating disease resistance. *Malus* 'Adams' and 'David' were among the most resistant taxa to natural infection and to artificial inoculations. Several phenolic compounds found in *Malus* spp. exhibited strong antibiotic activity in vitro against *E. amylovora*. Levels of endogenous phenolic compounds were analyzed by high-performance liquid chromatography (HPLC). Three unidentified constitutive compounds were correlated with resistance to fire blight among cultivars of flowering crabapples.

In the final study, three cultivars of flowering crabapple, 'Adams', 'Canary', 'Schmidtcutleaf', representing a range of resistance to fire blight were artificially inoculated to evaluate pathogen-induced changes in phenolic compounds over time. Seven major phenolic components were analyzed by HPLC. There were significant cultivar, inoculation, and time effects and various interactions among factors depending on the specific compound. Component A showed an inducible response and levels were higher in the resistant and moderately resistant cultivars following inoculation. Levels of component X were significantly lower in the highly susceptible cultivar, decreased in the moderately resistant cultivar following

inoculation, but were highest and remained unaffected following inoculation in the resistant cultivar.

This research provides information on methods for screening and evaluating for resistance, identification of resistant taxa, and insights into the role of endogenous phenolic constituents in host plant resistance to fire blight. These results will aid in further evaluation, selection, and improvement of cultivars with superior disease resistance.

HOST PLANT RESISTANCE TO FIRE BLIGHT (*ERWINIA AMYLOVORA*) IN THE ROSACEAE SUBFAMILY MALOIDEAE

by

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
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DEDICATION

In loving memory of you

who gave me life,

loved me unconditionally,

and introduced me to the wonderful world of plants.

To:

My mother
Patsy Elaine Bell
1945-1990

and my father
Daniel Andrew Bell
1939-2003

Personal Biography

Andrew C. Bell was born in Johnson City, Tennessee. He was raised in the nearby residential community of Colonial Heights, where he attended elementary and middle schools. In the mountains of eastern Tennessee, Andrew spent time with his family and friends hiking and camping in the Cherokee National Forest, where he developed great love for the natural world. He was introduced to horticulture at a very young age through the avid gardeners of his mother and father. Andrew attended elementary and middle school in Colonial Heights. He began working part-time at a garden center while attending Sullivan South High School where he graduated in 1990.

In the Fall of 1990, Andrew began pursuing a degree in civil engineering. Within two years, he realized that his passion was horticulture, not engineering, and enrolled in the Ornamental Horticulture and Landscape Design curriculum at the University of Tennessee, Knoxville. Andrew became active in student organizations and worked in the University's trial gardens and in a plant pathology research laboratory. He became interested in research and teaching through these experiences. Andrew graduated magna cum laude with a B.S. in Ornamental Horticulture and Landscape Design and a minor in Botany in 1995.

In the Fall of 1995, Andrew enrolled in a plant taxonomy master of science degree program run jointly by the University of Edinburgh and the Royal Botanic Garden Edinburgh, Scotland. In December 1996, Andrew earned an M.S. degree in the Biodiversity and Taxonomy of Plants from the University of Edinburgh. While

pursuing his masters degree, Andrew visited gardens and studied plants throughout Europe. Upon graduating from Edinburgh, he traveled to Australia for two months for a student sabbatical hosted by various botanical gardens. In January 1997, Andrew accepted a position at the Arnold Arboretum of Harvard University to supervise a one-year grant funded project to survey the Arboretum's shrub and woody vine collection.

In March 1998, Andrew was hired as a temporary research technician by Dr. Thomas Ranney at North Carolina State University's Mountain Horticultural Crops Research and Extension Center in Fletcher, North Carolina. In January 1999, Andrew began working on his Ph.D. under the direction of Dr. Ranney at NC State. During his graduate program, Andrew spent the summer months at the Mountain Horticultural Crops Research and Extension Center in Fletcher, where he conducted his dissertation research and spent the academic year on campus in Raleigh. Following graduation, Andrew will begin his career as the Associate Director of the North Carolina Botanical Garden at the University of North Carolina, Chapel Hill.

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During my graduate program at North Carolina State, I was very fortunate to have had Dr. Tom Ranney serve as my graduate advisor. I am honored to have been one of his graduate students. I am grateful for his mentoring and friendship. I would like to thank the rest of my graduate committee for their guidance and advice: Drs. Robert Lyons, Turner Sutton, and Dennis Werner. I received substantial and invaluable assistance with my research from Dr. David Danehower, Tom Eaker, and the staff at the Mountain Horticultural Crops Research Station. I would like to express my appreciation to all of the folks in Kilgore Hall who have helped me along the way. I could not have done this without your support and friendship. What a great place to spend five years! To my fellow graduate students, I thank you for all your support. I learned more from you than any course taken, and you have provided me with many good memories to last a lifetime.

To my brother Danny and my sister Sherry, thank you, and I love you dearly. Last but certainly not least, I want to acknowledge Sandy Cook and Michael Byrne. Thank you for everything, I love you both!

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Chapter 3. Changes in Endogenous Phenolic Composition among *Malus* Taxa Following Inoculation with *Erwinia amylovora*

GENERAL INTRODUCTION

The Rosaceae is a large and important family of woody and herbaceous plants. The family consists of over 100 genera that are subdivided into four subfamilies. Subfamily Maloideae is characterized by having a distinctive fruit, the pome. It is the fruit of some maloid genera that account for the economic importance and worldwide cultivation of this group of plants. The subfamily consists of approximately 1000 species in 30 genera that are widely distributed across the Northern Hemisphere and extend into parts of the Southern Hemisphere (Evans and Campbell, 2002). Several maloid taxa, apples (*Malus* Mill. spp.) and pears (*Pyrus* L. spp.) in particular, have long been cultivated and valued for their fruits. Currently, members of the Maloideae continue to be important and valued fruit crops. However, many are highly prized as garden ornamentals and have become a significant component of the nursery and landscape industry.

Cultivation of Maloid taxa as both ornamentals and as fruit crops can often be difficult due to their susceptibility to diseases and insects. Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al, is one of the most significant diseases of plants in the Rosaceae subfamily Maloideae. The first report of fire blight was published in 1794 based on observations made as early as 1780 (van der Zwet and Keil, 1979). This report provided a description of a "disorder" of apples and pears in an orchard in the Hudson Valley region of New York. However, at that time, bacteria were still not known to science and it would take 100 years before the causal organism of fire blight would be fully discovered, classified, and named.

Erwinia amylovora is a gram-negative bacterium that belongs to the family Enterobacteriaceae. The host range includes numerous genera in the Rosaceae but is more commonly associated with members of the subfamily Maloideae. The name "fire blight" was appropriately applied to this disease; when young actively growing shoots become infected, they curl at the tip and turn brown as if they have been burned with a flame. Since its discovery, fire blight has spread across the United States and into Canada and Mexico, and overseas to Europe, the Middle East, Japan, and New Zealand.

The pathogen typically enters a host plant through hydathodes in the hypanthium which is the nectarial disk that surrounds the base of the pistil (Thomson, 2000). Vegetative tissue, both leaves and stems, provide points of entry through stomata, lenticels, and wounds created by wind and hail. Initially, bacteria travel through intercellular spaces via physical pressure created by bacterial multiplication (Vanneste and Eden-Green, 2000). Beyond this point, there is no consensus among researchers regarding the pathway of internal movement in host tissue. Momol et al. (1998) reported that *E. amylovora* can move rapidly over long distances (from shoots to roots) in host tissue without causing any visible symptoms. The development and severity of infection depends largely on the interaction between the plant and the bacterial strain.

Despite over a century of research on fire blight, the host-pathogen interaction is still not fully understood. Momol and Aldwinckle (2000) reviewed studies on pathogen diversity and found that *E. amylovora* is not a diverse species like other

plant pathogenic bacteria. However, Paulin (2000) noted that numerous investigations have documented that strains vary in virulence, colony morphology, and sensitivity to antibiotics. For example, Norelli et al. (1986) reported some cultivars of *Malus* were highly resistant to *E. amylovora* strain 273 but were susceptible to strain E4001a. Clearly strain virulence and the host's ability to recognize a strain and initiate defense reactions are critical to host resistance.

Host plant resistance involves two primary lines of defense to protect them from pathogen invasion: structural and biochemical. Structural or physical barriers serve to prevent the pathogen from gaining entry or from migration in host tissue. The biochemical response is achieved via the action of constitutive (preformed) components and/or through synthesis of phytoalexins after infection has occurred (induced resistance). Secondary metabolites (phenolic compounds, terpenes, and alkaloids) present in host cells prior to infection have been shown to play a key role in some defense reactions (Agrios, 1997). However, in many cases, resistance is thought to be expressed after infection occurs and involves multiple mechanisms (Hammerschmidt and Nicholson, 1999).

A key component of induced resistance is the hypersensitive response. The hypersensitive response has been described as "the localized and rapid death of one or a few host plant cells in response to invasion by an avirulent isolate of a pathogen" (Hammerschmidt and Nicholson, 1999). The response is mediated by loss of membrane integrity in the host cell and accumulation of phenolic compounds. Although the hypersensitive response may be completely effective with some

pathogens, it may only serve as the first defense mechanism encountered in other plant-pathogen interactions. Other components of induced resistance are the production of active oxygen species and phytoalexins. Phytoalexins are antimicrobial compounds that are induced after infection (Hammerschmidt and Nicholosom, 2000). While multiple mechanisms may be involved in the defense reaction, several studies have shown that phytoalexins play a key role in preventing the migration of the pathogen in host tissue (Kuć, 1998).

Fire blight continues to cause significant economic damage to plants in both nursery and landscape plantings. Current control measures are very limited and expensive. Although many taxa are susceptible, there is considerable variation in resistance to this disease. The research presented in this dissertation was conducted to document the variation in resistance that exists within selected Maloid genera. An attempt was made to elucidate potential mechanisms of resistance and test the sensitivity of selected strains to secondary metabolites from *Malus* spp.

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Chapter 1

Resistance to Fire Blight

Among Flowering Pears and Quince

(In the format appropriate for submission to HortScience)

Resistance to Fire Blight Among Flowering Pears and Quince

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Resistance to Fire Blight Among Flowering Pears and Quince

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Abstract. Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is one of the most destructive diseases of plants in the Rosaceae subfamily Maloideae. Artificial inoculations, using *E. amylovora* strain E2002a, were conducted to determine levels of resistance to fire blight among taxa of flowering pears (*Pyrus* L. spp.) and quince (*Chaenomeles* Lindl. spp.). The level of resistance was measured as the length of the fire blight lesion as a percentage of overall shoot length. Considerable variation in resistance was observed among both pears and quince. *Pyrus ussuriensis* Maxim. 'Prairie Gem' was highly resistant with a lesion length of 1% of the total shoot length. *Pyrus calleryana* Decne. 'Bradford' was intermediate with a 50% lesion length while *P. calleryana* 'Chanticleer' was significantly more resistant with a lesion length of 31%. Nine taxa of flowering pears were highly susceptible and did not differ significantly from 100% disease severity (total shoot death). *Chaenomeles speciosa* (Sweet) Nak. 'Contorta' was highly resistant with a lesion length of 15%. Six taxa of flowering quince, including *C.* x

superba (Frahm) Rehd**.** ‘Cameo’, ‘Texas Scarlet’, and ‘Jet Trail’ were highly susceptible while nine other taxa showed intermediate resistance.

Flowering pears (*Pyrus* L. spp.) and flowering quince (*Chaenomeles* Lindl. spp.) are two genera in the Rosaceae subfamily Maloideae that include many species and hybrids with significant economic importance as nursery and landscape plants. Despite their popularity, cultivation of these genera can be difficult due to their susceptibility to fire blight.

The genus *Pyrus* consists of ≈22 species whose native ranges extend from Europe and North Africa to across the Asian continent (Bell and Hough, 1986). In addition to being cultivated as a fruit crop, some species of *Pyrus* are frequently used as landscape plants. For example, the callery pear (*Pyrus calleryana* Decne.) is one of the most common flowering trees used in landscape horticulture. The most notable and widely planted cultivar of callery pear is 'Bradford' which was introduced by the U.S. Department of Agriculture in the early 1960s. 'Bradford' and other cultivars of callery pear offer many desirable landscape qualities including early spring flowering, broad adaptability, excellent resistance to arthropod pests, and outstanding fall color. The popularity and use of flowering pears as landscape plants has increased significantly in recent decades.

The genus *Chaenomeles* consists of three species, two of which are native to China while the third is restricted to Japan. The flowering quince is closely related to pears, apples (*Malus* Mill. spp.), and the true quince (*Cydonia* Mill. spp.). Unlike its close relatives, flowering quince is not commonly cultivated as a fruit crop. However, Weber (1964) reported that the flowering quince has been cultivated for over 400 years and that more than 500 cultivars have been named and propagated.

Today, numerous cultivars of flowering quince are readily available in the horticultural industry. The flowering quince is highly prized as an early flowering shrub and is commonly used in landscapes.

Fire blight is a highly destructive disease caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al. This disease can be especially problematic in regions where environmental conditions for disease development are favorable, specifically where springtime weather is warm and wet (van der Zwet and Keil, 1979). Susceptible plants can be severely damaged or killed by fire blight in both nursery and landscape plantings. The economic importance and history of pears as a fruit crop has resulted in numerous attempts to document resistance among species and cultivars. van der Zwet and Keil (1979) summarized relative susceptibility of five important species of pears to fire blight. Furthermore, they provided fire blight resistance ratings for over 400 cultivars of pear fruit trees based on an extensive survey of the literature.

There have been few attempts to document resistance to fire blight among flowering pears. Fare et al. (1991) and McNeil et al. (1986) reported resistance ratings for selected cultivars of *Pyrus calleryana*. Both studies reported 'Redspire' and 'Aristocrat' as being more susceptible than 'Bradford', 'Capital', 'Fauriei', and 'Whitehouse'. Because the incidence of fire blight can be sporadic and vary from region-to-region and year-to-year, observations and results from field surveys can be inconsistent and highly variable. Plants that initially appear to be resistant may later be found to be susceptible when conditions are favorable for disease development. In

the aforementioned studies, artificial inoculations were not used and neither study documented which strain(s) of *E. amylovora* were present.

There is limited information on resistance to fire blight among taxa of flowering quince. Based on their survey of the literature, van der Zwet and Keil (1979) listed only one species, *Chaenomeles japonica* (Thunb.) Spach., and reported that it was "very susceptible" to blossom blight and "moderately susceptible" to twig blight.

Despite commercial importance to landscape horticulture, extensive information on fire blight resistance among flowering pears and quince is not readily available. Ornamental plant breeding programs could benefit greatly from screening studies that use specific clones and artificial inoculations. Artificial inoculations of actively growing shoots with virulent strains of *E. amylovora* provide an effective and consistent means for evaluating fire blight resistance (Aldwinckle and Preczewski, 1979). Therefore, the objective of this study was to determine levels of resistance to fire blight among a variety of flowering pear and quince taxa using artificial inoculations.

Materials and Methods

Pears. Twenty-seven taxa (Table 1) of containerized pears, arranged in a randomized complete block design with 4 to 12 replications, were screened for fire

blight resistance using artificial inoculations at the Mountain Horticultural Crops Research Station, Fletcher, N.C. This study included cultivars, clonal selections, and hybrids of *P. amygdaliformis* Vill., *P. betulifolia* Bunge., *P. calleryana*, *P. elaeagrifolia* Pall., *P. fauriei* Schneid., *P. koehnei* Schneid., *P. nivalis* Jacq., *P. pyrifolia* (Burm.) Nak., *P. regelii* Rehd., *P. salicifolia* Pall., and *P. ussuriensis* Maxim. All taxa were one-year budded plants on *P. calleryana* rootstocks grown in 14.2 L containers. Container substrate was 5 pine bark : 1 peat (by vol.) amended with 3 $kg \bullet m^{-3}$ micronutrients (Esmigran, The Scotts Co., Marysville, Ohio) and 5.3 $kg \bullet m^{-3}$ dolomitic limestone. Plants were placed in a lath house under 50% shade in April 2000 and fertilized with 50 g controlled-release fertilizer (Osmocote Plus, 15-9-12, The Scotts Co., Marysville, Ohio). Irrigation was applied as necessary.

The isolate of *E. amylovora* used in this study was strain (E2002a). This strain has previously been categorized and utilized to screen advanced selections of pears for resistance to fire blight (Bell, R.L., 1999, unpublished data and personal communication). The inoculum was prepared from 24 h cultures grown on nutrient agar plates at 26 °C. The cultures were rinsed with distilled water and bacterial suspension collected. The inoculum was adjusted to a concentration of $\approx 1 \times 10^7$ colony forming units (cfu)/mL. One to two actively growing shoots (subsamples) per tree were inoculated in May 2000. The two youngest leaves were bisected with a pair of scissors that had been dipped into the inoculum prior to each cut. The disease lesion length and total length of the current season's growth of the inoculated shoot were measured 40 d following inoculation. The severity of infection was expressed as

the length of the fire blight lesion as a percentage of overall shoot length. All data were subjected to analysis of variance procedures. Means for percent lesion length were separated by the Waller-Duncan test.

Chaenomeles. Twelve taxa (Table 2) of flowering quince were screened for fire blight resistance in May 2001. This study included taxa of *C. cathayensis* (Hemsl.) Schneid., *C. japonica*, *C. speciosa* (Sweet) Nak., and *C.* x *superba* (Frahm) Rehd. Plant material consisted of 1 year old container-grown rooted cuttings. Growing conditions, plant care, experimental design, inoculation procedure, and data analysis were conducted as described above. Inoculum for this study was *E. amylovora* strain E2002a (≈2 x 10^8 cfu/mL).

Results

Pears. The taxa included in this study showed considerable variation in resistance to fire blight with the severity of infection ranging from 1% to 100% of the current season's shoot growth (Table 1). Nine taxa were highly susceptible with extensive infection that did not differ significantly from 100%. These susceptible taxa included specific clones of *P. elaeagrifolia, P. fauriei, P. nivalis, P. pyrifolia,* and *P. salicifolia* as well as a number of advanced hybrid selections. At the other extreme, two taxa, *Pyrus usseriensis* 'Prairie Gem' and *Pyrus* 950104, a clone

derived from open pollination of a *Pyrus calleryana* x *Pyrus betulifolia* hybrid, were highly resistant with minimal infection that was not significantly different from 0%. Fifteen other taxa were intermediate with lesion length ranging from 16% to 81% of the annual shoot growth.

Chaenomeles. The taxa included varied significantly in resistance to *E. amylovora* strain E2002a (Table 2). Among the flowering quince taxa, *C. speciosa* 'Contorta' was highly resistant with a mean lesion length of 15%. Six of the 12 taxa included did not differ significantly from 100% and are highly susceptible. Five taxa were intermediate in susceptibility; *C. speciosa* 'Nivalis' was the least susceptible of this group while *C. speciosa* 'Mandarin' the most with lesion length of 37% and 68% respectively.

Discussion

Pears. Cultivars of *P. calleryana* differed significantly in their susceptibility to fire blight with *P. calleryana* 'Chanticleer' (syn. 'Cleveland Select') being more resistant to fire blight than *P. calleryana* 'Bradford', 'Whitehouse', 'Aristocrat', and 'Red Spire'. Our results were in general agreement with studies by Fare et al. (1991) and McNeil et al. (1986) in which 'Bradford', 'Fauriei', and 'Whitehouse' showed greater resistance than 'Aristocrat' and 'Redspire' under conditions of natural

infection. However, in this study, 'Whitehouse' was not significantly more resistant than either 'Aristocrat' or 'Red Spire'. Overall, 'Chanticleer' was the most resistant commercially available cultivar of callery pear included in this study. *Pyrus betulifolia* 'Dancer' and a clone of *P. regelii* exhibited a level of resistance similar to that of *P. calleryana* 'Chanticleer'.

The genetic diversity that exists within the genus *Pyrus* has been exploited for fruit tree breeding; however, many of these taxa also possess desirable ornamental characteristics. Some species vary in flowering time, heat and cold tolerance, leaf shape, tree form, and disease resistance. While considerable variation in resistance to fire blight exists in all species of pears, *P. ussuriensis* and *P. calleryana* have been reported to exhibit the most fire blight resistance (van der Zwet and Keil, 1979). The study reported herein included clonal selections of these species. One clone, *P. ussuriensis* 'Prairie Gem', was found to be very resistant.

The hybrid taxa included in this study were provided by the Landscape Plant Development Center, Chanhassen, Minn. The Center's ornamental pear breeding program has been successful in obtaining many new hybrid plants by utilizing a very diverse group of species as breeding parents (Hummel, 2000). Promising selections have been identified based on desirable ornamental characteristics, but no data regarding their resistance to fire blight have been recorded. One hybrid, *Pyrus* 950104, from this program was found to be very resistant to this virulent strain of *E. amylovora*. *Pyrus* 93-70-2 and 93-61-1 were significantly more resistant than 'Bradford', the industry standard for resistance.

Cultivars of *P. calleryana* are among the most widely planted flowering trees in much of the United States. Despite their popularity, these plants are often short lived in the landscape. Some cultivars are very weak wooded, have poor branch arrangement, and are susceptible to fire blight. Genetic diversity and interspecific crossability in the genus *Pyrus* has lead to the development of many new hybrid plants. The success of new hybrids is, in part, dependent on their resistance to fire blight. Results from this study document the variation in resistance that exists among many taxa of flowering pears.

Chaenomeles. There is very limited information available on resistance to fire blight among taxa of flowering quince; however, *C. japonica* has been documented as being susceptible to blossom infection (van der Zwet and Keil, 1979). Many cultivars of flowering quince are highly prized for their very early spring flowers, which are present when environmental conditions are too cool for disease development. Many susceptible plants may escape primary infection (blossom blight) due to their flowering habit. Secondary infection (twig and leaf blight) and blossom infection on sporadic blooms typically occurs later in the growing season when environmental conditions are favorable for disease development.

This study included a number of cultivars of flowering quince that are common and important to the horticultural industry. Only one cultivar, *C. speciosa* 'Contorta' was highly resistant. *Chaenomeles speciosa* 'Nivalis' and *C.* x *superba* 'Crimson and Gold' were moderately resistant (<50% lesion length). Although a

number of taxa in this study are very susceptible, they may escape infection in some years due to the erratic nature of this disease (van der Zwet and Keil, 1979).

This research utilized artificial inoculations which provides the most effective means to evaluate resistance to fire blight. Significant variation in resistance among flowering pears and quince was observed and highly resistant taxa were identified. Information from this study provides a basis for the selection and improvement of flowering pears and quince with superior disease resistance.

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Table 1. Fire blight ratings for taxa of flowering pears (*Pyrus* spp.) based on artificial inoculation.

Pyrus taxa	Severity of infection (% lesion length)
950104[y] (*P. calleryana* x *P. betulifolia*)	1[z]
P. ussuriensis 'Prairie Gem'	3
P. betulifolia 'Dancer'	16
P. regelii	22
93-70-2[y] (*P. calleryana* 'Chanticleer' x *P. elaeagrifolia*)	22
P. calleryana 'Chanticleer' (syn. 'Cleveland Select')	31
93-61-1[y] (*P. amygdaliformis* x *P. calleryana* 'Chanticleer')	32
91-42-1[y] (*P. amydaliformis* x *P. regelii*)	38
911014[y] (*P. usseriensis* x *P. regelii*)	42
93-15-1[y] (*P. elaeagrifolia* x *P. ussuriensis*)	44
P. calleryana 'Fauriei'	46
P. calleryana 'Bradford'	50
P. calleryana 'Whitehouse'	62
91-53-1[y] (*P. calleryana* 'Chantcleer' x *P. betulifolia*)	63
P. calleryana 'Aristocrat'	65
P. calleryana 'Red Spire'	69
93-17-3[y] (*P. elaeagrifolia* x *P. amygdaliformis*)	81
93-2-2y ((*P. calleryana* x *P. fauriei*) x *P. nivalis*)	87
P. fauriei 'Korean Sun'	89
P. elaeagrifolia 'Turkish Mist'	91
911010[y] (*P. ussuriensis* x *P. nivalis*)	92
93-32-4[y] (*P. salicifolia* 'Pendula' x *P. ussuriensis*)	94
P. pyrifolia	95
P. nivalis	97
P. koehnei 808	97
93-8-5[y] (*P. fauriei* x *P. salicifolia* 'Pendula')	98
P. salicifolia 'Pendula'	100
$LSD_{0.05}$	15

[y]Interspecific hybrid taxa

[z]Percentage of total shoot length diseased.

Table 2. Fire blight ratings for taxa of flowering quince (*Chaenomeles* spp.) based on artificial inoculation.

Chaenomeles taxa	Severity of infection (% lesion length)
C. speciosa 'Contorta'	15[y]
C. speciosa 'Nivalis'	37
C . x *superba* 'Crimson and Gold'	40
C. speciosa 'Dragon's Blood'	56
C. cathayensis	62
C. speciosa 'Mandarin'	68
C. speciosa 'Spitfire'	88
C. aponica 'Sargentii'	89
C . x *superba* 'Cameo'	91
C . x *superba* 'Texas Scarlet'	95
C. speciosa 'Toyo-Nishiki'	96
C . x *superba* 'Jet Trail'	98
$LSD_{0.05}$	22

[y]Percentage of total shoot length diseased.

Chapter 2

Variation in Endogenous Phenolic Compounds and Host Plant Resistance to Fire Blight Among *Malus* Taxa

(In the format appropriate for submission to
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Variation in Endogenous Phenolic Compounds and Host Plant Resistance to Fire Blight Among *Malus* Taxa

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Variation in Endogenous Phenolic Compounds and Host Plant Resistance to Fire Blight Among *Malus* Taxa

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Abstract. Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is one of the most destructive diseases of flowering crabapples (*Malus* Mill. spp.). Resistance to fire blight among a diverse collection of 49 taxa of flowering crabapples was evaluated based on natural infection over 3 years and artificial inoculations using three virulent strains of *E. amylovora.* Considerable variation in resistance was observed to both natural infection and artificial inoculations. *Malus sieboldii* 'Calocarpa', 'David', and 'Adirondack' showed no symptoms of natural infection and were the most resistance taxa to artificial inoculations. *Malus* 'Sinai Fire', 'Schmidtcutleaf' and *M. tschonoskii* were very susceptible to natural infection and artificial inoculations. Use of artificial inoculations provided a more consistent and rigorous approach for evaluating resistance than natural infections. Bioassays were conducted to evaluate the effects of phenolic compounds found in *Malus* spp. on the growth of *E. amylovora* and to elucidate their possible role in disease resistance. A number of phenolic compounds found in *Malus* spp., including phloridzin and phloroglucinol, were found to be inhibitors of *E. amylovora* in vitro, but did not

appear to confer resistance in vivo. Three unidentified phytochemical components were significantly correlated with resistance and warrant further study.

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is one of the most important diseases of rosaceous plants, particularly those members in the subfamily Maloideae. Susceptible plants, including taxa of flowering crabapples (*Malus* Mill. spp.), can be severely damaged or killed by fire blight in both nursery and landscape plantings. This disease can be especially problematic in regions where environmental conditions are favorable for disease development. Although many taxa are susceptible to fire blight, flowering crabapples vary considerably in resistance to this disease thus providing opportunities for selection and breeding of superior plants (Benson et al., 1991; Windham et al., 1997.)

Screening for resistance to fire blight among flowering crabapples has been limited and has been based primarily on observations of natural infection (Benson et al., 1991; Green, 1986; Nichols, 1983, 1986; Windham et al., 1997). Because the incidence of fire blight can be sporadic and vary from region-to-region and year-to-year, observations and results from field surveys can be inconsistent and highly variable. Plants that appear resistant initially may later be found to be susceptible when conditions are favorable for disease development. Artificial inoculations of actively growing shoots with a virulent strain of *E. amylovora* can provide an effective and consistent means for evaluating fire blight resistance (Aldwinckle and Preczewski, 1979; Bonn and Elfving, 1990).

In some instances, host plant resistance has been attributed to the action of secondary plant metabolites. The role of specific secondary compounds in resistance among pears (*Pyrus* L. spp) to fire blight has been investigated (Hildebrand, 1969;

Hildebrand and Schroth, 1964a). Arbutin, a phenolic glucoside, and its hydrolyzed product, hydroquinone, (the arbutin-hydroquinone complex) have been the focus of many studies that investigated the nature of resistance to fire blight among pears (Hildebrand, 1969; Hildebrand, 1970; Hildebrand & Schroth, 1964a,b; Powell & Hildebrand 1964). Among selected taxa, fire blight susceptible taxa of pears contained less arbutin compared to moderately resistant and resistant taxa (Hildebrand, 1969). Challice and Westwood (1972), however, concluded that there was no direct connection between phenolic constituents of pears and disease resistance. In a more recent study, Ryugo et al. (1990) reported that taxa of pears with higher concentrations of total phenolic compounds in their fruit showed greater resistance to fire blight.

Limited studies have investigated the role of phenolic compounds in resistance to fire blight among *Malus* spp. Plurad (1967) determined that phloridzin and phloretin exhibited in vitro antibacterial activity towards *E. amylovora* similar to that of arbutin in pears. Roemmelt et al. (1999) investigated the systemic movement of *E. amylovora* in apple. In inoculated plants, phenolic compounds accumulated in host tissue and appeared to inhibit the migration of bacteria. Furthermore, levels of phenolic acids were up to ten times higher in resistant taxa than in those taxa that exhibited pronounced symptoms.

Although a correlation between total phenolic concentration and resistance to fire blight may exist, total phenolic levels may not be as important as high levels of one or a few specific compound(s) that exhibit activity towards *E. amylovora*. The

objectives of this study were to 1) evaluate resistance to fire blight among selected taxa of flowering crabapples under natural conditions and by using artificial inoculations with virulent strains of *E. amylovora*, 2) test specific phenolic compounds found in *Malus* spp. and evaluate their effects on the growth of *E. amylovora*, and 3) measure constitutive levels of individual phenolic constituents among selected taxa of flowering crabapples representing a range of susceptibility to fire blight.

Material and Methods

Expt. 1 (Resistance Screening).

Forty-nine *Malus* taxa (Table 1), in a field plot arranged in a randomized complete block experimental design with three replications, were screened for resistance to fire blight at the Mountain Horticultural Crops Research Station, Fletcher, N.C. Trees were planted between March 1990 and March 1991.

The strains of *E. amylovora* used in artificial inoculations were provided by H. Aldwinckle and J. Norelli. All three strains used had been categorized previously as highly virulent among *Malus* taxa. The bacteria were maintained in 25% glycerol at –80 °C. Cultures were streaked onto nutrient agar dishes and dishes were incubated at 26 °C for 3 d prior to the inoculations. Culture dishes were washed with sterile distilled water to dislodge the bacteria from the agar surface, and the volume of the

resulting suspension was adjusted to 500 mL with distilled water. Dilution plating was used to determine the concentration of the inoculum.

Artificial Inoculations. On 20 May 1999, four arbitrarily selected shoots (subsamples) on separate major branches were inoculated on each tree with *E. amylovora* strain Ea273 at a concentration of ≈ 4.7×10^8 colony forming units (cfu)/mL. Inoculations were conducted by bisecting the two youngest leaves on actively growing shoots with scissors that had been dipped into the inoculum prior to each cut. Disease assessments were made 40 d after the initial inoculations. Lesion length and total length of the current season's growth were measured. The severity of infection was calculated as the length of the lesion as a percentage of overall shoot length.

Additional inoculations were conducted on 11 May 2000 using the same technique and field plot described above. However, at this time, three strains of *E. amylovora* (Ea273, E2002a, and E4001a) were used for the inoculum. There were three replications with four subsamples of each strain on each tree in a split plot treatment design. The inoculum concentration of each strain was ≈ 7.3×10^7 cfu/mL.

Natural Infection. Trees in the same field plot as described above were also evaluated based on naturally occurring fire blight infection. Disease severity was rated during Summers 994, 1995, and 1999 using the following system of Green (1986): 0= no evidence of fire blight, 1=few (1-3) branch tips infected, 2=numerous (>3) branch tips infected and a few (1-3) major branches infected, 3=several (2-3) major branches infected with considerable dieback, 4=major (>30%) portion of the

tree with dieback. Measurements of disease severity from the artificial inoculations and natural infections were subject to analysis of variance procedures and means separated by the Waller-Duncan test.

Expt. 2 (Bioassays).

Bioassays were conducted to determine the effects of selected phenolic compounds found in *Malus* taxa on the in vitro growth of *E. amylovora.* Strain E2002a was grown on nutrient agar for 24 h at 26 °C. Under aseptic conditions, 50 µL of a bacterial suspension (≈ 1 x 10^8 cfu/mL) was added to a 1.5 mL cuvette containing 1 mL of nutrient broth amended with 0, 1, 2.5, 5, 10, or 20 mM of a test compound. Cuvettes were arranged in a completely randomized design with six replications, cuvettes without *E. amylovora* served as additional controls. All cuvettes were capped and placed on a shaker and maintained at 26 °C. Absorbance measurements were taken initially and after at 8 h. Preliminary measurements indicated that bacterial growth after 8 h was still in a rapid linear phase and had not yet begun to plateau. The phenolic compounds tested were catechin, chlorogenic acid, p-coumaric acid, phloridzin, phloretin, phloretic acid, and phloroglucinol (Sigma-Aldrich, Co., St. Louis, Mo.).

Phloridzin and phloretin are highly insoluble in water and therefore the above protocol was modified according to MacDonald and Bishop (1952). All concentrations of both phloridzin and phloretin were dissolved in 1 mL of propylene glycol then adjusted to 20 mL with nutrient broth. The 5% propylene glycol nutrient

broth solution was then used as described above. We were unable to satisfactorily solulabilize rutin, therefore it was not tested. All data represented bacterial growth and were subject to analysis of variance procedures and means separated by the Waller-Duncan.

Expt. 3 (Endogenous phenolics analysis).

In Spring 2001, 10 taxa of flowering crabapples were selected to measure constitutive levels of phenolic compounds in leaf and stem tissue and to test for correlations with resistance. The taxa included in this study were subjected to artificial inoculations and corresponding, but separate, shoots were collected for chemical analysis. The taxa were selected to represent the wide of resistance that was documented from the 1999 and 2000 studies (Expt. 1). The same field plot described in Expt. 1 was used for this study. All data were subject to analysis of variance procedures and correlations were determined using stepwise, multiple regression analysis.

Artificial Inoculations. On 15 May 2001, four random actively growing shoots (subsamples) on three trees of each taxa were inoculated with strain E2002a at a concentration $\approx 1 \times 10^8$ cfu/mL. Inoculations, disease assessment, and data analysis were conducted as described in Expt. 1.

Chemical Analysis. On 16 May 2001, four actively growing shoots were collected from each tree, leaf and stem tissue were separated, and immediately frozen at –80 °C. Frozen tissue was lyophilized and ground to pass a 40-mesh screen. For each sample 200 mg of ground leaf or stem tissue for all taxa was placed in a 25 mL centrifuge tube and suspended in 4 mL of 85% methanol and sonicated for 2 h. The slurry was then centrifuged at 1000 g_n for 10 min. The supernatant was decanted and collected. The pellet was resuspended in 4 mL of 15% methanol, sonicated for 2 h then centrifuged for 10 min. The supernatant was decanted and combined with the first extraction. The extraction was adjusted to a total volume of 10 mL with 50% aqueous methanol prior to injection. Endogenous levels of individual phenolic compounds were analyzed by a reverse-phase high performance liquid chromatography (HPLC) method adapted from Fulcher et al. (1998). An isocratic solvent system of 40 methanol : 60 water with 0.2% o-phosphoric acid was used. Phenolic compounds were separated on an 5 μm Adsorbosphere C-18, 250 x 4.6 mm column (Alltech Associates Inc., Deerfield, Ill.) with a flow rate of 0.8 $mL \bullet min^{-1}$. Compounds were detected at 254 nm with an ultraviolet (UV) detector (Millipore Corp., Bedford, Mass.) and compared with commercial standards (Sigma-Aldrich Co., St. Louis, Mo.) using an external standard method. Samples of *Malus* 'Canary' and 'Centurion' extracts were analyzed by the North Carolina State University Department of Chemistry Mass Spectrometry Facility for confirmation of identity of phenolic components using liquid chromatography-mass spectrometry (LC-MS).

Concentrations of unknown components were calculated using the phloridzin standard.

Results

Expt. 1 (Resistance screening).

Artificial inoculations. Disease ratings varied considerably among taxa and ranged from 0% to 100% of total shoot length (Table 1). In 1999, *M.* 'Sinai Fire', 'Schmidtcutleaf' and *M. tschonoskii* were most susceptible and differed significantly from all other taxa. In 2000, these same taxa were highly susceptible to all three strains. Lesions extended into prior year's growth on some of the replicates of *M.* 'Sinai Fire', 'Schmidtcutleaf', 'Silver Moon', and *M. tschonoskii*. *Malus sieboldii* 'Calocarpa', and *M.* 'Adams', 'Adirondack', and 'David' were among the most resistant taxa over 2 years to strain Ea273 and to strains E2002a and E4001a in 2000.

Natural infection. The severity of fire blight infection was not high during any of the 3 years of the study. A total of 9, 14, and 22 taxa showed some infection for 1994, 1995, and 1999, respectively (Table 2). The degree of fire blight infection varied over 3 years and there were many instances where certain cultivars had no disease in some years, but severe infections in others. For example, *M. tschonoskii* and *M.* 'Brandywine' received a zero rating for both 1994 and 1995 but had mean disease ratings >2 for 1999. Because fire blight was not severe, differences among taxa were not great. However, *M.* 'Schmidtcutleaf', 'Silver Moon', and 'Sinai Fire' were generally more susceptible than the other taxa.

Expt. 2 (Bioassays).

All of the seven compounds tested inhibited *E. amylovora* to some extent (Fig. 1A-G). There was no significant reduction in growth for 1, 2.5, 5, 10 mM concentrations of catechin which behaved similar to the nontreated control (Fig 1A). The 20 mM concentration of catechin reduced bacterial growth by 90% compared to the control. The growth of *E. amylovora* was inhibited significantly by concentrations of chlorogenic acid compared to the control; however, no concentration inhibited growth by more than 50% (Fig. 1B). The 20 mM concentration reduced growth by 38%. All concentrations of *p*-coumaric acid were very inhibitory to bacterial growth (Fig. 1C). The 1 mM concentration reduced growth by 67%. The 2.5 and 5 mM concentrations were not significantly different from the 1 mM concentration. Bacterial growth was reduced by 98% with the 10 and 20 mM concentrations. Phloretic acid (Fig. 1D) and phloroglucinol (Fig. 1E) showed similar trends where reduction in growth >50% was achieved with the 10 mM concentration. Both compounds were very toxic to the bacterium at a concentration of 20 mM and inhibited growth by 99 and 95%, respectively. Phloridzin did not inhibit bacterial growth at the lowest concentration of 1 mM (Fig. 1F). Inhibition of growth at higher concentrations, 10 and 20 mM, was not significantly different but both were significantly more effective compared to all lower concentrations, reducing growth by 69% and 76%, respectively. Bacterial growth was reduced by 24% and 34% with the 2.5 and 5 mM concentrations, respectively. Phloretin exhibited significant antibacterial properties towards *E. amylovora* at all concentrations up to

10 mM (Fig. 1G). The lowest concentration, 1mM, reduced bacterial growth by 98% compared to the nontreated control.

Expt. 3 (Endogenous phenolics analysis).

Artificial inoculations. The taxa included in this experiment showed considerable variation in resistance to strain E2002a with the severity of infection ranging from 8 to 100% of the current season's shoot growth. *Malus* 'Adams' was most resistant with a lesion length of 8% while *M. tschonoskii* was the most susceptible with a lesion length of 100% of the shoot growth (Tables 3 and 4).

Endogenous levels of phenolics. Phloroglucinol, phloridzin, and rutin were found in both leaf (Table 3) and stem tissue (Table 4), while chlorogenic acid was found only in the stem tissue. The identification some of the phenolic constituents was not possible. An unidentified constituent, component X, was present in both leaf and stem tissue while unidentified components L and S were present in leaf and stem tissue, respectively (Tables 3 and 4). Preliminary analysis of samples using LC-MS suggested that component X is possibly an isomere of phloridzin. This assumption is based on fragmentation similarity of this component compared to that of phloridzin using LC-MS. The identification of components L and S was inconclusive.

The LC-MS analysis indicated the presence of an additional component that was not resolved in the HPLC analysis. This compound (molecular weight = 452 $g \bullet mol^{-1}$) co-eluted with phloridzin during the HPLC analysis. Thus, in this paper

concentrations of phloridzin are reported as the phloridzin complex to account for the presence of this additional component. In general, phloroglucinol and the phloridzin complex were found in high concentrations in both leaf and stem tissue. Chlorogenic acid was present in low concentrations in stem tissue; however, its presence in leaf tissue could not be confirmed. The isocratic HPLC solvent system utilized in this study did not provide clear resolution for determining the presence or concentrations of phloretic acid, catechin, *p*-coumaric acid, or phloretin and therefore they were not determined.

Although, phloroglucinol and the phloridzin complex were present in high concentrations in certain taxa, no correlation with resistance was found (Tables 3 and 4). Component X in stem tissue was correlated with disease resistance (Lesion Length (%) = 96.831 - 0.896X, r^2 = 0.7126, where X= concentration of component X in stem tissue). In leaf tissue, component X and component L were both correlated with disease resistance and their interaction was significant (Lesion Length (%) = 156.75 - 0.97X - 1.398L + 0.012XL, r^2 = 0.6122, where X= concentration of component X and L = concentration of component L in stem tissue).

Discussion

There was considerable agreement between the results from natural infection and controlled inoculations for some taxa. For example, *Malus* 'David',

'Adirondack', and *M. sieboldii* 'Calocarpa' showed no symptoms from natural infection during the 3 years of evaluation. This is consistent with the 0% lesion length observed following the artificial inoculations in 1999. *Malus* 'Schmidtcutleaf', 'Sinai Fire', and *M. tschonoskii* all showed symptoms of natural infection, particularly in 1999 when all three received a rating of at least 2, also consistent with high disease ratings from artificial inoculations.

There were other cases, however, where plants appeared to be resistant under natural conditions, but were susceptible when artificially inoculated. For example, *M.* 'Baskatong' and 'White Angel' showed no symptoms of natural infection for all 3 years, yet had mean lesion lengths ranging from 32% to 95% and 41% to 90%, respectively, depending on the year and the pathogen strain. Windham et al. (1997), reported *M.* 'White Angel' as "resistant" and *M.* 'Sinai Fire' as "moderately resistant" under natural conditions. In this study, *M.* 'White Angel' was susceptible to artificial inoculations while *M.* 'Sinai Fire' was highly susceptible under natural conditions and to artificial inoculations. Those cultivars reported as highly resistant in this study (i.e., 0% lesion length and rating = 0) were also reported as "resistant" by Windham et al. (1997). Differences in the virulence of the strain of *E. amylovora*, inoculum levels and variation in the bloom period and the weather conditions during bloom within and between years probably account for differences observed in this study and among studies at various other locations.

The consistent disease ratings for inoculations using the same strain (Ea273) over 2 years document the reliability of these results and the inoculation technique.

In general, there appeared to be an increase in susceptibility for most taxa in the 2000 study. This response might be attributed to tree vigor as influenced by fertility and environmental conditions. *Malus* 'David' was the most resistant taxa included in this study and was not affected by fire blight in either year tested. *Malus* 'Sinai Fire', 'Schmidtcutleaf', and *M. tschonoskii*, proved to be the most susceptible taxa in both years to all strains of *E. amylovora*. *Malus* 'Adirondack' and 'Adams' did not differ significantly from 0% infection to strain E2002a or strain Ea273 in both 1999 and 2000 studies.

Results from the bioassays documented that a number of phenolic compounds found in *Malus* spp. are potent in vitro inhibitors of *E. amylovora*. The antibacterial properties of phloridzin and phloretin reported herein confirm previous reports (Plurad, 1967). Given the activity of these compounds in vitro, the poor relationship between endogenous levels of certain phenolic constituents and disease resistance was unexpected. Although we were unable to quantify endogenous levels of all of the compounds tested in the bioassays, endogenous concentrations of phloroglucinol and phloridzin in whole leaf and stem tissue greatly exceeded levels that were very inhibitory when present in bioassays. Further catabolism of phloridzin to phloretin would result in even stronger inhibition. Failure of these compounds to confer in vivo resistance to *E. amylovora* suggests that mechanisms may exist to maintain separation between the bacteria and these compounds. Bacterial cells travel through intercellular spaces and may not initially come in contact with phenolic compounds that are present in the symplast. Noveroske et al. (1964) reported that phloridzin

occurred at levels far greater than its solubility in water; however, no crystals were apparent in plant cells. Absorption of these compounds into cell walls and membranes could also isolate them from bacterial cells until cells are ruptured.

The study of the relationship between phenolic compounds in *Malus* spp. and resistance to *E. amylovora* is difficult due to the diversity of phenolic compounds present in this genus and the structural similarity of many of these phenolics. Phloridzin is the main phenolic compound found in most *Malus* spp. (Williams, 1966). Phloretin, phloretic acid, and phloroglucinol are all enzymatic degradation products of phloridzin (Grochowska and Ciurzynska, 1979). However, phloridzin is partially or wholly replaced by a structurally similar glycoside, sieboldin, in some species including *M. floribunda* Siebold, *M. sieboldii* (Asami) Fiala, *M. toringo* Siebold ex De Vriese, and *M. sargentii* Redher (Williams, 1961; Fiala, 1994). In *M. trilobata*, phloridzin is completely replaced by the glycoside, trilobatin (Williams, 1961). Many cultivars varieties of *Malus* taxa are interspecific hybrids or chance seedlings of unknown parentage. Hybrid offspring from the same parent plant can have significant variation in concentrations of a specific phenolic compound. For example, Williams (1966) tested 256 open-pollinated seedlings from a single plant of *M*. x *zumi* Redher [syn. *M. sieboldii* (Asamia) Fiala.]. Phloridzin was present in the leaves of 44% of the seedlings while 56% contained phloridzin and sieboldin. In those seedlings that contained sieboldin, 20% had higher levels of sieboldin than phloridzin. Based on this report, sieboldin may be more widely distributed in present-day cultivars of *Malus* despite being restricted to only four species. The

component we initially identified by HPLC retention time and comparisons with an authentic standard as phloridzin was later determined to possess two components in samples of *M*. 'Canary' and 'Centurion' when analyzed using LC-MS. Based on preliminary LC-MS analysis, it is possible that this co-eluting component is sieboldin (molecular weight = 452 $g \bullet mol^{-1}$). Structurally, sieboldin is very similar to phloridzin and this would explain their co-elution as a single peak in the HPLC analysis. We were unable to resolve these two components in this study. Based on these analyses, the component in question is designated as the "phlordizin complex". This component may include only phloridzin or both phloridzin and sieboldin as in the case of 'Canary' and 'Centurion'.

In summary, flowering crabapples are one of the most economically important flowering trees produced in the United States; yet fire blight can be a serious disease on many of these taxa. Evaluation of fire blight resistance utilizing artificial inoculations demonstrated wide variation in resistance and identified numerous cultivars with resistance to multiple strains of *E. amylovora.* A number of phenolic compounds found in *Malus* spp., including phloridzin and phloroglucinol, were found to be inhibitors of *E. amylovora* in vitro, but did not appear to confer resistance in vivo. Three unidentified phytochemical components were significantly correlated with resistance and warrant further study. A greater understanding of host resistance can help facilitate evaluation, selection, and improvement of cultivars with superior disease resistance.

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Table 1. Fire blight ratings for taxa of flowering crabapples *(Malus* spp.) based on artificial inoculations with selected strains over 2 years.

Year:	1999	2000		
Strain:	Ea273	Ea273	E2002a	E4001a
Malus taxa				
'Adams'	1[z]	1	12	16
'Adirondack'	0	2	9	36
M. baccata 'Jackii'	4	14	46	56
'Baskatong'	32	75	95	90
Branzam' (Brandywine™)	39	51	78	63
'Callaway'	12	22	35	35
Camzam' (Camelot™)	7	16	73	69
'Canary'	17	33	33	38
'Candy Mint'	13	60	85	86
'Centurion'	4	10	24	17
'David'	0	1	1	6
'Dolgo'	10	27	51	35
'Donald Wyman'	61	60	93	97
'Doubloons'	28	35	57	64
M. floribunda	9	16	18	46
'Glen Mills'	14	19	94	93
Hargozam' (Harvest Gold™)	18	21	81	83
M. hupehensis	55	56	98	99
'Indian Summer'	8	1	22	27
'Jewelberry'	11	22	55	78
'Liset'	11	10	76	78
'Louisa'	19	10	23	27
'Mary Potter'	40	40	100	96
Mazam' (Madonna™)	50	66	100	97
'Molten Lava'	6	19	53	56
'Narragansett'	10	15	52	65
'Ormiston Roy'	7	4	47	56
'Pink Princess'	2	15	44	47
'Pink Satin'	6	17	22	69

Table 1 (continued).

'Prairie Maid'	22	91	100	88
'Prairifire'	7	43	93	79
'Professor Sprenger'	33	12	38	85
'Purple Prince'	11	52	91	98
'Radiant'	6	13	10	19
'Red Splendor'	23	19	63	54
'Robinson'	9	9	27	26
M. sargentii	10	48	83	59
Schmidtcutleaf' (Golden Raindrops™)	91*	100*	100*	100*
'Sentinel'	1	4	86	79
sieboldii 'Calocarpa'	0	1	24	28
'Silver Drift'	14	0	79	100
'Silver Moon'	61*	84*	99*	100*
'Sinai Fire'	100*	94*	100*	100*
'Snow Drift'	14	75	100	100
'Strawberry Parfait'	11	21	48	74
Sutyzam' (Sugar Tyme™)	3	1	41	35
M. tschonoskii	100*	100*	100*	100*
'White Angel'	41	44	90	70
'White Cloud'	4	NA	NA	NA
$LSD_{0.05}$	26	18	24	15

[z]Percentage of total shoot length infected.

[*]Lesion extended into prior years growth on some branches.

Table 2. Fire blight ratings for taxa of flowering crabapples (*Malus* spp.) based on natural infection.

	Year		
Malus taxa	1994	1995	1999
'Adams'	0.0[z]	0.0	0.0
'Adirondack'	0.0	0.0	0.0
baccata 'Jackii'	0.0	0.0	0.0
'Baskatong'	0.0	0.0	0.0
Branzam' (Brandywine™)	0.0	0.0	2.0
'Callaway'	0.0	0.0	0.0
Camzam' (Camelot™)	NA	0.0	0.0
'Canary'	0.0	0.0	0.0
'Candy Mint'	0.0	0.0	0.0
'Centurion'	0.0	0.0	1.0
'David'	0.0	0.0	0.0
'Dolgo'	0.0	0.0	0.3
'Donald Wyman'	0.0	0.3	0.5
'Doubloons'	0.3	0.7	0.7
M. floribunda	0.3	1.0	1.0
'Glen Mills'	0.0	0.0	0.3
Hargozam' (Harvest Gold™)	0.3	0.7	1.3
M. hupehensis	0.0	0.8	2.7
'Indian Summer'	0.0	0.0	0.0
Jewelberry'	0.0	0.0	0.0
'Liset'	0.0	0.0	0.7
'Louisa'	0.0	0.0	0.2
'Mary Potter'	0.0	1.0	1.3
Mazam' (Madonna™)	0.0	1.7	0.3
'Molten Lava'	0.0	0.0	0.0
'Narragansett'	0.0	0.0	0.0
'Ormiston Roy'	0.0	0.7	1.7
'Pink Princess'	0.0	0.0	0.0
'Pink Satin'	0.0	0.0	0.0

Table 2 (continued).

'Prairie Maid'	0.0	0.0	0.0
'Prairifire'	0.0	0.0	0.0
'Professor Sprenger'	0.0	0.3	0.3
'Purple Prince'	0.0	0.0	0.0
'Radiant'	0.0	0.0	0.7
'Red Splendor'	0.0	0.0	0.0
'Robinson'	0.0	0.0	0.0
M. sargentii	0.0	0.0	0.0
Schmidtcutleaf' (Golden Raindrops™)	1.0	2.0	2.3
'Sentinel'	0.3	0.7	0.0
M. sieboldii 'Calocarpa'	0.0	0.0	0.0
'Silver Drift'	0.0	0.0	0.7
'Silver Moon'	1.3	2.0	2.7
'Sinai Fire'	1.0	0.7	2.0
'Snow Drift'	0.0	0.3	0.3
'Strawberry Parfait'	0.3	0.0	0.0
Sutyzam' (Sugar Tyme™)	0.2	0.0	0.0
M. tschonoskii	0.0	0.0	2.5
'White Angel'	0.0	0.0	0.0
'White Cloud'	0.0	0.0	0.0
$LSD_{0.05}$	0.5	0.4	0.8

[z]Natural infection ratings: 0= no evidence of fire blight, 1=few (1-3) branch tips infected, 2=numerous (>3) branch tips infected and a few (1-3) major branches infected, 3=several (2-3) major branches infected with considerable dieback, 4=major (>30%) po

Table 3. Endogenous levels of individual phenolic compounds in leaf tissue among 10 crabapples (*Malus* spp.)

Taxa	Phenolic constituents (mmol•kg^{-1} dry wt)					Lesion length (%)
	Phloroglucinol	Phloridzin complex	Rutin	Component X	Component L	
M. 'Adams'	193.65	293.41	10.01	139.88	55.78	7.99[Z]
M. 'Centurion'	217.19	275.56	10.06	103.71	106.59	14.79
M. 'David'	150.14	343.22	7.56	98.78	92.79	24.61
M. floribunda	155.04	365.59	7.34	85.01	108.11	27.16
M. 'Canary'	248.16	232.50	12.93	173.45	67.64	39.06
M. 'Red Splendor	275.58	169.68	11.54	78.85	88.74	45.00
M. 'Harvest Gold'	166.84	291.11	8.31	69.52	62.69	45.11
M. 'Mary Potter'	214.50	333.03	5.48	61.22	63.68	77.02
M. 'Candy Mint'	250.09	442.24	8.24	16.52	44.99	78.65
M. tschonoskii	245.57	193.37	7.04	42.42	37.06	100.00
$LSD_{0.05}$	190.77	72.99	4.33	31.56	27.71	28.37
Correlation[Y]	NS	NS	NS	**	**	

[Z]Percentage of total shoot length diseased.

[Y]Correlation coefficient between selected compounds and percentage lesion length.

NS, *, ** Nonsignificant or significant at $P<0.05$ or 0.01, respectively.

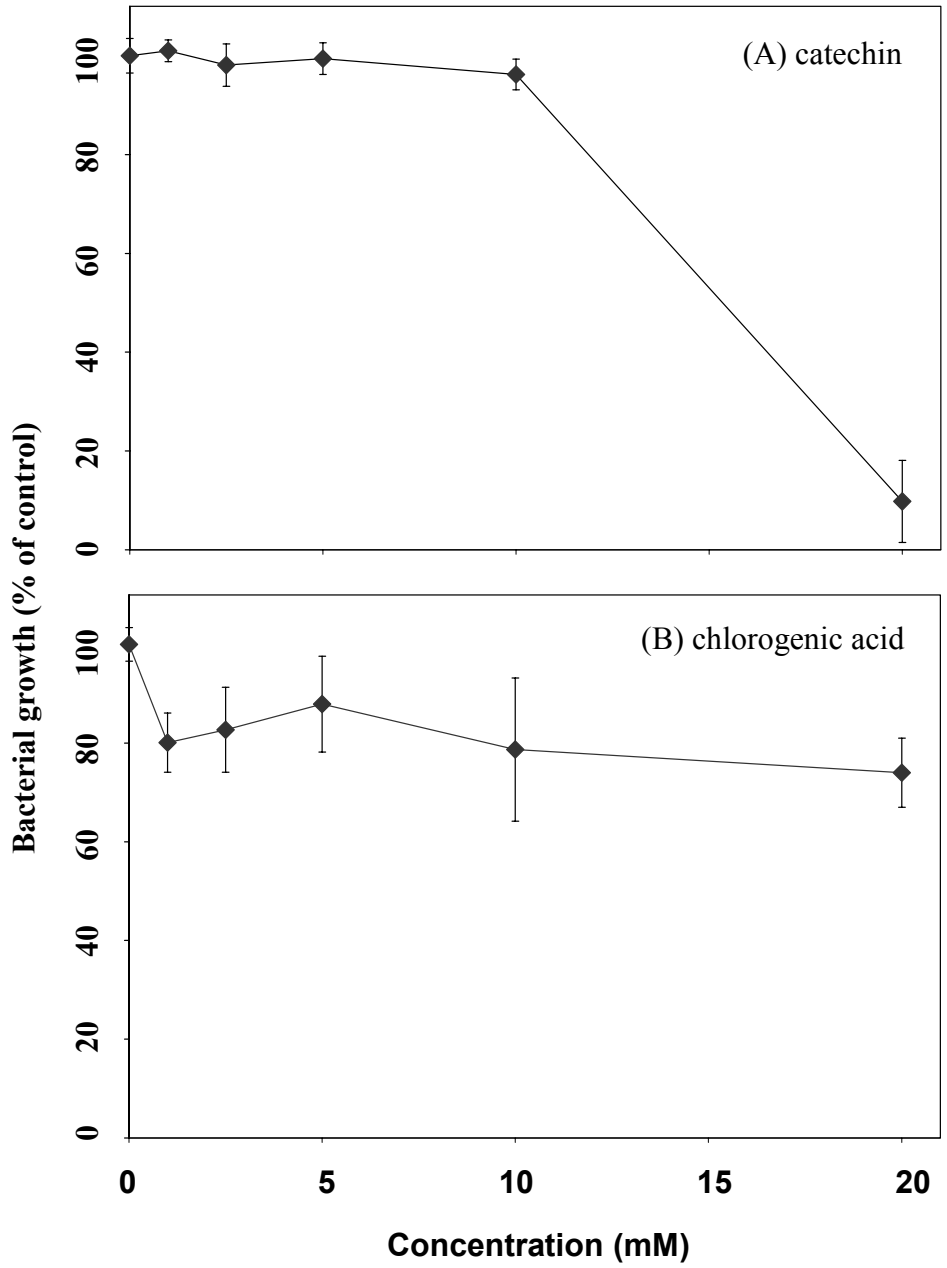
Table 4. Endogenous levels of individual phenolic compounds in stem tissue among 10 crabapples (*Malus* spp.)

Taxa	Phenolic constituents (mmol•kg^{-1} dry wt)						Lesion length (%)
	Phloroglucinol	Chlorogenic Acid	Phloridzin complex	Rutin	Component X	Component S	
M. 'Adams'	188.96	5.09	172.00	3.39	89.50	49.80	7.99[Z]
M. 'Centurion'	193.33	3.64	168.97	1.92	45.84	37.15	14.79
M. 'David'	140.61	1.57	218.25	1.04	59.44	38.93	24.61
M. floribunda	124.68	NA	281.63	1.68	58.81	29.07	27.16
M. 'Canary'	213.67	2.59	138.31	2.28	74.44	28.38	39.06
M. 'Red Splendor'	134.33	6.85	140.06	3.33	62.12	47.32	45.00
M. 'Harvest Gold'	154.26	NA	197.99	0.56	37.43	13.21	45.11
M. 'Mary Potter'	159.48	3.10	199.03	2.43	25.38	41.18	77.02
M. 'Candy Mint'	208.98	5.82	250.54	5.06	9.14	31.92	78.65
M. tschonoskii	167.34	9.58	202.84	1.77	6.76	52.33	100.00
$LSD_{0.05}$	71.68	2.68	66.28	1.85	16.56	21.35	28.37
Correlation[Y]	NS	NS	NS	NS	**	NS	

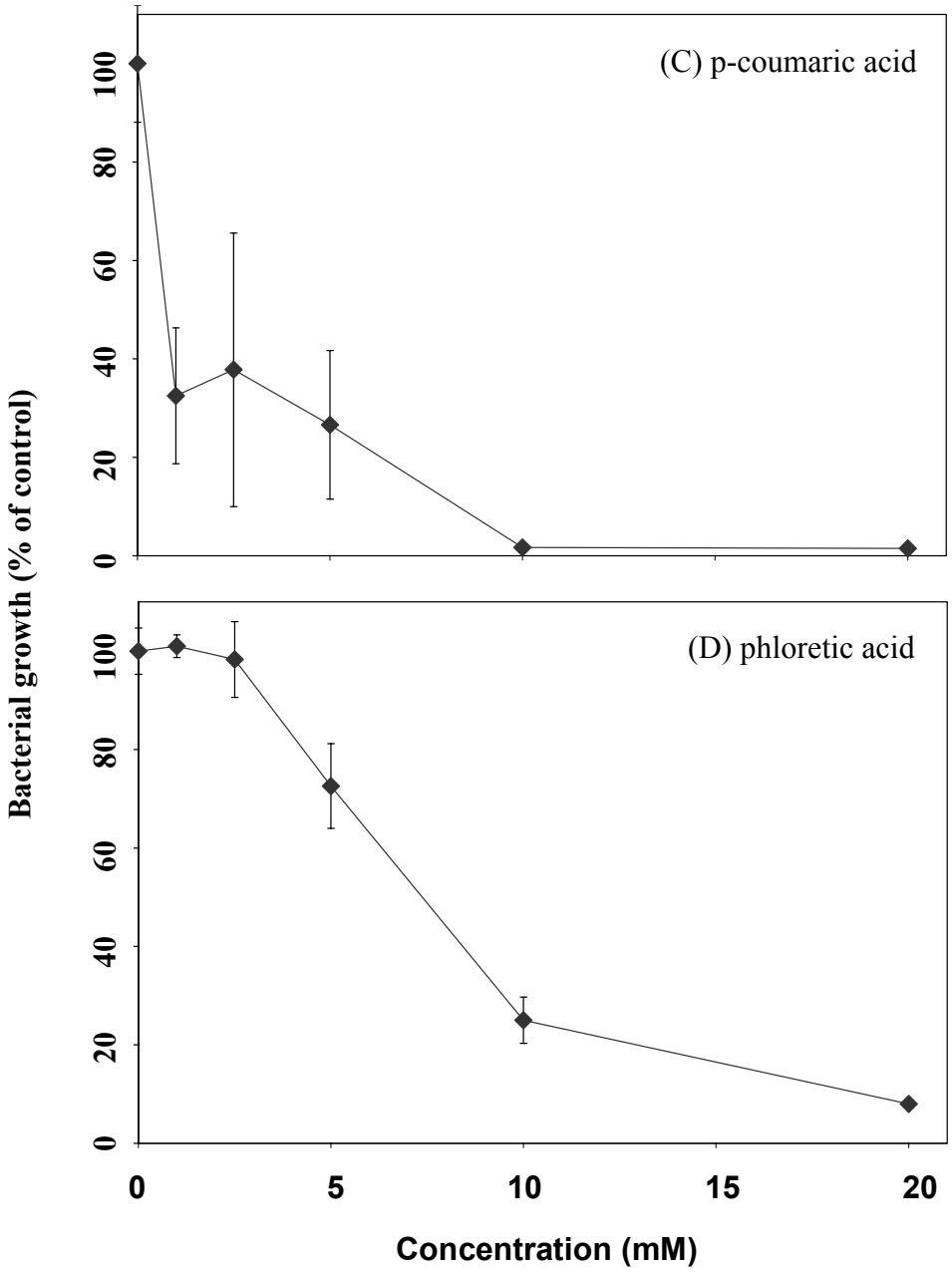
[Z]Percentage total shoot length diseased, NA=Data not available.

[Y]Correlation coefficient between selected compounds and percent lesion length.

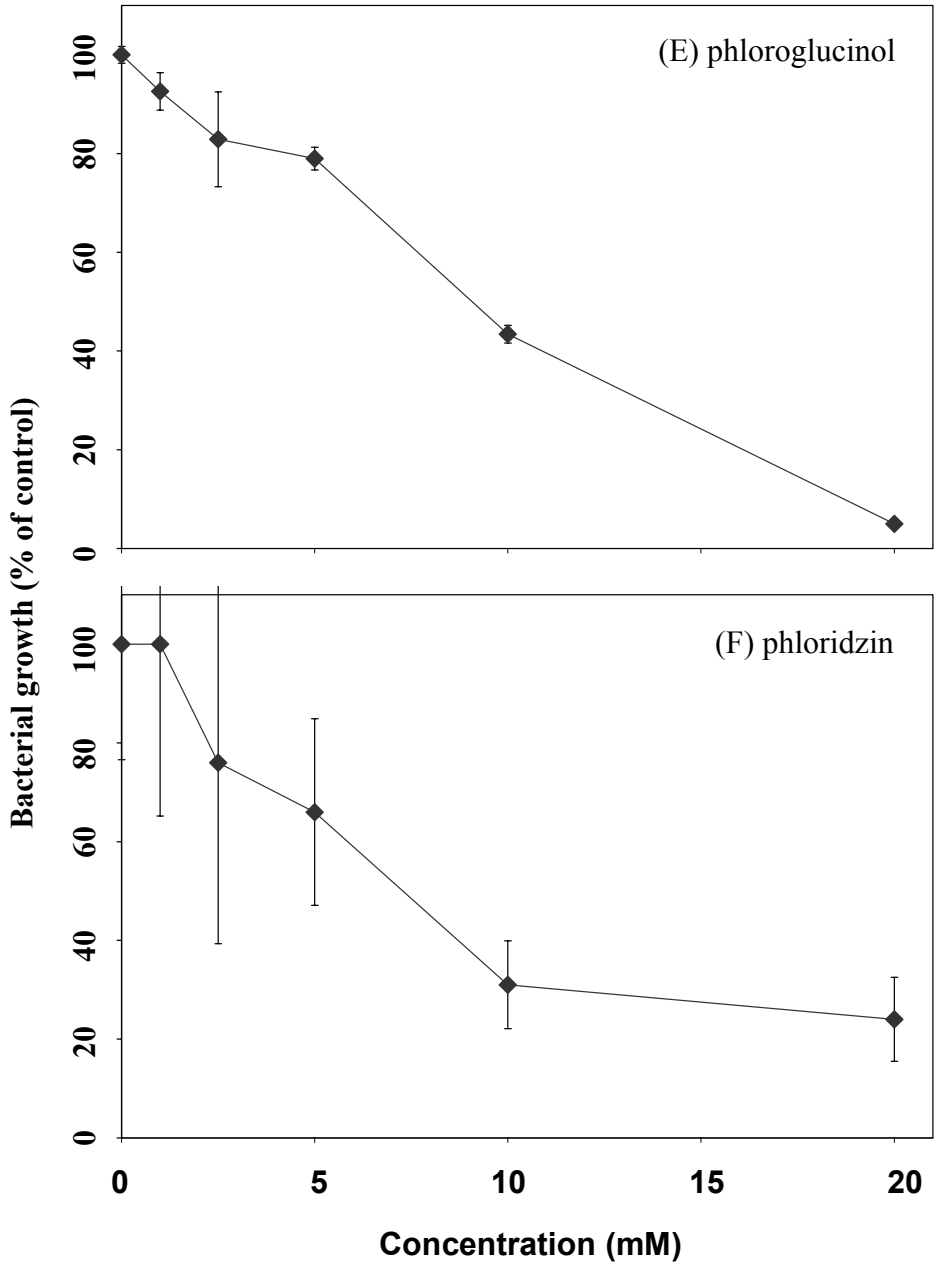
NS, *, ** Nonsignficant or significant at $P < 0.05$ and 0.01, respectively.



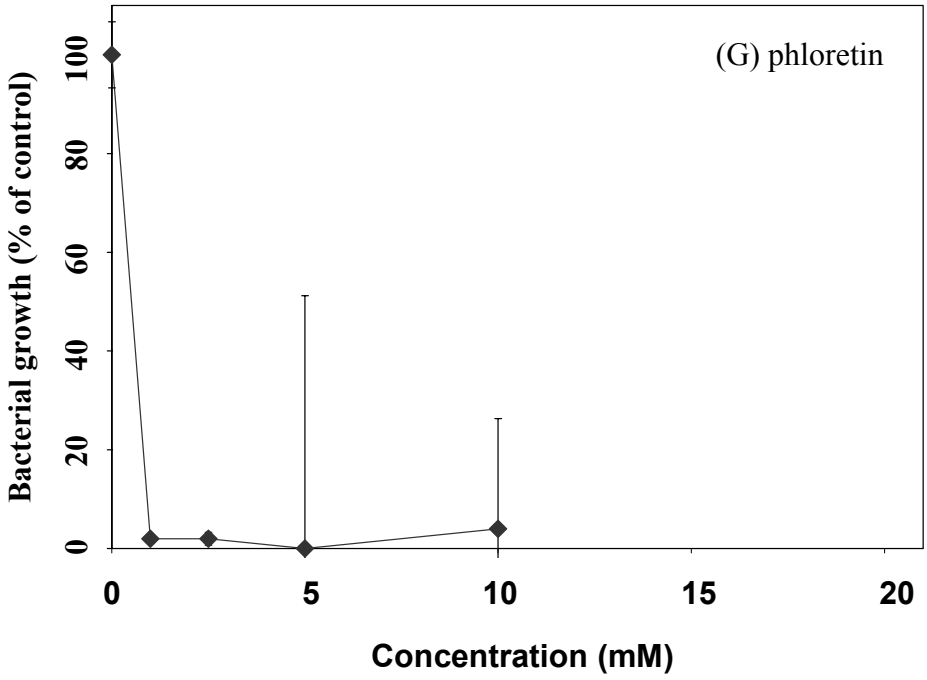
Figures 1A-G. Relative growth of *Erwinia amylovora* strain E2002a in nutrient broth amended with various concentrations of phenolic compounds after 8 h. Symbols represent means, n=6. Error bars represent $\pm$ 1 standard error of the mean of data for each concentration.



Figures 1A-G continued.



Figures 1A-G continued.



Figures 1A-G continued

Chapter 3

Changes in Endogenous Phenolic Composition among *Malus* Taxa Following Inoculation with *Erwinia amylovora*

(In the format appropriate for submission to HortScience)

Changes in Endogenous Phenolic Composition among *Malus* Taxa Following Inoculation with *Erwinia amylovora*

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Additional index words. disease resistance, fire blight, flowering crabapples, host plant resistance, induced resistance, phytoalexins, secondary metabolites

Abstract. Preformed and pathogen-induced phenolic compounds are known to play important roles in host plant resistance. Three cultivars of flowering crabapples (*Malus* Mill. spp.), representing a range of resistance to fire blight [*Erwinia amylovora* (Burrill) Winslow et al.], were inoculated with a virulent strain of *E. amylovora* to evaluate induced changes in phenolic composition over time. Seven major phenolic components including phloridzin, phloroglucinol, rutin, chlorogenic acid, and three unidentified compounds, were analyzed by high-performance liquid chromatography. Phenolic profiles varied among taxa, time, and in response to inoculation. For most compounds, there was no apparent relationship between their concentration and host plant resistance. However, an unidentified compound, component A, increased in the most resistant cultivar following inoculation. The constitutive levels of another unidentified compound, component X, were substantially higher in the more resistant taxa. Preliminary analysis by liquid chromatography mass spectrometry suggested that component X could be an isomere of phloridzin.

Erwinia amylovora (Burrill) Winslow et al., the casual agent of fire blight, is one of the most devastating pathogens of flowering crabapples (*Malus* Mill. spp.) and many other taxa in the Rosaceae. To date, applications of antibiotics provide the most effective control measures for this disease in orchard and nursery settings but are generally not practical for use in landscape plantings. Bell (2004) reported significant variation in resistance among 49 taxa of flowering crabapples to three virulent strains of *E. amylovora* based on artificial inoculations. Well documented resistance and a better understanding of host plant resistance can provide unique opportunities for breeding new plants with enhanced disease resistance.

Host plant resistance can sometimes be attributed to structural barriers and/or the activity of preformed secondary plant metabolites (Hammerschmidt and Nicholson, 1999). However, inducible responses following infection, including synthesis of phytoalexins, cell wall modifications, induction of *PR*-proteins, and the hypersensitive response, can also play important roles in defense reactions. Ultimately, host plant resistance is believed to be a complex process that involves multiple mechanisms (Hammerschmidt and Nicholson, 1999).

Some preformed (constitutive) phenolic compounds exhibit antimicrobial activity in vitro and are known to function as defense substances in some plant-pathogen interactions (Hammerschmidt and Schultz, 1996). In *Malus* spp., constitutive phenolic compounds have been reported to play an important role in resistance to apple scab [*Venturia inaequalis* (Cke.) Wint.] (Hunter, 1974; MacHardy, 1996). Bell (2004) measured endogenous levels of individual phenolic compounds

among selected taxa of flowering crabapples representing a range of resistance to fire blight. Several phenolic compounds measured, including phloridzin and phloroglucinol, were found to be inhibitors of *E. amylovora* in vitro, but did not appear to confer resistance in vivo. However, three unidentified phytochemical components were significantly correlated with resistance.

Following infection, the activity of phytoalexins becomes increasingly important in host plant resistance (Kuć, 1998). Phytoalexins are low-molecular weight antimicrobial compounds that are synthesized and accumulate in host tissue following exposure to microorganisms (Ebel, 1986). Kuć (1998) noted that some phytoalexins accumulate in both resistant and susceptible taxa; however; the accumulation of higher concentrations and the timing of their synthesis is critical for them to be effective.

Hrazdina et al. (1997) reported the synthesis and accumulation of a novel phenolic compound, malusfuran, in *Malus* taxa following inoculation with the fungus *Venturia inaequlais* (Cke.) Wint., the causal agent of apple scab. This compound was not present in either susceptible taxa or in noninoculated controls. Mayr et al. (1997) also provided strong evidence that phytoalexins were involved in resistance to apple scab among cultivars of apples. Leaves of an apple scab resistant cultivar were treated with a phenylalanine ammonium lyase (PAL) inhibitor then inoculated with the fungus. Severe symptoms developed on plants treated with the PAL inhibitor and no accumulation of flavanols occurred at the infection site. Nontreated, inoculated plants did not develop symptoms and a rapid accumulation of flavanols

occurred at the infection site and appeared to be functioning as phytoalexins. PAL, which catalyzes the conversion of phenylalanine to cinnamic acid, is a key enzyme in the biosynthesis of many phenolic compounds in *Malus* (Treutter, 2001).

Limited studies have investigated the role of phytoalexins in resistance to fire blight among *Malus* spp. Roemmelt et al. (1999a) reported that phenolic acids and simple phenols that accumulated after infection inhibited the migration of bacteria in host tissue suggesting phytoalexin activity. Furthermore, total levels of unspecified phenolic acids were up to 10 times higher in resistant taxa than in those taxa that exhibited pronounced symptoms.

Prohexadione-Ca, which was developed for commercial use to control excessive vegetative growth by disrupting the biosynthesis of gibberillins, has also been shown to increase resistance to fire blight among *Malus* taxa (Byers et al., 1997). This plant growth regulator does not possess antibacterial properties itself, but it alters flavonoid metabolism in treated plants (Roemmelt et al. 1999b). Several phenolic compounds, including luteoliflavan, a flavanoid which had not been reported previously in *Malus*, accumulated in plants following treatment. Prohexadione-Ca may induce novel defense mechanisms and/or enhance naturally occurring defense mechanisms employed against the fire blight pathogen.

Although significant variation in resistance to fire blight among *Malus* taxa has been documented, little is known about the mechanisms responsible for resistance to this disease. In light of recent information, the role of phytoalexins in resistance to fire blight among *Malus* taxa needs to be more thoroughly investigated. Therefore,

the objective of this study was to identify and measure levels of phenolic compounds in fire blight resistant, moderately resistant, and highly susceptible taxa of flowering crabapples (*Malus* spp.) prior to and over time following inoculation with a virulent strain of *E. amylovora*.

Materials and Methods

Three taxa from a field plot of 49 flowering crabapples taxa arranged in a randomized complete block design with three replications at the Mountain Horticultural Crops Research Station, Fletcher, N.C. were utilized for this study. The taxa selected were *M.* 'Adams' (resistant), *M.* 'Canary' (moderately resistant), and *M.* 'Schmidtcutleaf' (highly susceptible) (Bell, 2004). Trees were planted between March 1990 and March 1991.

Artificial Inoculations. On 7 May 2002, 15, randomly selected, actively growing shoots on separate major branches per replication were inoculated with *E. amylovora* strain E2002a at a concentration of 1×10^8 colony-forming units (cfu)/ml. Inoculations were conducted by the scissor technique as described by Bell (2004). Fifteen additional shoots per tree were cut with sterile scissors in a similar manner to the inoculation procedure and served as controls. Three inoculated shoots (subsamples) and three control shoots (subsamples) were collected from each of three

replicate trees at each harvest period. The distal 8 cm of the shoots were harvested at 0, 1, 2, 4, or 8 d after inoculation (DAI). Each harvest took place in the morning (8:00 to 10:00 AM). Leaves were removed and stems were frozen at -80 °C. The experiment design was an randomize complete block with a split split plot arrangement of treatments. The experimental design was split split plot design with cultivar as the main plot, time of harvest as the subplot, and treatment (control vs inoculated) as the sub-subplot.

Chemical Analysis. For each sample, 150 mg of lyophilized stem tissue were placed in a 25 mL centrifuge tube, extracted in 4 mL of 85% methanol, blended with a polytron homogenizer, sonicated for 2 h in a water bath then centrifuged at 1000 g_n for 10 min. The supernatant was decanted and collected. The pellet was resuspended in 4 mL of 15% methanol, sonicated for 2 h then centrifuged for 10 min. The supernatant was decanted and combined with the first extraction. The extraction was adjusted to a total volume of 10 mL with 50% aqueous methanol prior to injection.

Levels of individual phenolic compounds were analyzed by a reverse-phase high-perfermance liquid chromatography (HPLC) method adapted from Fulcher et al. (1998). An isocratic solvent system of 40 methanol : 60 water with 0.2% o-phosphoric acid was used. Phenolic compounds were separated on an 5µm Adsorbosphere C-18, 250 x 4.6 mm column (Alltech Associates Inc., Deerfield, Ill.) with a flow rate of 0.8 $mL \bullet min^{-1}$. Compound identity and quantity was detected at 254 nm with an ultraviolet (UV) detector (Millipore Corp., Bedford, Mass.) and compared with commercial standards (Sigma-Aldrich Co., St. Louis, Mo.) using an

external standard method. Concentrations of unknown components were calculated using the phloridzin standard.

Results

There were significant cultivar, inoculation, and time effects and various interactions among factors depending on the specific compound (Table 1). Seven major phenolic components were quantified and are listed in order of increasing retention times: phloroglucinol (≈3.9 min), chlorogenic acid (≈ 4.6 min), component A (≈5.6 min), component B (≈6.5 min), phloridzin (≈7.8-8.0 min), rutin (≈10.0-10.8 min), and component X (≈11.5-12.4 min) (Fig. 2). We were unable to quantify all peaks that were observed in the chromatographs (Fig. 2A-C).

There was a significant increase in phloroglucinol following inoculation compared to control (Fig. 1A). Averaged over all taxa and times, the concentration of phloroglucinol was 134 mM and 119 mM for inoculated and control samples, respectively. Component B and rutin were found in low concentrations and there were no noteworthy inoculation responses or interactions following inoculation (Fig. 1B-C).

A significant time x cultivar x inoculation interaction was observed for chlorogenic acid, component A, phloridzin, and component X (Table 1). Levels of chlorogenic acid more than doubled in inoculated *M.* 'Schmidtcutleaf' from 2 to 4 DAI while levels in inoculated *M.* 'Canary' were two times higher at 8 DAI

compared to 2 DAI (Fig. 1D). Levels of component A were higher in all inoculated taxa at 4 DAI compared to their controls (Fig. 1E). Levels of component A in inoculated *M*. 'Schmidtcutleaf' decreased between 4 and 8 DAI measuring 31 and 17 mM, respectively. At 8 DAI, inoculated *M*. 'Adams' and 'Canary' had the highest levels of component A with 26 and 32 mM, respectively. The noninoculated *M*. 'Adams' control had the lowest levels of component A at both 4 and 8 DAI.

Concentrations of phloroglucinol, phloridzin, and component X were the in highest concentrations among the compounds analyzed in this study (Fig. 1A and F-G). Phloridzin decreased over time in both inoculated *M*. 'Canary' and 'Schmidtcutleaf'. Within *M*. 'Schmidtcutleaf', levels of phloridzin were more than two times higher in the control compared to the inoculated treatment at 4 DAI. Levels of component X were substantially lower in *M*. 'Schmidtcutleaf' compared to both *M*. 'Canary' and 'Adams' at 0 DAI. Levels of component X decreased over time in inoculated *M*. 'Canary'. At 4 DAI, levels of component X were 119 mM and 56 mM for the control and inoculated *M*. 'Canary', respectively. In *M*. 'Adams', levels of phloridzin and component X were relatively unaffected following inoculation throughout the study.

Discussion

The present study documented significant changes in the levels of phenolic compounds in three taxa of flowering crabapples following inoculation with a virulent strain of *E. amylovora*. Levels of component A increased over time and were highest at 8 DAI in inoculated shoots of resistant *M.* 'Adams' and in moderately resistant *M*. 'Canary'. In highly susceptible *M.* 'Schmidtcutleaf', component A increased initially in inoculated shoots, then decreased. Given the observed trend, component A may possibly play a role in a secondary defense reaction to *E. amylovora*.

Phloroglucinol, which possesses strong antimicrobial activity in vitro towards strain E2002a (Bell, 2004), increased following inoculation demonstrating an inoculation induced response. However, levels of phloroglucinol at 8 DAI were similar for inoculated shoots of the resistant *M*. 'Adams' and susceptible *M*. 'Schmidtcutleaf' providing questionable evidence for a key role of phloroglucinol in the resistance of crabapples to *E. amylovora*. Chlorogenic acid increased in all taxa following inoculation. Like phloroglucinol, chlorogenic acid is a potent inhibitor of strain E2002a in vitro. In this study, however, levels of chlorogenic acid were highest in susceptible *M*. 'Schmidtcutleaf' at 4 DAI and lowest in resistant *M*. 'Adams' among inoculated treatments.

Phloridzin, the most abundant phenolic found in most *Malus* spp., and its aglycone, phloretin, also demonstrated antimicrobial activity towards strain E2002a

in vitro. In this study, degradation of phloridzin occurred over time in highly susceptible *M*. 'Schmidtcutleaf' and in moderately susceptible *M*. 'Canary' after inoculation. It is not clear whether the decrease in concentrations of phloridzin in *M*. 'Canary' and 'Schmidtcutleaf' over time following inoculation is due to direct activity of the pathogen, induced hydrolysis (possibly a defense reaction), or indirectly through tissue degradation as a result of disease development. The antibacterial properties of phloridzin and its related compounds and their catabolism following inoculation in *M*. 'Schmidtcutleaf' and 'Canary' but not in *M*. 'Adams' provides justification for additional investigation into their potential role in resistance of crabapples to *E. amylovora*.

Component X did not show an inducible response to inoculation. However, there were substantially higher concentrations of component X in shoots of the more resistant *M*. 'Adams' and 'Canary' than in *M*. 'Schmidtcutleaf'. Additional analysis by LC-MS conducted in a previous study (Bell, 2004) provided evidence that component X is potentially an isomere of phloridzin and also found that constitutive levels were correlated with resistance to fire blight.

Recent studies investigating the relationship between prohexadione-Ca treatment and an increase in resistance to fire blight among *Malus* taxa have provided additional information on the role of phenolic compounds in resistance to fire blight. Roemmelt et al. (2002), suggested that prohexadione-Ca triggers defense reactions similar to those induced by *E. amylovora.* An increase in phenylpropanoids was observed in tissue that was either treated with prohexadione-Ca or inoculated with *E.*

amylovora. Concentrations of phloretin increased 77% in tissue inoculated with *E. amylovora* and more than doubled in tissue treated with prohexadione-Ca. Their study, however, only included a fire blight susceptible taxa and therefore the results do not necessarily represent defense mechanisms that occur in naturally resistant taxa. We were unable to obtain clear resolution of phloretin in this study, though it is typically found in much lower concentrations and is a catabolism product of phloridzin.

It is becoming increasingly clear that phenolics can play a major role in the resistance of *Malus* spp. to several important diseases. Mayr et al. (1997) showed that apple scab resistant taxa develop disease symptoms when the biosynthesis of phenolic compounds is inhibited. Roemmelt et al. (1999a) reported that phenolic compounds accumulated and appeared to inhibit the migration of *E. amylovora* in host tissue. Our results herein provide information on variation in constitutive and inoculation-induced responses of diverse phenolic constituents found in flowering crabapples that vary in resistance to fire blight. Significant changes in phenolic profiles were observed in plant tissue following inoculation with a virulent strain of *E. amylovora*. In most cases there was little evidence suggesting that these induced changes contribute to enhanced resistance. However, component A increased in the most resistant cultivar following inoculation and warrants further study. Differences in constitutive levels of certain compounds may also be important in conferring resistance. Component X was substantially higher in more resistant taxa and is also deserving of further study.

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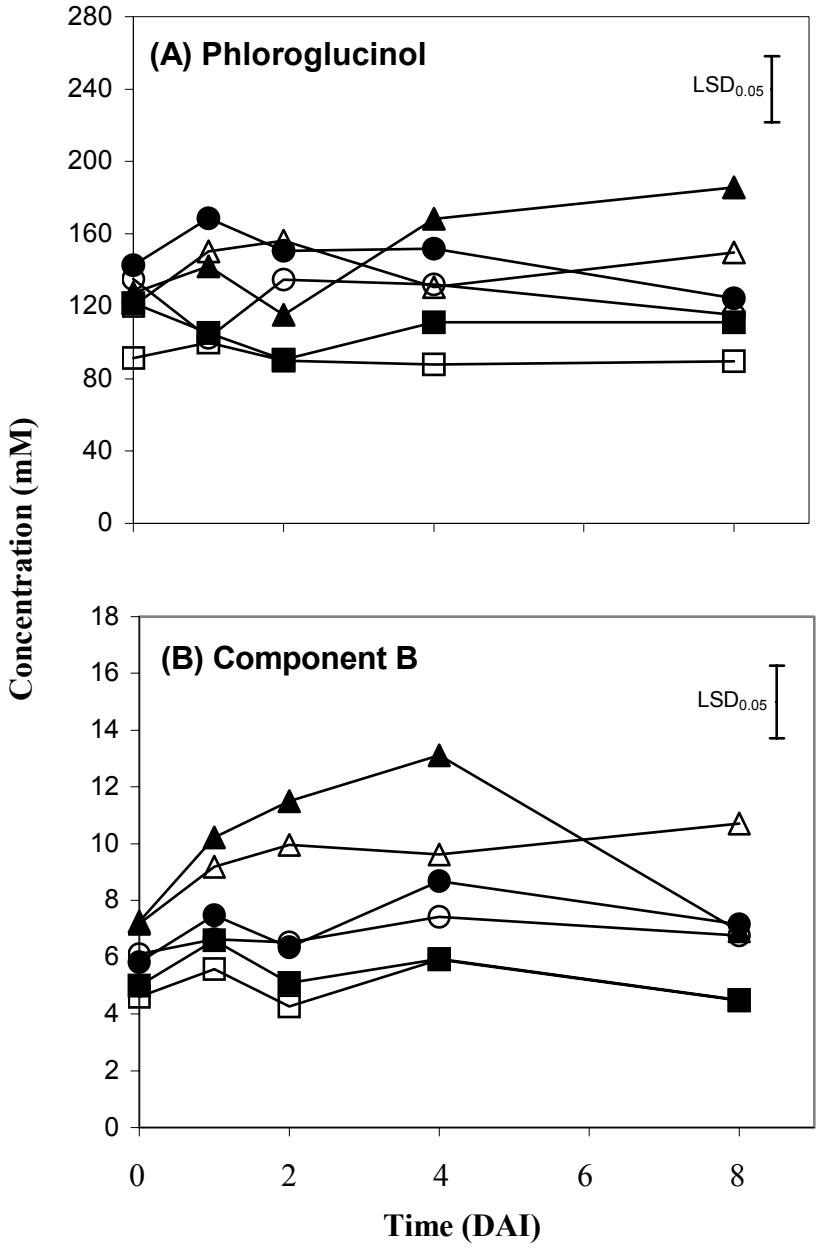
Table 1. Analysis of variance for phenolic compounds in *Malus* taxa in response to inoculation with *Erwinia amylovora.*[z]

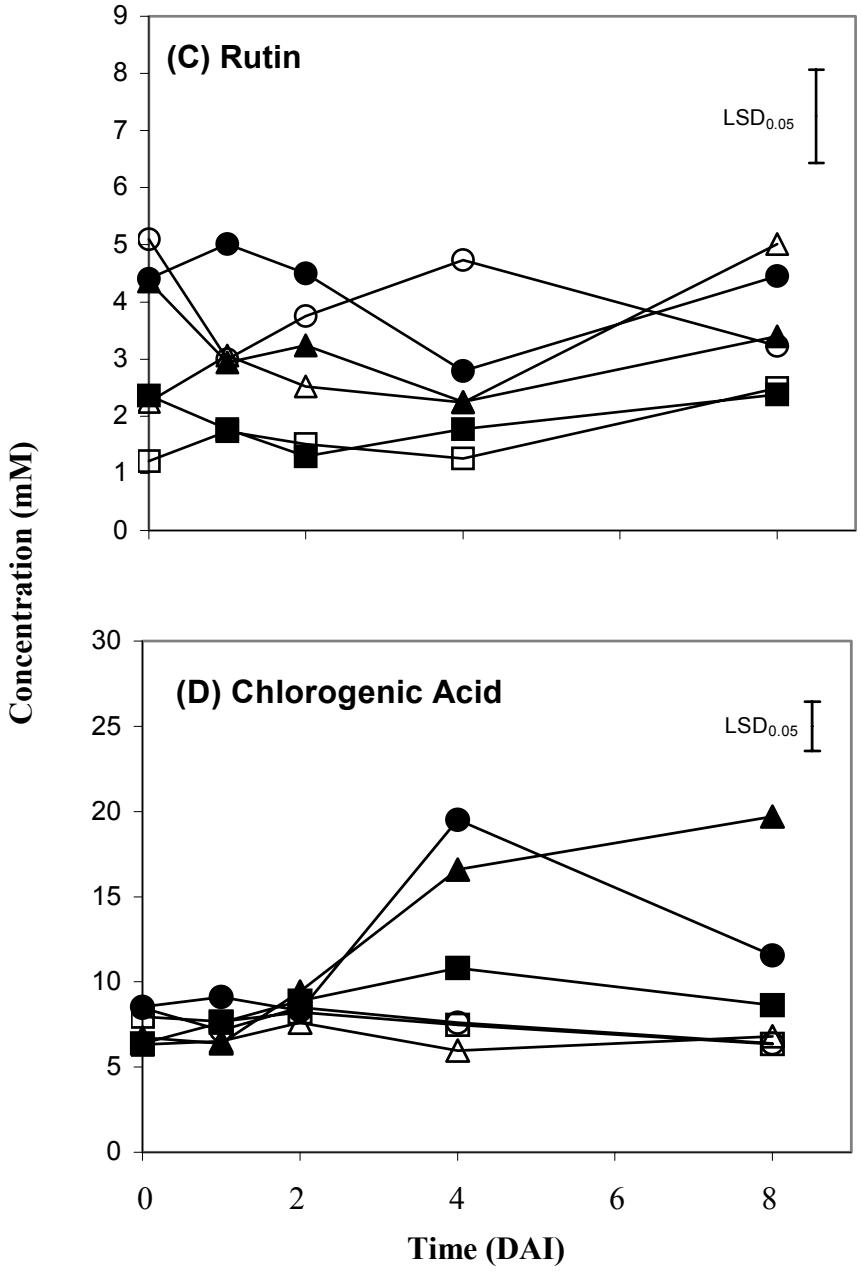
	df	Phloroglucinol	Chlorogenic Acid	Component A	Component B	Phloridzin	Rutin	Component X
Block (B)	2							
Cultivar (CV)	2	5.57^{NS}	0.90^{NS}	0.39^{NS}	12.30*	3.55^{NS}	6.86^{NS}	48.18**
B X CV (Error A)	4							
Inoculation (I)	1	15.02**	104.42**	6.95*	2.88^{NS}	84.56**	1.44^{NS}	31.59**
I X CV	2	1.60^{NS}	15.08**	6.16*	0.0^{NS}	8.23*	0.01^{NS}	26.33**
B X I X CV (Error B)	6							
Time (T)	4	0.45^{NS}	17.04**	4.48**	6.32**	38.06**	2.76*	18.43**
T X CV	8	2.14*	6.10**	2.27*	1.64^{NS}	4.02**	1.11^{NS}	8.77**
T X I	4	1.74^{NS}	24.92**	3.77**	1.99^{NS}	34.27**	1.40^{NS}	20.11**
T X CV X I	8	1.88^{NS}	3.93**	3.30**	1.44^{NS}	10.08**	2.80*	14.28**
Error C	48							

[z]Values are F-values from the analysis of variance.

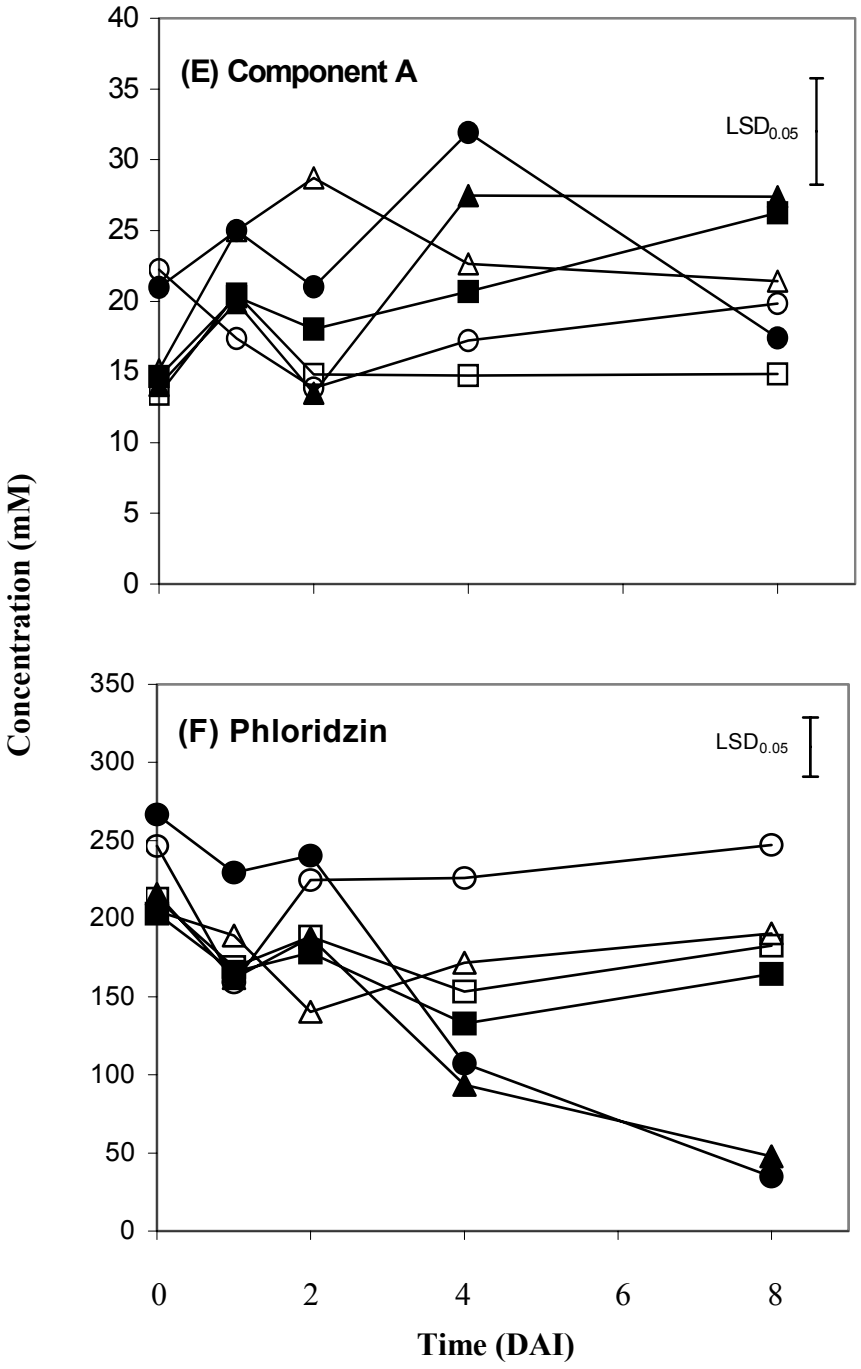
NS, *,** Nonsignificant or significant at P< 0.05 or 0.01, respectively.



Figures 1A-G. Changes in phenolic compound concentration in *Malus* taxa as a function of days after inoculation (DAI) with *Erwinia amylovora* strain E2002a. AC- 'Adams' control, AI- 'Adams' inoculated, CC- 'Canary' control, CI- 'Canary' inoculated, SC- 'Schmidtcutleaf' control, SI- 'Schmidtcutleaf' inoculated. $LSD_{0.05}$ for comparison among treatments within a given time.



Figures 1A-G. continued



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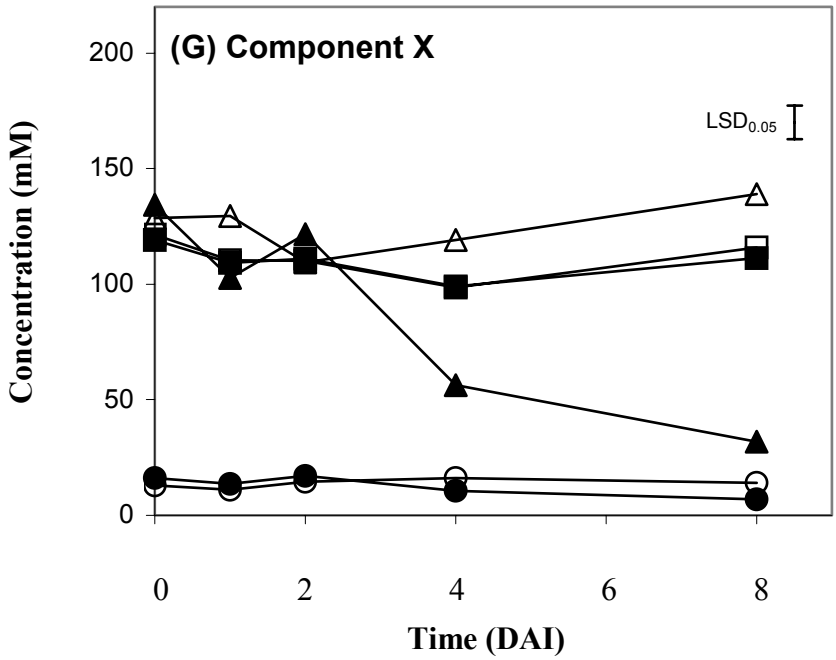

Figures 1A-G. continued

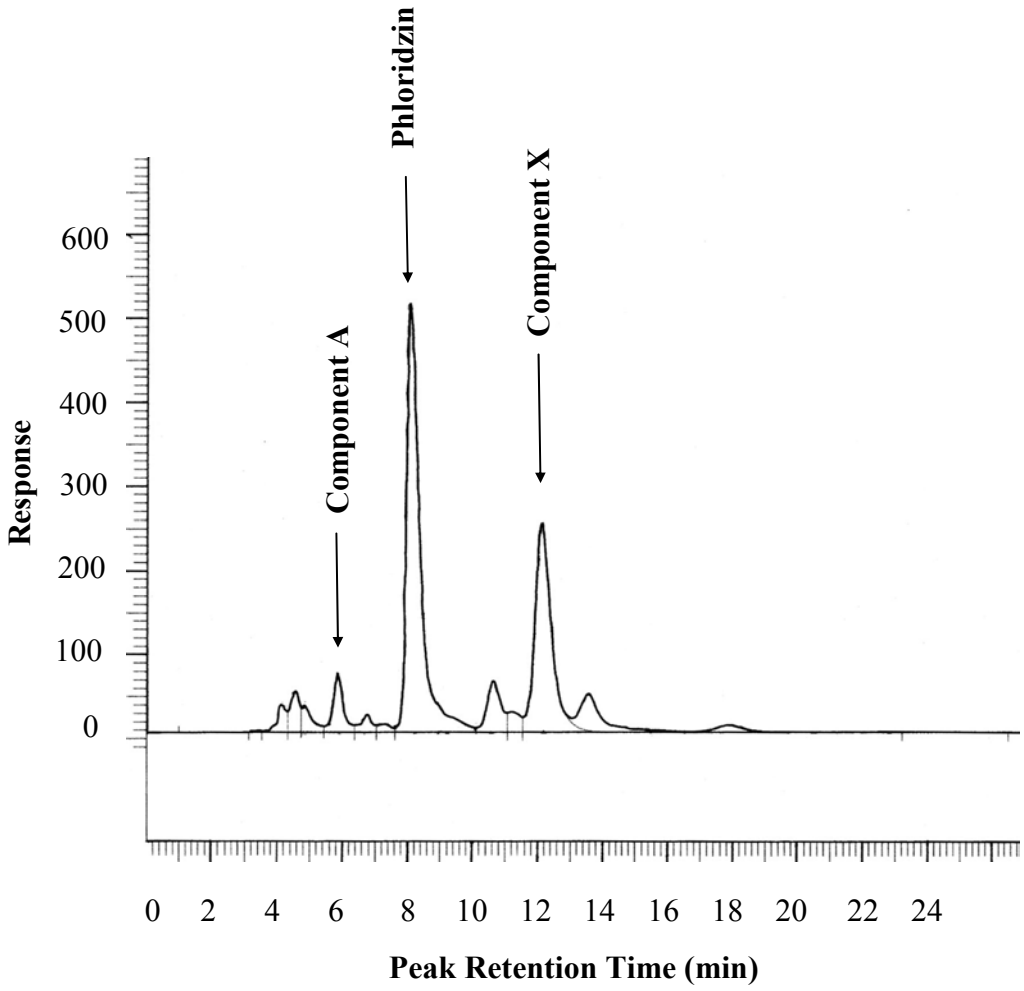

Fig. 2. Typical HPLC chromatograph for *Malus* ‘Adams’.