CONTROLLING APPLE ROOTSTOCK FIRE BLIGHT WITH RESISTANT ROOTSTOCKS AND STREPTOMYCIN RESISTANCE MANAGEMENT

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Fire blight, caused by the Enterobacterium *Erwinia amylovora*, is a devastating disease of apple (*Malus* spp.). Production losses are compounded due to fire blight's unique ability to attack all phases of apple development; including blossoms, shoots, and rootstocks. Incidence of rootstock blight, a lethal infection of the apple rootstock, has increased due to the adoption of high density planting systems, which rely on the susceptible dwarfing rootstock Malling 9 (M.9), to maintain tree size and productivity. Rootstock trials focusing on orchard performance and rootstock blight resistance have identified several rootstock selections, including Geneva® 41, Geneva® 935, and Budagovsky 9 (B.9), which have the potential to surpass M.9 in modern production systems. Previously B.9 rootstock was not recommended due to discrepancies concerning phenotypic variation in stool bed material and fire blight resistance. Microsatellite (SSR) analysis and inoculation assays verified the genetic uniformity of B.9 material. Rootstock evaluation revealed B.9 is highly susceptible to *E. amylovora* when leaf inoculated but highly resistant when woody tissue is directly challenged by the bacterium. B.9 resistance is influenced predominantly by the maturation of shoot tissue. Complete gain of resistance in relation to tissue development or maturation is evocative of age related resistance (ARR). The existence of ARR is supported by the cessation of lesion advancement; signified by a determinate lesion margin when rootstock suckers, young auxiliary shoots originating from rootstock tissue, intersect

with older, lateral root tissue. Due to the growth characteristics of apple rootstocks ARR would be a practical source of resistance in future breeding efforts.

Continued breeding of resistant planting material is essential due to the anticipated loss of streptomycin for the control of fire blight. Streptomycin resistance was initially discovered in 1971 and occurs in all major US apple production regions, excluding New York State. In 2002 streptomycin-resistant *E. amylovora* were discovered in New York, and successfully eradicated. Based on the resistance mechanism and circumstances surrounding the planting, it is probable streptomycin-resistant bacteria were imported on infected nursery stock. This incident demonstrates how easily streptomycin could be rendered ineffective, and the importance of resistant planting material in modern apple production.

BIOGRAPHICAL SKETCH

Nicole L. Russo was born Nicole LoGiudice in Holmdel New Jersey on 21, September 1978 to Joseph and Diane LoGiudice. Between 1996 and 2000 she completed her Bachelors Degree in Science at the land grant portion of Rutgers University, Cook College, now the School of Environmental and Biological Sciences. After completing her B.S. degree Nicole spend a year working at the Rutgers Vegetable Research Farm researching Easter lily production and cultivar improvement. In the summer of 2001 Nicole began her Ph.D. at Cornell University in the Department of Plant Pathology. She joined the lab of Dr. Herb S. Aldwinckle at the New York State Agricultural Station in Geneva, NY. In Dr. Aldwinckle's program Nicole focused on the use of disease resistant rootstocks to control rootstock blight, a lethal form of fire blight caused by the bacterium *Erwinia amylovora*. Her thesis research focused on the verification and description of fire blight resistance in the dwarfing rootstock Budagovsky 9 (B.9). Other work includes the screening of promising rootstock selections for fire blight resistance, along with the characterization of streptomycin-resistant strains of *E. amylovora* isolated from New York State apple orchards.

I would like to dedicate my doctoral thesis to my husband Eric, without whose love and support I never would have been able to accomplish this tremendous undertaking. Whether his support came in the form of a encouraging word or a gourmet meal at the end of a long commute he always did his best to put the needs of my education and commitments above his own. I am truly blessed to have met such a caring and devoted individual.

I would also like to dedicate this to my stepmother Kathleen, my sister Danielle, and my brother Alex, who manage to make my life both chaotic and worthwhile, and finally to my father, Joseph, who taught me the value of an education and the rewards of hard work.

Finally this is dedicated to my mother Diane. Although she was not able to witness many of my academic accomplishments she instilled in me the knowledge that with persistence, courage, and the ability to laugh at yourself, you could do anything or go anywhere you wished in life.

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I would also like to acknowledge my committee members Drs. Thomas J. Burr, Terence L. Robinson, and Stephen C. Winans. Their collective advice and support has proven invaluable to my research and education. Always available, their counsel has been both readily forthcoming and constructive.

Finally I would like to acknowledge Dr. Gennaro Fazio for his research contribution and continued guidance, Jay Osborne for technical support, and the members of the Aldwinckle lab, Marguerite Abbott, Ewa Borejsza-Wysocka, Włodek Borejsza-Wysocki, Megan Dewdney, Herb Gustafson, Shirley Lewis, Mickael Malnoy, and Nicole Werner, without whom this work would not be possible.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Budagovsky 9:	B.9
Malling series:	M.9, M.26, M.7, M.27
Geneva series:	G.11, G.16, G.41, G.65 G.935, G.30

CHAPTER 1

[*]FIELD EVALUATION OF 64 APPLE ROOTSTOCKS FOR ORCHARD PERFORMANCE AND FIRE BLIGHT RESISTANCE

Abstract

In 2002, apple rootstock trials using three scion cultivars were established at Geneva, NY to evaluate 64 apple (*Malus X domestica* Borkh.) rootstocks for horticultural performance and fire blight resistance. Field trials compared several elite Geneva® apple rootstocks, which were bred for tolerance to fire blight and *Phytophthora* root rot, to both commercial standards and elite rootstock clones from around the world. Three rootstocks performed well with all scion cultivars: 'B.9', 'Geneva® 935', and 'Geneva® 41'. All three rootstocks were similar in size to 'Malling 9' ('M.9') clones, but with elevated yield efficiency and superior resistance to fire blight. 'Geneva® 11' also performed very well with 'Golden Delicious' and 'Honeycrisp', with regard to yield efficiency and disease resistance. Resistant rootstocks greatly enhanced the survival of young trees, particularly with the susceptible scion cultivars 'Gala' and 'Honeycrisp'. Results demonstrate the ability of new rootstock clones to perform better than current commercial standards, reducing financial risk to producers, while promoting orchard health with enhanced disease resistance.

[*] Russo, N.L., T.L. Robinson, G. Fazio, and H.S. Aldwinckle. 2007. Field evaluation of 64 apple rootstocks for orchard performance and fire blight resistance. HortScience (*In Press*)

Introduction

Advancements in rootstock breeding and selection have revolutionized the manner in which apples are grown throughout the world. In modern production systems, selection of an appropriate rootstock is as important to the viability and success of a new planting as the choice of fruiting cultivar. Rootstocks affect a number of horticultural attributes including winter hardiness, fruit size, precocity, productivity, tree vigor, and disease resistance (Cummins and Aldwinckle, 1983; Momol et al., 1998; Westwood, 1988). Continued breeding and selection of novel rootstock cultivars promotes improved orchard performance while exploring new attributes that facilitate the health and stability of orchard systems.

Dwarfing rootstocks significantly reduce tree size, facilitating an increase in planting density (Ferree et al., 1993; Hampson et al., 2002, 2004a, 2004b; Robinson et al., 1991). Contemporary high-density orchards have tree densities of 1,200-7,000 trees•ha^{-1}. Planting densities of this magnitude reduce yield on a per tree basis but significantly increase the yield per unit area (Hampson et al., 2002, 2004a, 2004b) due to enhanced annual and lifetime light interception and maximized light partitioning within the canopy (Ferree et al., 1993; Robinson and Lakso, 1991; Robinson et al., 1991; Webster et al., 2000).

Although the economic benefit of high-density systems is clear (Robinson et al., 2007), a concern associated with specific dwarfing rootstocks is their susceptibility to rootstock blight, a discrete fire blight infection of the rootstock. Fire blight, caused by the bacterium *Erwinia amylovora* [(Burr.) Winslow et al.] is a common bacterial disease of rosaceous plants (Vanneste and Eden-Green, 2000). Fire blight affects multiple stages of tree development, and disease outbreaks can lead to considerable losses due to reduction in yield and tree replacement. Although most commonly associated with blossom or shoot infection, the rootstock phase of fire blight is

prevalent in young dwarf orchards (Robinson et al., 2006). Rootstock blight occurs when bacteria, which initially enter the tree via blossom or shoot infection, travel systemically through the vascular system into the rootstock without causing visible symptoms (Momol et al., 1998). Rootstock infection may also occur to a lesser extent through wounds and infected rootstock suckers (Vanneste and Eden-Green, 2000). The biological factors that induce disease development remain unclear, however once bacteria enter the rootstock, no cultural control or chemical treatment can prevent disease development (Norelli et al., 2003).

High-density systems rely mainly on the rootstock 'M.9', a highly productive dwarfing rootstock, which is particularly susceptible to rootstock blight. In heavy fire blight years under natural conditions, tree losses greater than 50% are common for orchards planted on 'M.9' rootstock (Ferree et al., 2002; Norelli et al., 2003; Robinson et al., 2006). Severe tree loss can be devastating to profitability in high-density systems where initial establishment costs are substantial. New high performance, disease resistant rootstocks are necessary to alleviate grower reliance on 'M.9' (Marini et al., 2006b).

The Geneva® rootstock series, originating from the Geneva NY Breeding Program, a joint venture between the USDA-ARS and Cornell University, are the leading fire blight resistant rootstocks commercially available (Johnson et al., 2001; Norelli et al., 2003). Geneva® rootstocks exhibit high cumulative yield efficiency in multiple size classes, combined with enhanced disease, and in some cases, insect, resistance (Autio et al., 2005a, 2005b; Cummins and Aldwinckle, 1983; Robinson et al., 2006). Norelli et al. (2003) determined 'G.16' and 'G.30' suffered 70% less rootstock blight-related tree mortality than either 'M.26' or 'M.9', in both inoculated and naturally infected field trials.

The objective of this work was to evaluate the Geneva® rootstocks as well as several elite rootstock clones from breeding programs around the world for both horticultural performance (dwarfing and yield efficiency) and resistance to rootstock blight when grafted to three economically important scion cultivars, 'Gala', 'Honeycrisp', and 'Golden Delicious'. Horticultural performance data were collected and evaluated by Jason Osborne and Terence Robinson. Orchard performance trials were conducted in combination with rootstock blight resistance evaluations.

Materials and Methods

In 2002, duplicate, replicated rootstock trials were planted at two locations at the New York State Agricultural Experiment Station, Geneva, NY. The two trials were separated by 1000 m. One of the plots was used for evaluation of horticultural performance of rootstock clones and the other to evaluate rootstock resistance to rootstock blight. Within each plot, three sub-plots were planted each with a different scion cultivar ('Royal Gala', 'Golden Delicious', and 'Honeycrisp'). For each sub-plot a randomized complete block experimental design was used. There were 19 rootstock clones with 'Gala', 46 with 'Golden Delicious', and 22 with 'Honeycrisp'. Rootstock clones included appropriate Malling rootstock controls and other rootstocks of interest from around the world (Table 1.1).

Table 1.1 Apple rootstocks grown for 5 years with ‘Gala’, ‘Golden Delicious’, or ‘Honeycrisp’ as the scion at Geneva, New York.

Rootstock	Scion cultivars used in trials			Origin of rootstock	Dwarfing class[y]
B.9-NE	Gala	Golden Delicious	Honeycrisp	Michurinsk, Russia	3
B.9-OR	Gala	Golden Delicious	Honeycrisp	Michurinsk, Russia	3
CG.2406		Golden Delicious		Geneva, USA[z]	2
CG.3007	Gala	Golden Delicious	Honeycrisp	Geneva, USA	6
CG.4002		Golden Delicious		Geneva, USA	8
CG.4004		Golden Delicious		Geneva, USA	6
CG.4011		Golden Delicious		Geneva, USA	3
CG.4013		Golden Delicious	Honeycrisp	Geneva, USA	5
CG.4202		Golden Delicious		Geneva, USA	5
CG.4288		Golden Delicious		Geneva, USA	4
CG.4814		Golden Delicious		Geneva, USA	5
CG.5030		Golden Delicious		Geneva, USA	6
CG.5463		Golden Delicious		Geneva, USA	8
CG.5890		Golden Delicious		Geneva, USA	7
CG.6006		Golden Delicious		Geneva, USA	7
CG.6143		Golden Delicious		Geneva, USA	6
CG.6210		Golden Delicious	Honeycrisp	Geneva, USA	6
CG.6253		Golden Delicious		Geneva, USA	7
CG.6589		Golden Delicious		Geneva, USA	8
CG.6874		Golden Delicious		Geneva, USA	7
CG.6879		Golden Delicious		Geneva, USA	6
CG.6969		Golden Delicious		Geneva, USA	6
CG.7073		Golden Delicious		Geneva, USA	7

Table 1.1 (Continued)

Rootstock	Scion cultivars used in trials			Origin of rootstock	Dwarfing class[y]
CG.8534		Golden Delicious		Geneva, USA	8
G.11		Golden Delicious	Honeycrisp	Geneva, USA	3
G.16		Golden Delicious	Honeycrisp	Geneva, USA	4
G.41	Gala	Golden Delicious	Honeycrisp	Geneva, USA	3
G.65			Honeycrisp	Geneva, USA	2
G.935	Gala	Golden Delicious	Honeycrisp	Geneva, USA	5
JM.1	Gala			Morioka, Japan	6
JM.2	Gala			Morioka, Japan	7
JM.7	Gala			Morioka, Japan	5
JTE-B		Golden Delicious		Czech Republic	3
JTE-C		Golden Delicious		Czech Republic	8
JTE-D		Golden Delicious		Czech Republic	7
M.26EMLA	Gala	Golden Delicious	Honeycrisp	East Malling, UK	5
M.26NAKB	Gala			East Malling, UK	5
M.27EMLA			Honeycrisp	East Malling, UK	2
M.9			Honeycrisp	East Malling, UK	3
M.9Burg756	Gala			East Malling, UK	4
M.9EMLA			Honeycrisp	East Malling, UK	4
M.9NAKBT337	Gala	Golden Delicious	Honeycrisp	East Malling, UK	3
M.9Nic8			Honeycrisp	East Malling, UK	3
M.9Nic29	Gala		Honeycrisp	East Malling, UK	4
M.9Pajam1			Honeycrisp	East Malling, UK	4
M.9Pajam2			Honeycrisp	East Malling, UK	4

Table 1.1 (Continued)

Rootstock	Scion cultivars used in trials		Origin of rootstock	Dwarfing class[y]
M.7	Golden Delicious		East Malling, UK	6
MM.106	Golden Delicious		East Malling, UK	7
MM.111	Golden Delicious		East Malling, UK	7
Marubakaido	Golden Delicious		Japan	8
NAGA	Golden Delicious		Japan	8
Ottawa 3	Golden Delicious	Honeycrisp	Ontario, Canada	4
P.14	Gala		Skierniewice, Poland	6
P.22		Honeycrisp	Skierniewice, Poland	2
PiAu-36-2	Gala		Pillnitz, Germany	6
PiAu-51-11	Gala		Pillnitz, Germany	3
PiAu-51-4	Gala		Pillnitz, Germany	7
PiAu-56-83	Gala	Golden Delicious	Pillnitz, Germany	6
Supporter 4	Gala	Golden Delicious	Honeycrisp	Pillnitz, Germany	6
V.1	Golden Delicious		Vineland, Canada	6
V.2	Golden Delicious		Vineland, Canada	3
V.3	Golden Delicious		Vineland, Canada	3
V.4	Golden Delicious		Vineland, Canada	6
V.7	Golden Delicious		Vineland, Canada	4

[y] Rootstocks dwarfing class is a range from 1-10 representing with 1=10 and 10=100% the size of a tree on a full vigor seedling rootstock. Size classification according to Johnson et al. (2001).

[z] Cornell University-USDA-ARS, New York State Agricultural Experiment Station, Geneva, NY

With ‘Gala’, there were 7 single tree replications of each rootstock while with both ‘Golden Delicious’ and ‘Honeycrisp’ there were 10 single tree replications of each rootstock clone. ‘Gala’ trees were grown at Treco nursery, Woodburn, OR, and the ‘Golden Delicious’ and ‘Honeycrisp’ trees were grown in a nursery at the New York State Agricultural Experiment Station, Geneva, NY. The horticultural plot had a tree spacing of 2.5 x 4.5 m while the fire blight plot had a spacing of 1 x 3 m. The two plots were planted on fine sandy loam soil with 4% organic matter. Both plots had previously been planted to apples and were fumigated with Telone C-17 (Dow AgroSciences LLC, Indianapolis, IN) (375 l•ha^{-1}) in early Sept. 2001, the fall before planting. Trees were planted, with bud union height 10 cm above the soil line, in early May 2002 and were minimally pruned at planting. The leader was not headed but lateral branches, if present, were shortened by 1/3. A support trellis was installed in mid-summer 2002. Trees were trained to the Vertical Axis system (Robinson, 2003), which included leaving the leader unheaded and removing only 1-2 large vigorous lateral branches each year. Branches were removed at the point of origin on the trunk using an angle cut. Trees received 60 kg N•ha^{-1} as ammonium nitrate each spring at bud break and 120 kg K_2O•ha^{-1} as KCl each November. Trees were not irrigated. In 2002, 2003, 2004, and 2006 adequate rainfall was received each month of the growing season (>75 mm•month^{-1}). In 2005, moderate drought occurred in late June and July. Trees were defruited in the first two years (2002 and 2003) then allowed to crop in 2004-2006. In 2004, trees were hand thinned to a single fruit per cluster while in 2005 and 2006 trees were chemically thinned by spraying them with 5 mg•ha^{-1} NAA (Fruitone-N, AMVAC Chemical Corp., Los Angeles, CA) tank mixed with 600 mg•ha^{-1} Carbaryl (Sevin XLR, Bayer Crop Sciences, Research Triangle Park, NC) using 935 l water•ha^{-1} at 10 mm fruit size. Chemical thinning was effective and no additional hand thinning was necessary.

In the horticultural plot, fruit number and fruit weight were recorded per tree in 2004-2006. At the end of the experiment (Nov. 2006) tree survival, tree circumference, tree height, canopy width in two compass directions, and number of root suckers per tree were recorded. Canopy volume was calculated assuming a conical canopy shape. The distance below the bottom branch to the soil was not included in the volume calculation. Data were analyzed separately for each scion cultivar, with replicate as a random effect and rootstock as fixed effect, using SAS Proc Mixed procedure ($y_{ij} = m + r_i + s_j + e_{ij}$) (SAS Institute, Cary, NC). Means were adjusted for missing trees using the LSMeans procedure. Mean separation was determined using Least Significant Difference with a P value of 0.05.

In the disease resistance plot, a subset of fifty-five dwarf and semi-dwarf rootstocks were compared for their sensitivity to rootstock blight infection. In 2005, trees were inoculated at 60% bloom using a backpack sprayer containing $1x10^7$ cfu/ml of *E. amylovora* strain E4001a (Ea266) in potassium phosphate buffer (0.05M). Strain E4001a was selected based on its virulence and ability to overcome certain sources of resistance (Norelli and Aldwinckle, 1986; Norelli et al., 1987). Percent infection was measured by recording the proportion of infected blossom clusters out of fifty randomly selected blossom clusters for each inoculated tree. Incidence of rootstock blight infection was based on the presence of diagnostic symptoms, primarily bacterial ooze emitted from the rootstock. Subsequent tree death and/or premature reddening of tree foliage confirmed frequency of rootstock blight. Trees were evaluated for rootstock blight symptoms on 21 July, 10 Aug., 6 Oct., and 19 Oct. 2005. Data were analyzed with logistic regression to determine likelihood of developing rootstock blight using a P value of 0.05. Based on the parameters of logistic regression, rootstock clones with no observed rootstock blight were excluded from analysis, and designated resistant for that particular scion rootstock combination.

Table 1.2. Horticultural performance of apple rootstocks grown for 5 years with either 'Gala', 'Golden Delicious' or 'Honeycrisp' as the scion at Geneva, New York.

Cultivar	Rootstock[w]	Tree height (m)	Tree width (m)	Canopy volume (m^3)	Trunk cross-sectional area (cm^2)	Root suckers	Tree survival[x] (%)	Cumulative yield (kg)	Cumulative yield efficiency (kg/cm^2 TCA)	Mean fruit size[y] (g)
Gala	B.9-NE	2.7	2.2	3.0	12.5[z]	3.7	100	24.9	1.99	149
	G.41	3.5	2.4	4.4	19.1	0.0	100	25.6	1.38	150
	B.9-OR	3.1	2.4	4.3	19.8	1.0	100	24.6	1.32	151
	G.935	3.2	2.8	5.2	22.1	5.7	70	43.8	1.69	142
	M.9NAKBT337	3.2	2.4	4.2	23.6	1.2	100	18.8	0.82	153
	M.26EMLA	3.2	2.4	4.5	24.9	0.8	80	12.6	0.52	143
	PiAu-51-11	3.1	2.4	4.3	27.8	0.7	100	11.6	0.39	154
	M.26NAKB	3.2	2.6	4.7	28.1	0.8	100	17.8	0.70	152
	M.9Nic29	3.3	2.8	5.9	29.6	2.0	80	25.5	0.85	154
	M.9Burg756	3.6	3.0	7.1	30.7	1.3	100	16.0	0.48	165
	JM.7	3.7	2.8	6.9	33.6	0.0	100	31.4	0.90	151
	Supporter 4	3.6	3.0	7.0	36.3	0.0	100	20.1	0.49	162
	JM.1	3.5	2.6	5.5	36.9	0.0	100	25.3	0.70	166
	P.14	4.1	3.2	9.4	41.4	0.0	80	15.1	0.31	146
	PiAu-36-2	3.9	3.0	8.0	44.9	0.5	100	12.9	0.27	151
	PiAu-56-83	3.7	3.0	8.0	44.9	1.2	100	10.8	0.20	139
	PiAu-51-4	4.1	3.0	8.2	52.2	0.0	100	16.4	0.28	145
	JM.2	3.7	2.6	6.1	52.5	0.0	100	46.5	0.77	152
	CG.3007	4.1	3.2	9.9	66.9	1.0	100	17.1	0.23	139
	LSD p≤0.05	**0.4**	**0.4**	**2.0**	**11.6**	**2.5**	**30**	**14.5**	**0.53**	**16**

Table 1.2 (Continued)

Cultivar	Rootstock[w]	Tree height (m)	Tree width (m)	Canopy volume (m^3)	Trunk cross-sectional area (cm^2)	Root suckers	Tree survival[x] (%)	Cumulative yield (kg)	Cumulative yield efficiency (kg/cm^2 TCA)	Mean fruit size[y] (g)
Golden Delicious	CG.2406	2.2	1.0	1.9	12.9	0.5	85	9.5	0.73	182
	CG.4013	2.5	1.0	2.1	17.3	2.2	95	11.4	0.66	164
	V.2	2.7	1.1	2.8	18.4	1.3	100	12.3	0.68	169
	V.3	2.7	1.0	2.2	19.4	1.0	68	8.0	0.48	171
	B.9-NE	2.7	1.1	3.1	20.0	9.0	100	20.3	1.03	197
	G.16	2.7	1.0	2.6	21.0	0.0	100	13.8	0.66	173
	CG.4011	2.7	1.2	3.5	21.5	5.1	90	21.5	1.04	186
	Ottawa 3	2.8	1.1	3.6	22.7	2.8	100	11.2	0.72	171
	B.9-OR	2.7	1.2	3.1	23.0	1.3	80	14.6	0.69	176
	G.11	2.6	1.1	2.8	23.1	2.2	85	12.1	0.52	188
	G.41	2.8	1.1	2.8	23.6	0.0	100	11.6	0.49	202
	M.9T337	2.8	1.1	2.8	24.0	1.1	80	6.5	0.25	180
	JTE-B	2.9	0.9	2.1	25.2	0.7	100	4.7	0.38	197
	V.7	2.7	1.3	3.8	26.9	0.0	100	7.6	0.31	141
	CG.4288	2.9	1.2	3.7	27.1	6.4	100	16.2	0.63	175
	CG.3007	2.7	1.2	3.3	28.8	0.3	100	15.5	0.54	181
	G.935	2.9	1.2	4.0	28.8	1.0	95	14.4	0.50	185
	CG.4814	2.9	1.2	4.2	29.3	3.7	100	13.4	0.46	183
	CG.6143	2.9	1.3	4.5	29.4	4.9	80	14.1	0.48	173
	CG.4202	3.2	1.2	4.0	29.6	0.0	95	9.6	0.32	181

Table 1.2 (Continued)

Cultivar	Rootstock[w]	Tree height (m)	Tree width (m)	Canopy volume (m^3)	Trunk cross-sectional area (cm^2)	Root suckers	Tree survival[x] (%)	Cumulative yield (kg)	Cumulative yield efficiency (kg/cm^2 TCA)	Mean fruit size[y] (g)
Golden Delicious	Supporter 4	3.1	1.2	4.1	30.5	0.3	100	5.4	0.21	198
	CG.6969	2.9	1.2	3.9	31.1	1.9	100	19.9	0.65	183
	CG.4004	3.0	1.2	4.7	32.9	1.2	100	14.0	0.42	184
	CG.6879	3.0	1.3	4.7	33.3	4.9	100	14.8	0.43	171
	CG.5030	3.1	1.3	4.6	33.4	10.7	100	13.0	0.43	180
	CG.6210	3.3	1.2	4.9	34.0	2.3	100	12.1	0.36	185
	M.26EMLA	3.0	1.2	3.6	35.3	0.1	90	7.5	0.21	183
	CG.7073	3.3	1.1	3.3	35.9	0.0	100	0.3	0.01	130
	MM.106	3.2	1.1	3.8	36.6	0.7	100	12.6	0.34	179
	CG.6874	3.2	1.2	4.7	36.8	2.7	100	17.1	0.46	190
	CG.6006	3.1	1.4	5.7	38.9	1.7	100	22.9	0.58	169
	MM.111	3.3	1.0	3.4	38.9	3.0	88	5.6	0.15	168
	V.1	3.0	1.3	4.3	40.3	0.0	100	11.5	0.28	193
	CG.5890	3.2	1.2	4.2	40.4	1.5	100	13.9	0.35	195
	M.7	3.2	1.2	3.9	41.3	9.5	100	3.6	0.09	180
	CG.6253	3.3	1.4	5.9	44.8	0.7	100	11.7	0.26	182
	V.4	3.5	1.1	3.9	46.6	3.0	67	3.5	0.12	177
	JTE-D	3.4	1.1	4.2	47.3	0.0	94	4.8	0.10	172
	NAGA	3.0	1.2	4.5	49.9	0.3	100	7.8	0.16	172
	CG.8534	3.7	1.3	5.5	52.5	0.0	90	4.4	0.08	175

Table 1.2 (Continued)

Cultivar	Rootstock[w]	Tree height (m)	Tree width (m)	Canopy volume (m^3)	Trunk cross-sectional area (cm^2)	Root suckers	Tree survival[x] (%)	Cumulative yield (kg)	Cumulative yield efficiency (kg/cm^2 TCA)	Mean fruit size[y] (g)
Golden Delicious	Marubakaido	3.7	1.3	5.2	56.3	0.0	100	9.5	0.17	183
	CG.6589	3.4	1.4	5.9	57.6	0.0	100	3.6	0.06	165
	CG.5463	3.9	1.3	6.1	60.5	0.3	100	2.7	0.04	163
	PiAu-56-83	3.5	1.4	6.6	62.3	0.0	100	6.6	0.11	172
	CG.4002	3.6	1.4	6.6	66.2	1.8	100	8.1	0.12	170
	JTE-C	3.6	1.2	5.6	71.0	0.0	100	5.1	0.07	159
	LSD p≤0.05	**0.3**	**0.5**	**1.5**	**10.4**	**5.0**	**31**	**6.0**	**0.25**	**20**
Honeycrisp	P.22	1.9	0.6	0.8	7.1	1.2	90	8.9	1.40	262
	G.65	2.0	0.7	0.8	8.0	2.1	100	12.5	1.76	261
	B.9-NE	2.3	0.8	1.2	9.0	4.6	100	13.2	1.50	285
	B.9-OR	2.3	0.8	1.3	9.7	1.9	100	16.5	1.71	293
	M.27	2.3	0.8	1.5	11.7	3.5	100	9.6	1.04	300
	G.11	2.6	1.0	2.4	13.1	0.6	100	22.5	1.72	292
	G.41	2.8	1.0	2.5	14.1	0.7	90	22.8	1.55	320
	M.9NAKBT337	2.7	0.9	2.0	14.7	2.1	100	10.7	0.73	308
	M.9Pajam1	2.7	0.9	1.9	15.3	2.0	90	13.3	0.82	310
	M.9	2.5	1.0	1.9	15.5	2.3	100	11.7	0.73	299
	M.9Nic29	2.6	0.9	2.1	15.5	3.5	100	11.6	0.83	286
	Supporter 4	2.7	0.9	2.1	15.6	1.9	100	10.3	0.64	313
	CG.4013	2.7	0.8	1.7	15.6	3.8	100	12.2	0.74	265

Table 1.2 (Continued)

Cultivar	Rootstock[w]	Tree height (m)	Tree width (m)	Canopy volume (m^3)	Trunk cross-sectional area (cm^2)	Root suckers	Tree survival[x] (%)	Cumulative yield (kg)	Cumulative yield efficiency (kg/cm^2 TCA)	Mean fruit size[y] (g)
	M.9EMLA	2.6	1.0	2.1	15.7	4.3	100	14.3	0.89	301
	Ottawa 3	2.7	1.0	2.3	16.2	1.7	100	12.2	0.71	302
	CG.3007	2.9	0.9	2.1	16.5	0.7	100	15.3	1.17	263
	M.26	2.7	0.9	2.0	16.9	1.4	100	16.1	0.96	302
	G.935	2.9	1.2	3.4	17.2	2.1	100	28.2	1.59	279
	M.9Nic8	2.7	0.9	2.1	17.3	1.8	100	16.7	0.93	299
	G.16	2.7	1.0	2.2	17.4	0.2	100	19.4	1.14	288
	M.9Pajam2	2.8	1.0	2.6	19.0	5.5	100	14.2	0.73	319
	CG.6210	3.1	1.2	4.3	26.1	3.4	100	36.2	1.35	319
	LSD p≤0.05	**0.3**	**0.1**	**0.6**	**3.8**	**3.0**	**11**	**7.1**	**0.50**	**23**

[w] Rootstocks ranked by increasing trunk cross-sectional area for each cultivar.

[x] Refers to tree death unrelated to experiment, cause undetermined

[y] Cropping was not excessive in 2004-2006. As a result, mean fruit size was not adjusted for crop load

[z]Means are Lsmeans from SAS Proc Mixed Procedure. Least Significant Difference indicated by LSD p≤0.05.

Results

Orchard Performance

'Gala' as the scion. 'Gala' trees with the smallest trunk cross-sectional area (TCA) were on 'B.9' sourced from The Netherlands ('B.9'-NE), 'B.9' sourced from Oregon ('B.9'-OR), 'G.41', 'G.935', and 'M.9NAKBT337' (Table 1.2). There was no significant difference between trees on 'B.9'-OR and 'B.9'-NE. The vigorous clones of 'M.9', 'M.9Burg756' and 'M.9Nic29', produced trees larger than 'M.9NAKBT337' similar in size to 'M.26' but the difference after 5 years was not significant. Trees with 'M.26NAKB' were not significantly different from those with 'M.26EMLA'. Among the JM rootstocks, 'JM.7' and 'JM.1' were the most dwarfing and produced trees similar in size to the vigorous clones of 'M.9', while 'JM.2' produced trees significantly larger. Among the PiAu rootstocks, 'PiAu-51-11' was the most dwarfing and produced trees similar to 'M.9Nic29' while trees with other three PiAu stocks ('PiAu-51-44', 'PiAu-36-2', and 'PiAu-56-83') were significantly larger. Among the Geneva® rootstocks, 'G.41' was the most dwarfing followed by 'G.935', which produced trees similar in size to 'M.9T337'. 'CG.3007' produced trees significantly larger than other Geneva® and CG rootstocks, and 'CG.3003' trees were the largest in the trial.

Tree canopy volume measurements and TCA measures were generally correlated (Figure 1.1). Exceptions included trees on 'P.14' and 'M.9Burg756', which had larger canopies than predicted based on their TCA, while 'PiAu-51-11' and 'PiAu-51-4' produced trees with smaller canopies than predicted.

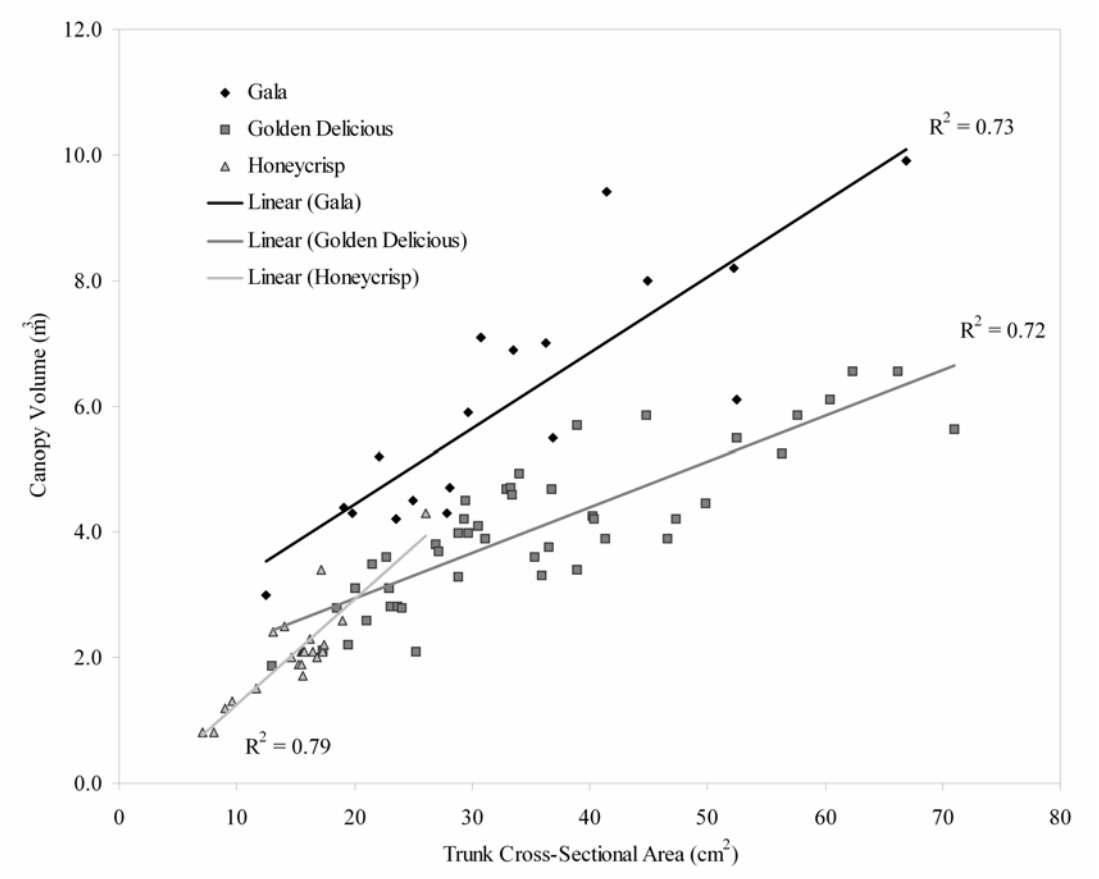

Figure 1.1 Relationship of trunk cross-sectional area and canopy volume of 64 apple rootstocks with 3 scion cultivars after 5 years.

The greatest number of root suckers (4-6) was recorded with 'G.935' and 'B.9'-NE (Table 1.2). The majority of rootstocks had few, if any, root suckers. Tree survival did not differ significantly among rootstocks, but 'G.935' had the lowest survival overall (Table 1.2).

The greatest cumulative yield was with 'JM.2' (46 kg) followed by 'G.935' (44 kg) and 'JM.7' (31 kg) (Table 1.2). The various clones of 'M.9' and many of the other rootstocks had intermediate yield while the PiAu stocks, 'P.14', and 'M.26' had the lowest yield.

The greatest cumulative yield efficiency (yield adjusted for tree size) was with trees on ‘B.9’-NE, ‘G.935’, ‘G.41’, and ‘B.9’-OR, followed by ‘JM.7’, ‘M.9Nic29’, ‘M.9NAKBT337’, ‘JM.1’, ‘JM.2’, and ‘M.26NAKB’. Clones of ‘M.9’ and ‘M.26’ along with ‘Supporter 4’ had intermediate yield efficiency while the PiAu stocks and ‘P.14’ had the lowest yield efficiency. Yield efficiency was negatively correlated with TCA (Figure 1.2). Exceptions included ‘B.9’-NE, 'G.935', and ‘JM.2’, which had higher yield efficiencies than predicted from their TCA, while ‘PiAu-51-11’ had lower yield efficiency than predicted from its TCA.

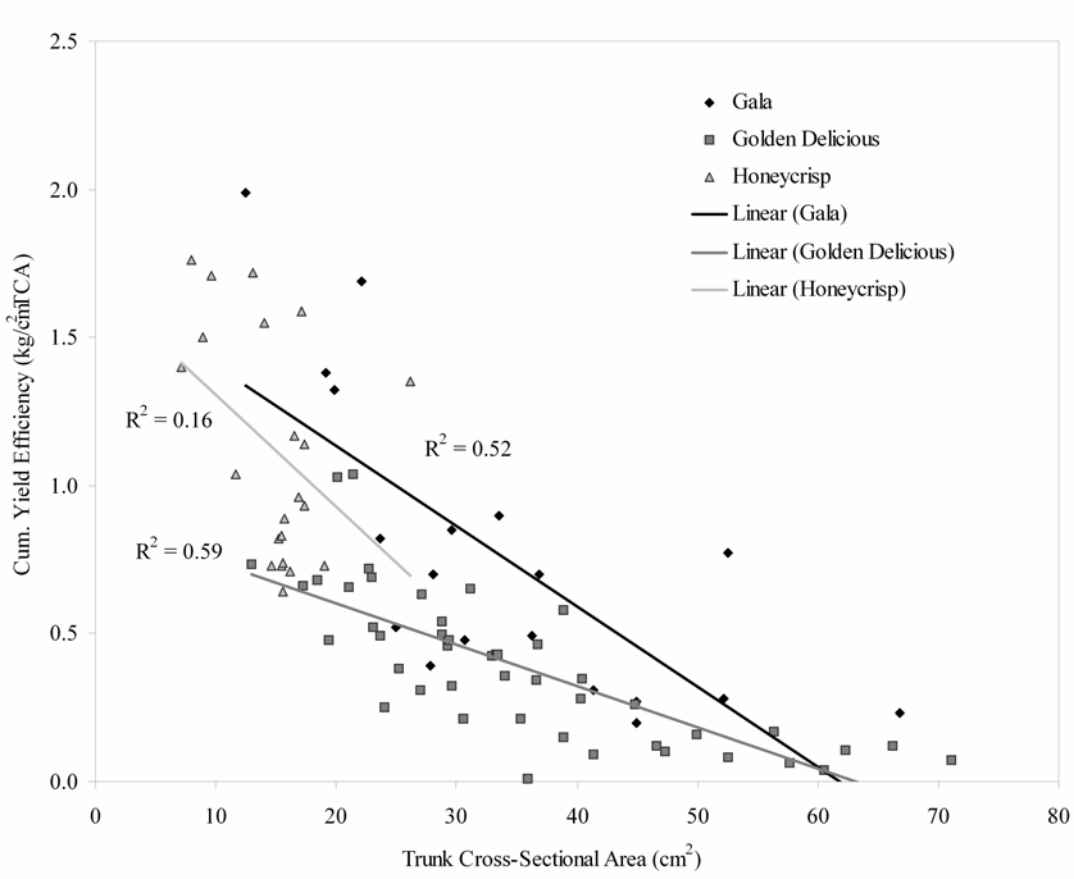

Figure 1.2 Relationship of trunk cross-sectional area and cumulative yield efficiency of 64 apple rootstocks with 3 scion cultivars after 5 years.

Average fruit size was largest with ‘JM.1’, ‘M.9Burg756’, and ‘Supporter 4’ while ‘CG.3007’, ‘PiAu-56-83’, ‘G.935’, and ‘M.26EMLA’ had the smallest fruit size (Table 1.2). The remaining rootstocks had intermediate fruit size that did not significantly differ from each other.

‘Golden Delicious’ as the scion. ‘Golden Delicious’ trees with the smallest TCA were on ‘CG.2406’ (Table 1.2). Trees, similar to ‘M.9’, were on ‘CG.4013’, ‘V.2’, ‘V.3’, ‘B.9’-NE, ‘G.16’, ‘CG.4011’, ‘Ottawa 3’, ‘B.9’-OR, ‘G.11’, ‘G.41’, ‘JTE-B’, and ‘V.7’. There was no significant difference between trees on ‘B.9’-OR and ‘B.9’-NE. A third group was similar in size to ‘M.26’ and included ‘G.935’, ‘Supporter 4’, 'CG.4814', ‘CG.4202’, ‘CG.6210’, 'CG.6969', with 7 lesser known CG rootstocks. A fourth group, comparable in size to ‘M.7’ and ‘MM.111’ trees, included ‘V.1’, ‘V.4’, ‘JTE-D’, 'CG.6874', 'CG.6006' and 2 lesser-known CG rootstocks. The most vigorous group included ‘Marubakaido’, ‘PiAu-56-83’, ‘JTE-C’, and 4 CG rootstocks.

Tree canopy volume measurements and TCA measures were generally correlated (Figure 1.1). Exceptions included ‘CG.6006’, which had a larger canopy than predicted from its TCA while ‘JTE-B’ had a smaller canopy than predicted from its TCA.

The greatest number of root suckers (9-10) was recorded with ‘CG.5030’, ‘M.7’, and ‘B.9’-NE (Table 1.2). ‘CG.4288’, ‘CG.4011’, ‘CG.6879’, and ‘CG.6143’ had 4-6 root suckers while the remaining rootstocks had fewer than 3. Tree survival was significantly lower than 100% with ‘V.3’ and ‘V.4’. Tree survival for the remaining rootstocks did not differ significantly from 100% (Table 1.2).

The greatest cumulative yield (19-23 kg/tree) was with trees on ‘CG.6006’ followed by ‘CG.4011’, ‘B.9’-NE, and ‘CG.6969’ (Table 1.2). An intermediate yielding group included ‘CG.6874’, ‘CG.4288’, ‘CG.3007’, ‘CG.6874’, ‘B.9’-OR, ‘G.935’, ‘CG.5890’, ‘G.16’, ‘CG.4814’, ‘CG.5030’, ‘MM.106’, ‘V.2’, ‘G.11’,

‘CG.6210’, ‘CG.4013’, ‘CG.6253’, ‘G.41’, ‘V.1’, and ‘Ottawa 3’. The lowest yielding group included ‘M.9’, ‘M.26’, ‘V.3’, ‘V.7’, ‘Supporter 4’, ‘MM.111’, ‘M.7’, ‘V.4’, and many others. Many of the trees planted with vigorous rootstocks had low yield.

The greatest cumulative yield efficiency generally was with the most dwarfing rootstocks. The rootstocks with the highest yield efficiency were ‘CG.4011’, ‘B.9’-NE, ‘CG.2406’, ‘Ottawa 3’, ‘B.9’-OR, ‘V.2’, ‘CG.4013’, ‘CG.6969’, ‘CG.4288’, ‘CG.3007’, ‘G.11’, ‘G.935’, and ‘G.41’. All of the Malling rootstocks (‘M.9’, ‘M.26’, ‘M.7’, ‘MM.106’, and ‘MM.111’) and ‘Supporter 4’ had intermediate to low yield efficiency while the PiAu stocks and ‘P.14’ had the lowest yield efficiency. Yield efficiency was negatively correlated with TCA (Figure 1.2). Exceptions included ‘B.9’-NE, 'CG.4011', and ‘CG.6006’, which had higher yield efficiencies than predicted from their TCA, while ‘M.9T337’, 'Supporter 4', 'CG.7073', and 'M.7' had lower yield efficiency than predicted from their TCA.

Average fruit size was largest with ‘JM.1’, ‘M.9Burg756’, and ‘Supporter 4’ while ‘CG.3007’, ‘PiAu-56-83’, ‘G.935’, and ‘M.26EMLA’ had the smallest fruit size (Table 1.2). The remaining rootstocks had intermediate fruit size and did not differ significantly from each other.

‘Honeycrisp’ as the scion. ‘Honeycrisp’ trees with the smallest TCA were on ‘P.22’, ‘G.65’, ‘B.9’-NE, ‘B.9’-OR, and ‘M.27’. A slightly larger group, similar in size to ‘M.9’, included ‘G.11’, ‘G.41’, ‘Supporter 4’, ‘CG.4013’, and 3 clones of 'M.9' (T337, Pajam1 and Nic29). A third group which was similar in size to ‘M.26’ included ‘Ottawa 3’, ‘CG.3007’, ‘G.935’, ‘G.16’, and the vigorous ‘M.9’ clones (Nic8 and Pajam2), (Table 1.2). ‘CG.6210’ was significantly larger than other CG rootstocks and was the largest rootstock in the trial.

Tree canopy volume measurements and TCA measures were generally correlated (Figure 1.1). Exceptions included 'G.935' which had a larger canopy volume than predicted from its TCA.

The greatest number of root suckers (3-6) was recorded with ‘M.9Pajam2’, ‘B.9Europe’, ‘M.9EMLA’, ‘CG.4013’, ‘M.27’, ‘M.9Nic29’, and ‘CG.6210’. The remaining rootstocks had fewer than 3 root suckers. Tree survival did not differ significantly among rootstocks (Table 1.2).

The greatest cumulative yield (36.2 kg) was with ‘CG.6210’ followed by ‘G.935’, ‘G.41’, ‘G.11’, and ‘G.16’. 'P.22' and 'M.27' had the lowest yield. The remaining rootstocks had intermediate yield.

The rootstocks with the highest yield efficiency were ‘G.65’, ‘G.11’, ‘B.9’-OR, ‘G.935’, ‘G.41’, ‘B.9’-NE, ‘P.22’, and ‘CG.6210’. The remaining rootstocks did not differ in cumulative yield efficiency, but ‘Supporter 4’, ‘M.9’, and ‘M.9NAKBT337’ had the lowest overall yield efficiency. Yield efficiency was negatively correlated with TCA (Figure 1.2). Exceptions included 'CG.6210', 'G.935', 'G.11', 'G.41', ‘B.9’-OR, and ‘G.65’, which had higher yield efficiencies than predicted from their TCA, while ‘M.9’, 'Supporter 4', 'CG.4013', and 'Ottawa 3' had lower yield efficiency than predicted from their TCA.

Average fruit size was largest with ‘G.41’, ‘CG.6210’, 'M.9Pajam2', ‘Supporter 4’, 'M.9Pajam1', 'M.9T337', ‘Ottawa 3’, ‘M.26’, and ‘M.27’, while ‘G.65’, ‘P.22’, ‘CG.3007’, and ‘CG.4013’ had the smallest fruit size. All ‘M.9’ rootstocks had large fruit size except for ‘M.9Nic29’. The two clones of 'B.9’ had smaller fruit size than 'G.41' or 'CG.6210'. The remaining rootstocks had intermediate fruit size and did not differ significantly from each other (Table 1.2).

Rootstock blight experiment

In 2004 a natural epidemic of fire blight developed in the test orchard, and several ‘Gala’ and ‘Honeycrisp’ trees developed rootstock blight as a result. Trees with rootstock infections were recorded and removed prior to the 2005 season. Shoot blight was pruned out of the orchard at the end of 2004 and did not affect the 2005 inoculation trial. Incidence of blossom infection in 2005 was uniform across all three cultivars. Symptoms were first observed on 21 July 2005 and new infections continued to develop through October 2005. ‘Gala’ and ‘Honeycrisp’ suffered severe shoot blight as a result of the blossom inoculation. The canopies of these two cultivars were largely destroyed by fire blight such that during the winter 2006 pruning, 94% of ‘Gala’ and 60% of ‘Honeycrisp’ trees had most of the canopy removed, regardless of rootstock infection. The cultivar ‘Golden Delicious’ had noticeably less shoot blight and no trees were removed in 2006. The degree of rootstock mortality was likewise elevated in cultivars ‘Gala’ and ‘Honeycrisp’ compared with ‘Golden Delicious’. Based on these observations and the low number of rootstocks shared between cultivars, data from ‘Gala’ and ‘Honeycrisp’ trees were combined and analyzed separately from ‘Golden Delicious’. Logistic regressions indicated the probability of developing rootstock blight was significantly affected by rootstock for both ‘Gala/Honeycrisp’ and ‘Golden Delicious’ cultivars at P=0.05 (Table 1.3). The effect of scion and the interaction of scion and rootstock on rootstock blight were not significant for the 'Gala/Honeycrisp' analysis.

Table 1.3. Effect of rootstock on the probability of developing rootstock blight.

		‘Gala’ / ‘Honeycrisp’		
	Df	Deviance	Likelihood ratio test	Pr (Chi)
NULL		240.48		
Scion	1	242.04	1.56	0.21
Rootstock	11	282.70	42.21	<0.0001*
Scion × Rootstock	3	243.57	3.09	0.38
		‘Golden Delicious’		
	Df	Deviance	Likelihood ratio test	Pr (Chi)
NULL		67.90		
Rootstock	6	84.80	16.91	0.01*

*Significant at $p \leq 0.05$

‘Gala’ and ‘Honeycrisp’ as the scion. Twelve rootstock cultivars were found to have elevated probability of developing rootstock blight with ‘Gala’ or ‘Honeycrisp’ as the scion (Table 1.4). Susceptible rootstocks included all four ‘M.9’ clones (Burg756, EMLA, NAKBT337, and Nic29), the three ‘M.26’ clones (M.26, EMLA, NAKB), as well as ‘Ottawa 3’, ‘P.22’, ‘JM.2’, ‘Supporter 4’, and ‘M.27’. Eight rootstocks had a significantly lower probability of developing rootstock blight, and two rootstocks were designated resistant since they had no rootstock infection. Among these, ‘PiAu-51-4’ and ‘P.14’ were slightly more resistant to rootstock blight than ‘PiAu-56-83’. There was no significant difference between ‘B.9’-OR and ‘B.9’-NE. All of the Geneva® rootstocks evaluated had high levels of resistance to rootstock blight.

Table 1.4. Effect of rootstock on probability of developing rootstock blight with either 'Gala' or 'Honeycrisp' as the scion.

Rootstock	Mean blossom infection 2005 (%)	Rootstock blight (2004)	Rootstock blight (2005)	Tree total	Proportion infected	Standard error
M.26	60	1	13	15	0.93	0.06*
M.9NAKBT337	89	1	5	7	0.86	0.13*
Ottawa 3	70	1	15	19	0.84	0.08*
M.9EMLA	76		15	19	0.79	0.09
M.26EMLA	85	1	5	8	0.75	0.15*
M.26NAKB	88		6	9	0.67	0.16*
P.22	80	2	10	16	0.75	0.11*
JM.2	95	1	2	5	0.60	0.22*
M.9Nic29	85		4	7	0.57	0.19*
M.9Burg756	87	1	4	9	0.56	0.17*
Supporter 4	81		12	25	0.48	0.10*
M.27	86		5	20	0.40	0.11*
PiAu-56-83	79		2	9	0.22	0.14
G.935	71		1	8	0.13	0.12
G.11	81		2	17	0.12	0.08
G.65	83	1	0	10	0.10	0.09
G.41	84		1	26	0.04	0.04
P.14	90		1	10	0.10	0.09
B.9-NE	70	1	0	19	0.05	0.05
B.9-OR	73		1	29	0.03	0.03
PiAu-51-4	82		0	9	n.a.[z]	
G.16	72		0	18	n.a.	

*Significant probability of developing rootstock blight.

[z]n.a. = Not analyzed. No rootstock blight recorded during 2004-2005 seasons.

'Golden Delicious' as the scion. There was a marked reduction in rootstock blight with 'Golden Delicious' as the scion compared to either 'Gala' or 'Honeycrisp'. Only three rootstocks had elevated probability of developing rootstock blight, 'M.26', 'Ottawa 3', and 'M.9EMLA', reflecting the results for 'Gala' and 'Honeycrisp'. Of the forty-two rootstocks tested, thirty-five failed to develop any observed rootstock blight symptoms despite high percentages of flower infection (Table 1.5). As a group, the Geneva® rootstocks as well as the Vineland and JTE series demonstrated high levels of resistance to rootstock blight. As with 'Gala' and 'Honeycrisp' there was no significant difference between 'B.9'-OR and 'B.9'-NE with regard to disease resistance. Development of rootstock blight was not significantly affected by scion cultivar in nine out of the ten rootstocks that were evaluated in both cultivar groups. 'M.26', 'M.9EMLA', and 'Ottawa 3', had less overall rootstock blight with 'Golden Delicious' as the scion. Conversely, 'Supporter 4' was found to be highly susceptible with 'Gala' and 'Honeycrisp' as the scion, but had no observed rootstock blight with 'Golden Delicious' as the scion.

Table 1.5. Effect of rootstock on probability of developing rootstock blight with 'Golden Delicious' as the scion.

Rootstock	Mean blossom infection 2005 (%)	Rootstock blight (2005)[y]	Tree total	Proportion infected	Standard error
M.26	74	6	9	0.67	0.16*
Ottawa 3	70	6	10	0.60	0.15*
M.9EMLA	84	4	10	0.40	0.15*
G.11	79	2	10	0.20	0.13
CG.4288	78	1	10	0.10	0.09
CG.6210	76	1	10	0.10	0.09
B.9-NE	68	1	10	0.10	0.09
B.9-OR	68	0	10	n.a.[z]	
G.41	77	0	7	n.a.	
G.16	65	0	10	n.a.	
G.935	77	0	10	n.a.	
CG.2406	73	0	10	n.a.	
CG.3007	78	0	10	n.a.	
CG.4002	77	0	10	n.a.	
CG.4004	72	0	10	n.a.	
CG.4013	70	0	10	n.a.	
CG.4202	79	0	9	n.a.	
CG.4814	71	0	9	n.a.	
CG.5030	74	0	10	n.a.	
CG.5463	80	0	10	n.a.	
CG.5890	75	0	10	n.a.	
CG.6006	81	0	10	n.a.	
CG.6143	79	0	10	n.a.	
CG.6253	73	0	10	n.a.	
CG.6589	86	0	7	n.a.	

Table 1.5 (Continued)

Rootstock	Mean blossom infection 2005 (%)	Rootstock blight (2005)[y]	Tree total	Proportion infected	Standard error
CG.6874	75	0	10	n.a.	
CG.6969	78	0	10	n.a.	
CG.8534	79	0	10	n.a.	
JTE-B	80	0	9	n.a.	
JTE-C	82	0	10	n.a.	
JTE-D	80	0	8	n.a.	
M.7	66	0	10	n.a.	
Marubakaido	81	0	10	n.a.	
MM.106	68	0	10	n.a.	
NAGA	85	0	6	n.a.	
PiAu-56-83	83	0	9	n.a.	
Supporter 4	77	0	9	n.a.	
V.1	70	0	10	n.a.	
V.2	78	0	10	n.a.	
V.3	79	0	10	n.a.	
V.4	86	0	10	n.a.	
V.7	80	0	10	n.a.	

[*]Significant probability of developing rootstock blight.

[y]No tree death recorded in 2004 from rootstock blight

[z]n.a. = Not analyzed. No rootstock blight recorded during 2005 season.

Discussion

Results from the three cultivars tested varied slightly, but overall rootstock responses with regard to size control and yield efficiency were consistent across cultivars (Autio et al., 2006ab). Cumulative yield efficiency provided a uniform method for comparing rootstock productivity. The close correlation between canopy volume and TCA (Figure 1.1), with few exceptions, supported the use of TCA as a comprehensive measure of tree size for trees that had not been containment pruned. Based on the relationship between canopy size and production potential, it was not unexpected that the most dwarfing rootstocks had the highest yield efficiency. These results support previous research where smaller canopy volume coupled with higher tree density increased cumulative yield potential of an orchard site (Hampson et al., 2002, 200a4, 2004b; Robinson and Lakso, 1991). However, there were notable exceptions to the rule that dwarfing rootstocks are the most yield efficient. 'CG.6006' with 'Golden Delicious' and 'CG.6210', and 'G.935' with 'Honeycrisp', all semi-dwarfing rootstocks, had higher yield efficiency than expected for their tree size. Similarly several dwarfing rootstocks showed lower than expected yield efficiency with 'Honeycrisp'. 'Honeycrisp' as expected was biennially bearing during the course of the experiment, and therefore requires further testing to validate the effect of rootstock on yield efficiency. It should be noted that high yield efficiency, although important, must not be achieved at the expense of fruit size, which significantly affects crop value. During the course of this trial, however, crop load was not excessive and there was no significant relationship between yield efficiency and fruit size.

Our results indicate that several new dwarfing rootstocks exceed the productivity of M.9, which has been the world standard. High-density orchards with these rootstocks should produce greater yields, thus reducing costs per kg of fruit (Hampson et al. 2002; Robinson and Lakso, 1991). The few semi-dwarfing rootstocks that had

higher than expected yield efficiency would allow higher yielding moderate density orchards than previously possible.

Some of the fire blight resistant rootstocks evaluated demonstrated considerable tolerance to rootstock blight during the 2004 and 2005 field seasons. Rootstock was the main factor influencing the development of rootstock blight, but a greater level of rootstock blight was observed with 'Gala/Honeycrisp' trees than with 'Golden Delicious'. 'Gala' and 'Honeycrisp' are both highly susceptible cultivars, which suffered severe shoot infection as a result of the 2005 inoculation. 'Golden Delicious' in comparison, previously described as intermediately susceptible to fire blight (Gardner et al., 1980), had less severe scion infection and lower incidence of rootstock blight. Rootstocks 'M.9' and 'M.26' each experienced a 30% reduction in disease incidence when planted with 'Golden Delicious' compared with 'Gala' or 'Honeycrisp'. Based on these observations rootstocks evaluated only using 'Golden Delicious' as the scion require additional examination before an accurate assessment of rootstock blight sensitivity can be made. The effect of scion cultivar on rootstock blight development clearly demonstrates the need for fire blight resistant rootstocks when planting susceptible cultivars. Conversely, fire blight 'tolerant' rootstocks may provide a measure of protection against rootstock blight when moderately susceptible scion cultivars are being considered.

The Malling rootstocks have persisted as the standard dwarfing rootstocks for over fifty years. 'M.9' clones performed well in orchard trials but slight variation was observed with regard to tree size and cumulative yield efficiency. The more vigorous 'M.9' clones, including 'M.9Burg756', 'M.9Nic29', and 'M.9Pajam2', produced larger than expected trees with reduced yield efficiency. Marini et al. (2006a) reported slight variation in tree size and yield among 'M.9' clones, but discrepancies were largely insignificant and varied by location. 'M.9' clones had satisfactory yield

efficiency but were often inferior to more advanced rootstock selections (Table 1.2) as well as far more susceptible to fire blight (Table 1.4, 1.5). As a group, the Malling rootstocks were highly susceptible to rootstock blight, with 'M.26' and 'M.9' suffering tree loss between 56 and 93% when grafted to a highly susceptible scion cultivar.

All of the Geneva® rootstocks evaluated had significantly lower probability of developing rootstock blight than the standard Malling rootstocks. Even 'G.11', previously described as fire blight tolerant (Norelli et al., 2003), had significantly less overall rootstock blight, even with the highly susceptible cultivars 'Gala' and 'Honeycrisp'. 'G.41' and 'G.935' performed exceedingly well with all cultivars, producing trees comparable in size to less vigorous 'M.9' clones with greater cumulative yield efficiency. 'G.41' and 'G.935' also maintained good fruit size, although 'Gala' fruit size was reduced with 'G.935'. 'G.16', with tree size comparable to more vigorous 'M.9' clones, had moderate yield efficiency. The main concern with 'G.16' remains its sensitivity to latent viruses, which necessitates the use of virus-free scion wood at budding (Johnson et al., 2001). Several unreleased CG rootstocks, particularly 'CG.4011' and 'CG.4013', showed considerable promise for future release, although further evaluation is necessary to verify orchard performance and disease resistance.

'B.9' rootstock from nurseries in both Oregon and The Netherlands produced trees comparable in size to the less vigorous 'M.9' clones. Although average fruit size was comparable to 'M.9', cumulative yield efficiency exceeded 'M.9' clones for all three cultivars. 'B.9' also demonstrated high levels of resistance to rootstock blight development, demonstrating its potential for sites with a history of fire blight infection. This is in contrast to initial reports that indicated 'B.9' was highly susceptible to fire blight. Those evaluations were done by inoculating 'B.9' plants

directly rather than by inoculating a scion cultivar grafted on ‘B.9’ (Cummins and Aldwinckle, 1983; Norelli et al., 2003; Travis et al., 1998). Our data support the findings of Norelli et al. (2003) that showed significant resistance of ‘B.9’ to rootstock blight in field plantings. Anecdotal evidence from commercial orchards supports the resistance of ‘B.9’ to rootstock blight when tested as a grafted tree. This anomaly of susceptibility as a non-grafted plant but resistance as a rootstock is the subject of ongoing research.

Plant material of ‘B.9’ from The Netherlands and US nursery suppliers was virtually identical in tree size, yield, fruit size, and disease resistance, but ‘B.9’-NE produced significantly more rootstock suckers than ‘B.9’-OR, with all cultivars. Slight variation may exist in the ‘B.9’ population accounting for this discrepancy and other unexplained differences in nursery stock (Norelli et al., 2003). These data support anecdotal reports from nursery growers that ‘B.9’ is not completely genetically uniform.

Several of the Japanese JM rootstocks had promising results. All three JM rootstocks, ‘JM.1’, ‘JM.2’, ‘JM.7’, had high cumulative yield efficiency and good fruit size. Unfortunately only ‘JM.2’ was included in the disease resistance trial, where it proved susceptible to rootstock blight.

The PiAu rootstocks, which originated from the Dresden Pillnitz breeding program in Germany, including ‘Supporter 4’ produced trees larger than expected. Of the four rootstocks tested only ‘PiAu-51-11’ produced a tree comparable to ‘M.9’ in size. As a group the PiAu rootstocks were moderately resistant to rootstock blight, but their low yield efficiency negates the usefulness of these rootstocks in dwarf production systems.

The Vineland rootstocks, from Ontario, Canada, produced a wide range of tree sizes with varying levels of productivity. Three rootstocks ‘V.2’, ‘V.3’, and ‘V.7’

produced trees similar in size to ‘M.9’ while ‘V.1’ and ‘V.4’ were sized closer to ‘M.26’. One major disadvantage of the Vineland series was lower than expected yields. One rootstock, ‘V.2’, demonstrated significant promise producing a tree equivalent in size to ‘M.9’ with high cumulative yield efficiency. Consistent with other Vineland rootstocks, ‘V.2’ was highly resistant to rootstock blight, but resistance evaluation was only done with the cultivar Golden Delicious. These results support work by Cline et al. (2001) and Ferree et al. (2002) in which the Vineland series maintained a significant level of resistance to fire blight in inoculated and naturally infected field trials.

In these studies, tree loss due to rootstock blight was considerable. High losses using conventional rootstocks emphasize the need for novel rootstock selections that promote good orchard performance coupled with functional disease resistance. Disease resistant rootstocks are a reliable and cost-effective method to enhance the survival of young trees during initial years of orchard establishment (Cline et al., 2001; Schupp et al., 2002). Several rootstock selections evaluated during the course of this study show considerable promise as alternatives to ‘M.9’ in future plantings.

These results represent the combined orchard performance and rootstock blight resistance data of 64 apple rootstocks after five years of orchard evaluation. Five years is often too short a time to critically evaluate rootstock performance. A complete summary after ten years should provide more conclusive information regarding the influence of rootstock on orchard performance.

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CHAPTER 2

FIRE BLIGHT RESISTANCE OF BUDAGOVSKY 9 APPLE ROOTSTOCK

Abstract

Erwinia amylovora, the causal agent of fire blight, can also cause a fatal infection of apple rootstocks known as rootstock blight. Budagovsky 9 (B.9) apple rootstock is reported to be highly susceptible to rootstock blight, although multiple field trials report B.9 to be resistant to rootstock blight infection. Conflicting results may stem from genetic variation in the clonal B.9 population, based on phenotypic differences in rootstock material. Genetic testing, using twenty-three microsatellite loci, confirmed the clonal uniformity of B.9 in commerce. Variation in growth habit between B.9 rootstocks originating from two nurseries has also been discounted as a source of disease resistance. Inoculation of grafted and non-grafted shoot tissue versus woody rootstock tissue has revealed the existence of a novel resistance phenotype. B.9 rootstock are susceptible to leaf inoculation by *E. amylovora*, statistically similar to the susceptible rootstock Malling 9 (M.9). Conversely, inoculation assays targeting mature tissue reveal a high degree of resistance in B.9, whereas M.9 remains susceptible. Although the mechanism by which B.9 develops resistance is unknown, our results support the development of adult plant resistance in B.9 apple rootstock. Durable fire blight resistance correlated with tissue development could be a valuable tool for rootstock breeders.

Introduction

Fire blight caused by the bacterium *Erwinia amylovora* [(Burr.) Winslow et al.], is a devastating disease of rosaceous plants. Present in over 40 countries, fire blight is a constant threat to apple (*Malus* X *domestica* Borkh.) production worldwide. Most commonly associated with blossom and shoot blight, *E. amylovora* may also cause an infection of the apple rootstock known as rootstock blight (41). Rootstock cankers develop rapidly, stimulating an untreatable and lethal infection (22,27). In recent years rootstock blight has generated considerable financial losses due to lost production and cost of replanting. Most years, rootstock blight is sporadic resulting in isolated tree death, but under severe fire blight conditions tree losses of 50% and greater have been reported (8,31).

Mounting reports of rootstock blight can be attributed to the increased use of susceptible dwarfing rootstocks in high-density orchards. High-density orchards require less land, accelerate cropping, generate higher cumulative yields, and produce a greater percentage of premium fruit, providing a vital economic advantage in a competitive industry (7,12,32). M.9 rootstock, the industry standard dwarfing rootstock, is particularly susceptible to rootstock blight. Robinson et al. (31) and Ferree et al. (8) reported significantly higher levels of tree mortality for M.9 rootstock, compared to rootstock selections moderately or fully resistant to fire blight. Fire blight resistant apple rootstocks are the only known method of preventing rootstock blight. Once bacteria breach the plant surface, no cultural or chemical control can prevent disease development (27).

Dwarfing rootstocks conferring desirable horticultural traits and disease resistance are crucial for the advancement of the apple industry. Budagovsky 9 (B.9) is a lesser-known, but increasingly popular, dwarfing rootstock similar in size class and productivity to M.9 (2). Historically, B.9 had been reported as susceptible to fire

blight infection (4,6) when inoculated as a non-grafted rootstock. However B.9 has exhibited a significant level of rootstock blight resistance in naturally infected and artificially inoculated field trials using grafted trees (8,27,31,35,37). Contrary to similar trials Travis et al. (40) reported that B.9 was highly susceptible to infection by *E. amylovora* when grafted in combination with six scion cultivars. In this particular experiment, rootstock infection was evaluated as scion lesions progressed into rootstock tissue and not as an isolated infection, which is the principal characteristic of rootstock blight. Norelli et al. (27) conducted experiments simultaneously comparing infection of shoot-inoculated, non-grafted rootstocks with rootstock blight development in blossom-inoculated grafted trees. Results indicated that B.9 was susceptible to shoot inoculation but resistant to rootstock blight development as grafted trees. This was the first reported case of differential fire blight resistance in apple rootstock material, when acting as a scion or grafted rootstock.

Phenotypic variation in B.9 plants from different nurseries has been widely reported within the horticultural community. B.9 rootstock plants sourced from European nurseries have flatter branches and more trailing growth than B.9 plants sourced from USA nurseries. Since B.9 is clonally propagated this variation likely originated from a small mutation early in the commercialization process either in the USA or Europe. Such phenotypic differences in clonally propagated rootstocks are well known with more than 20 strains of the common M.9 rootstock (44).

Genetic differences in B.9 from European and US nurseries may account for the observed variation in morphological characteristics as well as in fire blight resistance. Simple sequence repeats (SSRs) have been extensively used to verify the genetic relationships among *Malus* species and hybrids. SSR markers are PCR based, polymorphic in nature, and easily reproducible. Distributed across the *Malus* genus, SSRs facilitate the systematic comparison of genetic identity and relatedness

(10,14,15). Guilford et al. (11) differentiated 21 closely related apple cultivars using a minimum of 3 SSR markers. Over two hundred SSR sequences are currently available for genetic fingerprinting of apple (10,11,14,15,17,28,38).

Although B.9 has been shown to be susceptible to fire blight as a non-grafted plant and resistant as a grafted tree it is not known if this resistance is due to observed genetic variation between rootstock sources or due to a novel resistance mechanism. The development of rootstock blight is closely associated with the ability of bacteria to migrate systemically through host tissue and colonize rootstock tissue (22,27). The objectives of this study were to clarify conflicting reports dealing with the resistance of B.9 rootstocks to *E. amylovora* and to better understand the nature of fire blight resistance. Verification of B.9's genetic identity and fire blight resistance will support recommendation of B.9 as a resistant apple rootstock to succeed M.9 in high-density systems. Bacterial migration and resistance assays were performed to determine the effect phenotypic variants of B.9 had on bacterial movement *in vivo* and subsequent rootstock colonization.

Materials and Methods

Molecular marker analyses

B.9 rootstock material, designated B.9-OR and B.9-NE, was obtained from TRECO, Inc. and Janssen Bros. Nursery, respectively. Additional apple (*Malu*s spp.) tissue, including three cultivated rootstocks, Malling 8 (M.8) the maternal parent of B.9 (2), M.9, and Robusta 5 (*M*. x robusta) and four wild accessions, two *Malus sieversii* (Ledeb.) M. Roem, 'Niedzwetzkyana' (GMAL 3563.g and GMAL 3781.c), *M. pumila* (Miller) 'Niedzwetzkyana' (PI 589225) and one *M. sylvestris* (L.) Miller, with uncharacteristic red pigmentation (PI 392302), were acquired through the USDA-ARS Plant Genetic Resources Unit's collection of *Malus* germplasm in Geneva, NY.

Wild *Malus* accessions of diverse genetic background were chosen to represent the unknown paternal parent of B.9, 'Red Flag.'

Total genomic DNA was isolated from young leaves via the Wizard® Genomic DNA Extraction Kit (Promega, Madison WI). Additional polysaccharide precipitation of B.9 DNA was conducted according to Rhodes (33). A total of twenty-three SSR markers distributed over *Malus's* seventeen linkage groups were evaluated, including twenty SSR loci previously described by Liebhard et al. (17) [CH02a08z, CH02c02b, CH02c09, CH02c11, CH02d08, CH02g09, CH03a04, CH03d08, CH04c06, CH04c07, CH04g07, CH05d08, CH05e03, CH05e06, CH05f04], Silfverberg-Dilworth et al. (38) [Hi11a03], and Hokanson et al. (14) [GD12, GD96, GD100, GD162], as well as three previously undisclosed markers [GD6, GD136, GD158] (Hokanson, unpublished data). SSR markers amplified in 15 μl PCR reaction mixtures containing 20 ng genomic template DNA, 2 mM $MgCl_2$, 3 μl 5X Flexi Buffer (Promega, Madison WI), 0.04mM dNTP's, 0.25 units GoTaq® DNA Polymerase (Promega, Madison WI), and 0.32 μM of each primer. PCR reactions were denatured at 95°C for 5 min followed by 30 cycles at 95°C for 30 s, primer annealing at 52.3°C, 57.7°C, 61.1°C, or 63.6°C (14,17,38) for 45 s, 72°C primer elongation for 45 s and a 7 min extension at 72°C. Amplified products were analyzed using an ABI Prism 310 Genetic Analyzer, GeneScan program (Applied Biosystems, Inc.). Band size and allele binning were based on internal size standard (ROX™, Applied Biosystems Inc) using Genotyper ver. 3.7 (Applied Biosystems, Inc). Genetic distance estimates were calculated using a Jaccard coefficient as described by Staub et al. (39) and Landry & Lapointe (16). Cluster analysis and phenogram were computed using the numerical taxonomy program NTSYS-pc, ver. 2.01 (34).

Plant Material

In 2002, duplicate orchard plots were planted at two locations at the New York State Agricultural Experiment Station, Geneva, NY. One plot used grafted and non-grafted plants in 3 gal containers with field soil, while the second plot was planted in the ground at uniform tree spacing (1 x 3 m). In both plantings featherless trees were headed back to 56 cm, and branches, which developed after heading, were trained below horizontal in the summer of 2002 to promote early flowering. Four scion cultivars, 'Gala', 'Jonagold', 'Gingergold', and 'Red Yorking', were planted in combination with three rootstocks B.9-NE, M.9, and Geneva® 16 (G.16), except for 'Gingergold' which was not available on G.16. The rootstock B.9-OR was only available grafted to 'Gala'. Twelve scion/rootstock combinations were planted in total. Non-grafted rootstocks, B.9-NE, B.9-OR, M.9, and G.16, were planted at the same time as the grafted trees and under identical conditions.

Shoot Inoculation of Non-grafted Rootstocks

Non-grafted rootstocks B.9-NE, B.9-OR, M.9, and G.16 were simultaneously grown under field and greenhouse conditions. In 2003 vigorously growing shoots were inoculated by transversely bisecting the two youngest leaves with scissors dipped in a suspension of *E. amylovora* strain E4001a (Nal^rRp^r) ($1x10^7$ cfu/ml) in potassium phosphate buffer (PPB) (0.05 M) (22,24). Strain E4001a (Nal^rRp^r) was chosen based on its virulence and selective antibiotic markers (25,26). Up to five shoots were inoculated per rootstock, with individual rootstocks as the unit of replication. Control plants were mock inoculated with PPB (0.05 M). Lesion length was recorded, when lesions ceased progressing as a percent of the current years shoot length, described as percent infection, and used as a measure of susceptibility (24). Mean percent lesion length was analyzed using one-way analysis of variance; means were adjusted for

missing trees and significance determined using least square means at a P value of 0.05.

Bacterial Migration

The influence of rootstock variety on bacterial migration was assessed by inoculating the above ground portion of grafted and non-grafted trees followed by sequential sampling of host tissue for the presence of *E. amylovora*. Due to inconsistent flowering in 2003, five shoots per tree were inoculated to ensure a high level infection, bisecting the two youngest leaves transversely with scissors dipped in a suspension of *E. amylovora* strain E4001a (Nal^rRp^r) ($5x10^8$ cfu/ml) in PPB (0.05 M) (22). Lesion length was evaluated as a percent of the current year's shoot growth to verify cultivar susceptibility. In 2004 flowering was consistent across all cultivars. Trees at 60% of full bloom were sprayed with *E. amylovora* strain E4001a (Nal^rRp^r) ($1x10^8$ cfu/ml). Inoculum concentration was reduced to normalize infection levels from 2003 to 2004, based on more favorable conditions for fire blight infection. Infected blossoms were recorded as a percent of total blossoms. Trees were randomly sampled when scion lesions ceased progressing, approx. six weeks after inoculation. Eight trees in 2003, and fourteen in 2004, per scion combination were sampled; not all combinations were available for all replicates. Trunks were surface sterilized with 0.5% NaOCl and rinsed with distilled water. Bark samples weighing between 0.3 and 0.5 g were taken at three points along the trunk, 50 cm above the graft union, 5 cm above the graft union, and 5 cm below the graft union, using a cork borer. The cork borer was sterilized between isolations. Bark tissue was ground in 2 ml PPB (0.05 M) and 100 µl aliquots were plated on LB media amended with rifampicin (50 µg/ml) and nalidixic acid (50 µg/ml). Plates were incubated for 48 hr at 28°C and subsequently washed with 2 ml sterile water (22). PCR reactions, 50 µl total volume, were carried

out using 2.5 μM primers A and B (1), 12.5 mM $MgCl_2$, 5 μl PCR Reaction Buffer (Promega, Madison WI), 0.1 mM dNTP's, 0.625 units Taq® DNA Polymerase (Promega Madison WI), and 10 μl of bacterial sample. Probability of bacterial incidence at each isolation point was determined using logistic regression.

Direct Inoculation of Grafted and Non-grafted Rootstocks

B.9-NE and B.9-OR rootstocks were evaluated for resistance to direct inoculation by *E. amylovora* in 2005 and 2006. In 2006 the field planting was substituted for container trees due to an incomplete number of replicates. Artificial wounds, 14 mm in diameter made with a cordless drill, were positioned 5 to 10 cm below the graft union for grafted trees, and 10 cm above the soil line for non-grafted rootstocks. Wounds were inoculated with 100 μl of *E. amylovora* strain E4001a in PPB (0.05 M) at 10^5,10^7, and 10^9 cfu/ml in 2005, and 10^7, and 10^9 cfu/ml in 2006. Control plants treated with PPB (0.05 M). Treatment 10^5 cfu/ml was eliminated in 2006 based on the limited availability of trees and lack of a significant treatment effect the previous season. Development of typical rootstock blight lesions and tree viability were assessed at the end of the experiment. The probability of developing rootstock blight symptoms for each rootstock/treatment combination was determined using logistic regression.

Results

Microsatellite analysis

All twenty-three SSR markers generated multiple alleles when amplified with genomic DNA from nine *Malus* accessions. The number of alleles ranged from three to eight with a mean value of 5.8 alleles per individual locus. Cluster analysis using genetic distance estimates, based on twenty-three SSR profiles clearly distinguished the nine *Malus* accessions in this study (Figure. 2.1).

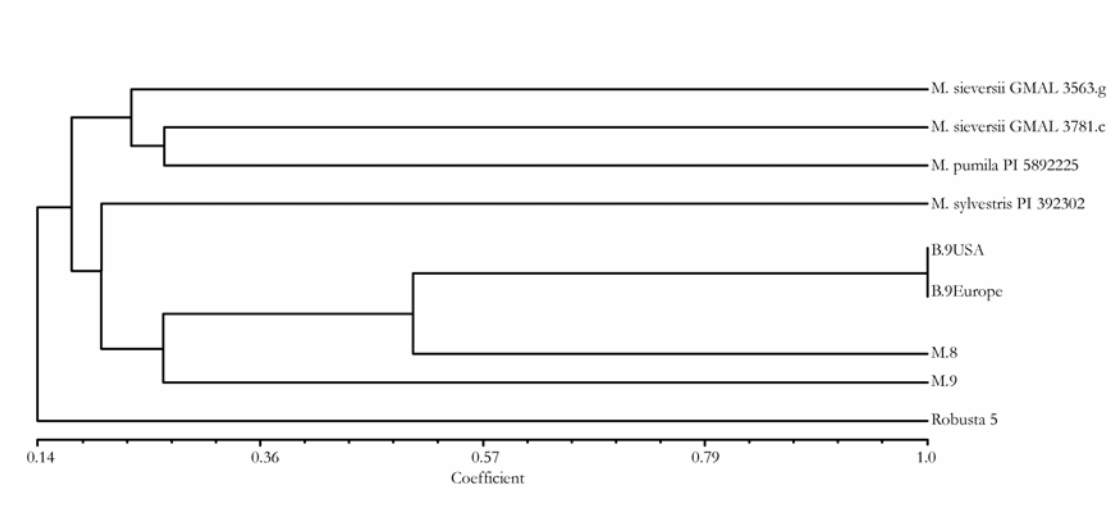

Figure 2.1 Genetic distance estimates of nine *Malus* accessions and rootstock cultivars analyzed in this study. Genetic distance coefficient determined using Jaccard analysis.

Limited sample size and diversity of *Malus* accessions prevented the segregation into well-defined clusters although two broad groups could be distinguished. The two *M. sieversii* (GMAL 3563.g and GMAL 3781.c) selections and the *M. pumila* accession form one group. Remote genetic similarity between *M. pumila* and the *M. sieversii* accessions (GD = 0.2) is not unexpected. *M. sieversii* is generally recognized as the ancestral 'wild' apple from which domesticated apple originated, although *M. pumila*

may have validity as the binomial for the domesticated apple (13,18). The second group includes both B.9 samples, and the Malling rootstocks, M.8 and M.9. Two other accessions, Robusta 5, an *M. baccata* (L.) Borkh. open pollinated variety (6), and a red leaved genotype labeled *M. sylvestris,* a 'wild' apple species common in Europe, show very little genetic affinity to any of the other genotypes analyzed. It is probable that the alleged *M. sylvestris* was misidentified during collection based on the uncharacteristic red leaf color. Based on our parameters, B.9-OR and B.9-NE were not genetically disparate (GD = 1.0), indicating a clonal relationship. M.8 was closely related to B.9-OR and B.9-NE (GD = 0.5) verifying the reported parental relationship. None of the wild Asian accessions were closely related to B.9 rootstock, providing no indication as to the paternity of this cultivar.

Bacterial Migration

In 2003 and 2004 severe fire blight developed on orchard trees resulting from blossom and shoot inoculation. Since scion cultivar did not influence the detection of bacteria at any of three isolation points in either 2003 or 2004, findings were grouped by rootstock cultivar. Detection of bacteria at 50 cm above the graft union was not significantly different for either scion or rootstock in 2003 or 2004, suggesting bacteria migrated from the inoculation site regardless of rootstock or scion genotype (Table 2.1, 2.2). Due to uncontrolled deer feeding and spray drift, controls were excluded from the analysis. The utilization of antibiotic resistant strains ensured that any bacteria isolated from apple tissue resulted from artificial inoculation.

Table 2.1. Effect of scion and rootstock on bacterial detection at three isolation points in 2003.

			50 cm above graft union			5 cm above graft union			5 cm below graft union		
	Df	Residual Df	Deviance	Residual deviance	P(>|Chi|)	Deviance	Residual deviance	P(>|Chi|)	Deviance	Residual deviance	P(>|Chi|)
NULL		11		7.54			21.89			7.06	
Scion	3	8	1.15	6.38	0.76	1.09	20.79	0.78	2.45	4.61	0.48
Rootstock	3	5	1.78	4.61	0.62	7.27	13.53	0.06*	0.38	4.23	0.94
Scion X rootstock	5	0	4.61	<0.0001	0.47	13.53	<0.0001	0.02**	4.23	<0.0001	0.52

[a*] Significant at P = 0.06

[b**] Significant at P = 0.02

Table 2.2. Effect of scion and rootstock on bacterial detection at three isolation points in 2004.

			50 cm above graft union			5 cm above graft union			5 cm below graft union		
	Df	Residual Df	Deviance	Residual deviance	P(>|Chi|)	Deviance	Residual deviance	P(>|Chi|)	Deviance	Residual deviance	P(>|Chi|)
NULL		11		14.71			12.38			16.49	
Scion	3	8	5.63	9.08	0.13	1.87	10.50	0.60	0.60	15.90	0.90
Rootstock	3	5	3.61	5.47	0.31	10.50	<0.0001	0.01*	12.89	3.00	0.005*
Scion X rootstock	5	0	5.47	<0.0001	0.36	<0.0001	<0.0001	1.00	3.00	<0.0001	0.70

[a*] Significant at P = 0.01

In 2003 *E. amylovora* was recovered from all rootstock cultivars, regardless of the fire blight susceptibility or resistance of the genotype (Figure 2.2A). Results indicate that bacteria were able to survive for an indeterminate period of time in rootstock tissue regardless of fire blight susceptibility.

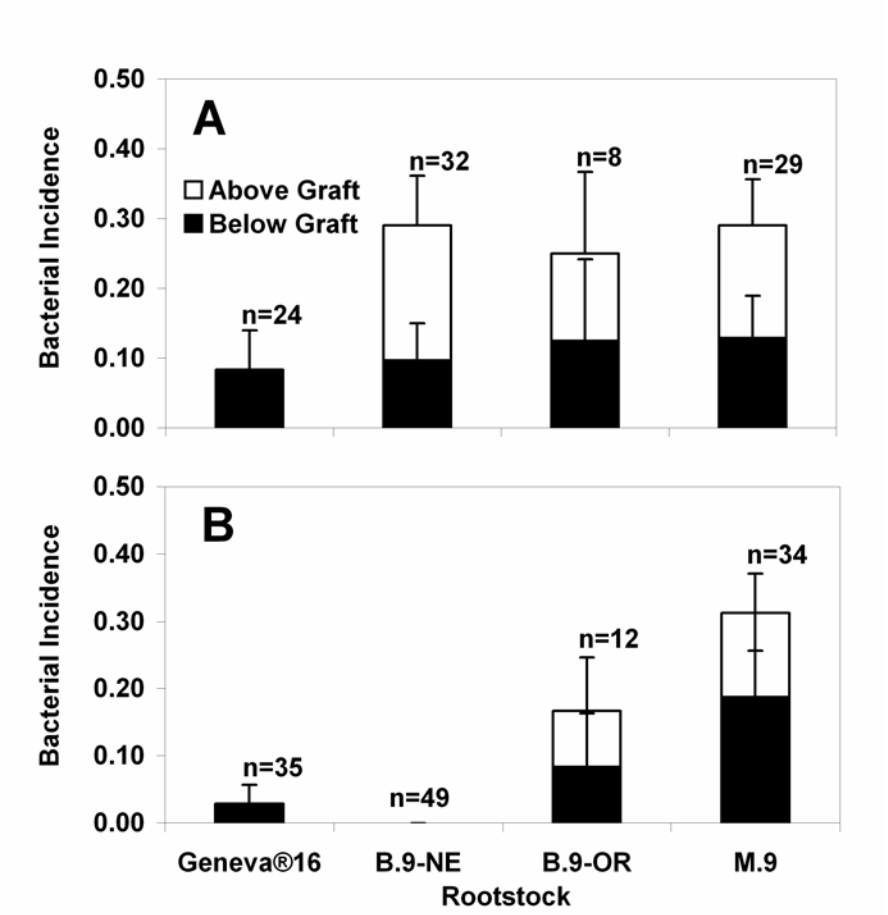

Figure 2.2 Proportion of rootstocks that tested positive for *E. amylovora* in **A**, 2003 and **B**, 2004. Positive detection based on the presence of 1kb fragment of ubiquitous *E. amylovora* plasmid pEa29. Trees were assayed 5 cm below the graft union (solid bars), 5 cm above the graft union (open bars), and 50 cm above the graft union (data not shown). Bacterial presence is indicative of bacterial migration not symptom development. No rootstock blight symptoms were observed during the 2003 and 2004 seasons.

B.9-NE and B.9-OR did not differ significantly in the frequency of rootstocks positive for *E. amylovora* above or below the graft union. Rootstock did not significantly affect bacterial movement in 2003, except for G.16, which had an absence of bacteria directly above the graft union (Table 2.1). The results from 2004, however, contrast with findings from the previous year. Rootstock had a significant effect on bacterial incidence both 5 cm above and 5 cm below the graft union (Table 2.2). Effect of rootstock on bacterial survival was only suggested for the susceptible rootstock M.9, which had a significantly higher incidence of detection than for G.16 or for either B.9 strain (Figure 2.2B). As in 2003, no bacteria were found directly above the graft union on G.16 rootstocks indicating a localized rootstock effect on scion susceptibility. Bacteria were detected 5 cm above and below the graft union in an intermediate number of B.9-OR rootstocks, significantly less than in M.9 but greater than in G.16. No bacteria were detected directly above or below the graft union in trees grafted on B.9-NE rootstocks in 2004 (Figure 2.2B). These results are inconsistent given that trees grafted onto B.9-NE suffered severe scion infection and *E. amylovora* were detected 50 cm above the graft union verifying initial bacterial migration.

Shoot Inoculation of Non-grafted Rootstocks

B.9-NE and B.9-OR displayed characteristic phenotypic differences in both the field and greenhouse experiments. B.9-OR possessed an erect growth type while B.9-NE had a spreading growth habit with weeping branch angles. Despite growth differences in B.9 plant material, fire blight sensitivity was similar between B.9-NE and B.9-OR in both field and greenhouse evaluations of non-grafted, own-rooted rootstocks (Figure 2.3). Both B.9-NE and B.9-OR were intermediately susceptible to fire blight infection and did not differ significantly in mean disease rating. In the field

evaluation, disease ratings were elevated for all rootstocks except M.9, which had significantly less disease, but at 70% infection remained highly susceptible to fire blight. B.9-NE had a significantly higher mean disease rating than B.9-OR, but neither B.9 rootstock was significantly different from the susceptible control, M.9. No infection was detected in mock-inoculated controls (data not shown).

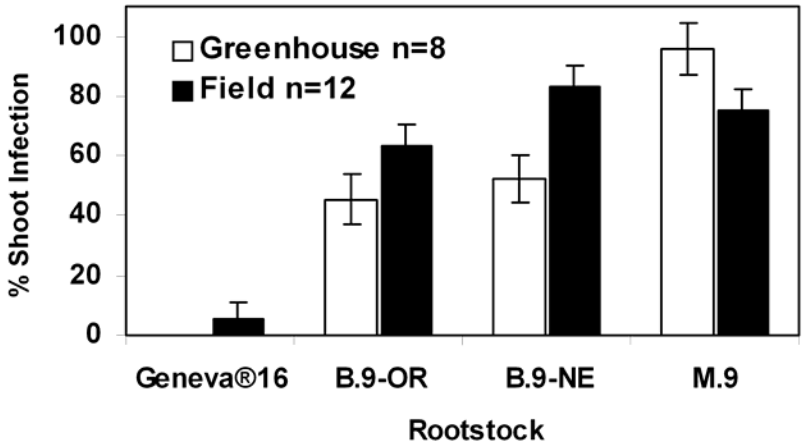

Figure 2.3 Percent shoot infection of four non-grafted rootstock cultivars inoculated by bisecting the two youngest leaves. Open bars represent rootstocks inoculated in the greenhouse while solid bars represent rootstocks inoculated in field plantings.

Direct Inoculation of Grafted and Non-grafted Rootstocks

Disease symptoms resulting from direct inoculation with *E. amylovora* included the production of bacterial ooze accompanied by a dark sunken lesion, which developed over a period of two months. It is important to note that symptom development was not indicative of tree mortality, which would be typical with naturally occurring rootstock infection. Rootstocks B.9-NE and B.9-OR, and to a greater extent G.16, demonstrated the ability to recover from rootstock infection whereas M.9 had high tree mortality at the end of the experiments in both 2005 and

2006 (Table 2.3). Neither treatment level or scion cultivar significantly affected symptom development in 2005, but scion cultivar was slightly significant in 2006 (Table 2.4). Based on the overwhelming significance of rootstock in both years, treatment and scion cultivar were combined for all rootstocks. Results from the two years were consistent; the only irregularity being increased disease incidence in 2006 (Figure 2.3B). B.9-NE and B.9-OR did not differ from the resistant rootstock G.16 in either year while the susceptible rootstock M.9 had significantly higher disease incidence (Figure 2.3) and tree mortality (Table 2.4). There was no significant difference in grafted and non-grafted rootstocks indicating the grafted phenotype does not directly influence B.9's resistance to rootstock blight. No symptom development was observed in mock-inoculated controls (data not shown).

Table 2.3. Effect of scion, treatment, and rootstock in 2005 and 2006, on the development of rootstock blight symptoms when grafted and non-grafted rootstocks were directly inoculated with *E. amylovora*.

	2005				2006			
			Likelihood				Likelihood	
	Df	Deviance	ratio	Pr (Chi)	Df	Deviance	ratio	Pr (Chi)
NULL		31.93				30.04		
Scion	3	35.13	3.20	0.36	3	38.22	8.18	0.042 *
Rootstock	3	78.92	46.99	<0.001**	3	59.39	29.35	<0.001**
Treatment	2	36.29	4.36	0.11	1	30.36	0.33	0.57

*significant at a P =0.05

** significant at a P =0.001

Table 2.4. Percent tree mortality resulting from direct inoculation of grafted and non-grafted rootstock tissue in 2005 and 2006.

	2005		2006	
	Grafted	Non-grafted	Grafted	Non-grafted
M.9	15	7	63	100
Geneva®16	0	0	10	0
B.9-NE	0	0	0	20
B.9-OR	0	0	25	12.5

Discussion

Microsatellite analysis is a powerful tool for systematic comparison and identification of apple cultivars despite complicated genetic relationships. Routine examinations of germplasm collections often reveal duplicate selections and otherwise misidentified apple cultivars (21). Misidentification occurs as a result of uncontrolled open pollination, handling mistakes, and incorrect labeling (30). Coupled with the close relatedness and phenotypic similarity of most rootstock clones, the genetic verification of plant material is often necessary.

Comparing 23 microsatellite loci, no genetic differences were found between B.9-OR and B.9-NE rootstock material. Based on these results we conclude that B.9 rootstock material, propagated independently in Europe and the US, are equivalent rootstock clones. Microsatellite analysis verified the parental status of M.8 rootstock, but failed to provide evidence of the origin of the unidentified parental cultivar, historically referenced as 'Red Flag' (Krasny Shtandard in Russian). A complete characterization of the apple germplasm collection would provide broader insight into the hereditary relationships between popular rootstocks and their 'wild' relatives.

Although microsatellite results verify the clonal relationship of B.9 they fail to explain the observed phenotypic variation of the B.9 rootstock currently in commerce. Phenotypic variation may be explained through the inadvertent selection of B.9 subclones by different nurseries. A 'subclone' is a term used to describe clonal rootstocks selected from within a cultivar for economically relevant attributes. M.9 reportedly has at least 26 subclones that vary in precocity, productivity, and tree vigor (20,43). The genetic bases of differences are likely minor mutations, which have previously proved difficult to identify using microsatellite analysis. Gianfranceschi et al. (10) failed to distinguish 'Red Delicious' and 'Starking' a somatic mutant with improved color. Monte-Corvo et al. (23) were similarly unsuccessful in the discrimination of 5 'Rocha' pear subclones using multiple bioinformatics approaches, despite obvious phenotypic differences. Although subclone selection is important in regards to vigor and stool bed propagation, it has not been linked to significant variation in disease resistance (20,31).

Bacterial migration from localized fire blight lesions into rootstock tissue is a crucial step in the development of rootstock blight. Momol et al. (22) previously described the ability of bacteria to migrate from the scion into the rootstock using the susceptible cultivar M.26. Although this study verified the ability of *E. amylovora* to traverse great distances, it did not investigate the effect genetically diverse rootstocks have on bacterial migration. Despite differences in experimental years, bacteria were clearly able to migrate and survive in rootstock tissue for an indeterminate amount of time, regardless of rootstock susceptibility/resistance. The presence of bacteria in resistant rootstocks as well as susceptible cultivars implies migration into the rootstock is not the limiting factor in the development of rootstock blight. Inconsistent results in 2003 and 2004 make it difficult to assess the effect, if any, that phenotypic variants of B.9 exert on bacterial migration. The reason for such seasonal variation is not clear.

Weather conditions throughout the experiment were similar with regard to average monthly temperature and rainfall (Climatological Benchmark Station No. 33031840, Geneva NY). Inoculation method varied between 2003 and 2004, but previous studies have utilized both methods with equivalent results (22,27). The absence of bacteria in B.9-NE rootstocks in 2004 may indicate an underlying effect divergent growth habits assert on bacterial movement. There is no conclusive evidence that this variation affects field susceptibility, however, as no rootstock blight symptoms were observed in B.9 rootstocks from either nursery source during the 2003 and 2004 seasons.

There was general agreement with regard to level of *E. amylovora* infection between B.9-NE and B.9-OR rootstocks throughout our experiments. Similar levels of fire blight susceptibility/resistance support microsatellite evidence that commercially available B.9 rootstock is clonal in nature. These results also support the conclusion that the two divergent growth forms identified in B.9 do not directly influence resistance to fire blight. Instead results from two sets of inoculation experiments support an emerging theory of differentially expressed fire blight resistance when B.9 is leaf inoculated versus the direct inoculation of rootstock tissue.

Non-grafted, leaf-inoculated B.9-OR and B.9-NE rootstocks displayed high levels of infection in both greenhouse and field experiments, signifying that growth condition does not influence fire blight sensitivity. These results support previous work by Norelli et al. (27), which established B.9 susceptibility to fire blight infection when leaf inoculated. Ostensibly these results validate the classification of B.9 as susceptible to fire blight, but further experiments have revealed a more intricate resistance phenomenon. Our results obtained from direct inoculation of non-grafted and grafted rootstock tissue contradict previous experiments that have shown a high degree of resistance when B.9 rootstocks are inoculated indirectly via the scion in a

grafted tree. The existence of a novel resistance phenotype would rationalize conflicting reports of B.9 resistance (8,27,31).

Age related resistance (ARR) or ontogenic resistance is a possible explanation of contradictory experimental findings. ARR is the phenomenon by which plant tissues gain resistance to plant pathogens as either a function of time or relating to a specific phase of tissue development (9,19,29). ARR has been described in multiple plant systems and is closely correlated with physiological stages of plant development including the onset of flowering, senescence, or the transition from vegetative to reproductive stage (29,36). Ontogenic resistance to apple scab, caused by the fungus *Venturia inaequalis*, has previously been characterized in apple, and is signified by the suppression of fungal sporulation in mature apple leaves (19). In B.9 rootstocks the physiological process of hardening off, or the transition from green tissue to mature wood, may trigger an innate defense response that could explain B.9's unusual resistance response.

Another possible explanation for B.9 resistance is the effect wound position exerts on the development of rootstock blight. Rootstock blight consistently and unexplainably develops directly below the graft union, away from more metabolically active regions of the plant where hormones accumulate and influence plant growth and development. Increased auxin accumulation has already been linked to elevated gall production in grapevines due to *Agrobacterium* infection (5).

Regardless of the mechanism, it is clear that B.9 rootstock tissue displays a high level of resistance to fire blight infection and rarely develops typical rootstock blight symptoms when planted as a grafted or budded tree in commercial or experimental plantings (8,27,31). Based on our results we can conclude resistance is due neither to substantial genetic variation in source material nor from the inability of *E. amylovora* to migrate into the rootstock. B.9's novel resistance contradicts known

fire blight resistance, which has been linked to several QTLs, and is constant throughout the life of the apple tree (3). Not much is known about the parentage of B.9. A thorough investigation of the apple germplasm collection could lead to the discovery of new resistance phenotypes that have been overlooked by the accepted method of fire blight resistance screening. Better understanding of the basis of resistance would be a valuable tool for rootstock breeding programs, providing a novel avenue of research in the development of resistant plant material.

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CHAPTER 3

FACTORS INFLUENCING THE RESISTANCE OF BUDAGOVSKY 9 APPLE ROOTSTOCK TO FIRE BLIGHT

Abstract

Rootstock blight, a fatal form of fire blight caused by the bacterium *Erwinia amylovora*, is an increasing threat to orchard longevity and productivity in high-density apple production systems. Fire blight resistant rootstocks are the only reliable option to prevent rootstock blight development. Budagovsky 9 (B.9) dwarfing rootstock displays varying resistance to fire blight at different stages of plant development. This unique resistance phenotype is reminiscent of age related or ontogenic resistance. To verify the presence of age related resistance in B.9; reciprocal grafts were utilized to determine effect of wound position on tissue susceptibility and B.9 plants were inoculated at different developmental stages to assess disease severity in diverse tissue types. Rootstock suckers were also evaluated for disease susceptibility and the ability to act as a conduit for bacterial movement into B.9 rootstock. Reciprocal grafting experiments clearly demonstrated that wound position and the graft union, are not factors in B.9 resistance to fire blight. B.9 tissue was found to be highly susceptible during the first year of tissue growth prior to the transition from green to woody tissue. Woody tissue lost susceptibility to fire blight infection resulting in almost complete resistance. Rootstock suckers, while susceptible to infection, did not act as an entry point for bacteria into the rootstock; furthermore rootstock sucker infections ceased progressing at the junction of green and woody tissue. These results verify previous reports of B.9 resistance to rootstock blight while providing further evidence for the existence of age related resistance.

Introduction

Fire blight, caused by the Enterobacterium *Erwinia amylovora* [(Burill.)Winslow et al.], affects all stages of apple growth and development. Fire blight outbreaks often result in considerable monetary losses, due to blossom death, shoot blight, and tree mortality (24). Reports of tree death attributed to fire blight have risen in recent years, largely due to an increase in the incidence of rootstock blight. Rootstock blight is a discrete fire blight infection of the apple rootstock that is almost always fatal (3,12,13,15). Rootstocks become infected either through the systemic movement of bacteria from scion infections into the rootstock, direct infection of abiotic/biotic wounds, or via infection of rootstock suckers: adventitious shoots originating from rootstock tissue (15,24).

Escalation in rootstock blight severity is correlated with the proliferation of high-density orchard systems, which have relied heavily on the susceptible dwarfing rootstock M.9 to reduce tree size and enhance production. Rootstock blight severity is compounded when M.9 is planted in combination with susceptible fruiting cultivars, and tree losses over 50% have been reported (7,17). Presently, almost all the leading apple cultivars in the world market are susceptible or very susceptible to fire blight. The only control option proven to be effective against rootstock blight development is the utilization of fire blight resistant rootstocks (15).

Budagovsky 9 apple rootstock is a cold-hardy dwarfing rootstock with yield efficiency and size comparable to some M.9 rootstock clones (4,6,18). B.9 has repeatedly demonstrated high levels of field resistance to *E. amylovora* infection in both inoculated and naturally infected orchard trials (7,17,18,20). Previous investigation into B.9 resistance has revealed a unique resistance phenotype, distinct from previously identified fire blight resistance in *Malus* (5,10). Russo et al. (18) verified the susceptibility of non-grafted B.9 rootstock tissue when actively growing

leaves were directly challenged with fire blight. The level of susceptibility in young leaf tissue was comparable to the susceptible rootstock M.9. Conversely, the inoculation of four-year-old woody trunks of both grafted and non-grafted B.9 rootstock tissue indicated that B.9 was significantly less susceptible than M.9, and the degree of resistance to direct inoculation was not distinguishable from the resistant control Geneva®16. Resistant apple varieties normally exhibit a range of resistance to *E. amylovora* but that range is constant throughout the life of the plant (15,10). van der Zwet and Miller (23) identified individual fruiting cultivars that varied in degree of susceptibility between tissue types, specifically in the severity of blossom or shoot infection. However none of variation in resistance described by van der Zwet and Miller (23) compared to the complete gain of resistance observed in older B.9 tissue. B.9 resistance appears to be contingent on, as of yet, unidentified developmental or physiological processes.

B.9's differentially expressed resistance could be explained as a form of age related resistance, otherwise known as adult plant or ontogenic resistance. Age related resistance (ARR) is a generalized term for whole plants or plant parts that gain disease resistance at specific developmental stages or over a linear period of time (16). ARR has been previously described in several plant-pathogen interactions including rice/*Xanthomonas campestris* pv. *oryzae*, wheat/ *Puccinia recondite* f.sp. *tritici*, tobacco/*Peronospora tabacina* (16). Only two perennial plant species, grape (*Vitus* spp.) and apple (*Malus* spp.), have been described as displaying any form of ARR. Mature grape berries display ontogenic resistance to powdery mildew, caused by *Uncinula necator*, and downy mildew, caused by *Plasmopora viticola* (8). In *Malus* spp. a form of ARR occurs in aging leaf tissue, inhibiting infection by the apple scab fungus *Venturia inaequalis* (11,21) and by the cedar apple rust fungus,

Gymnosporangium juniperi-virginianae (1). Schwabe (21) determined that apple leaves lose the ability to support fungal sporulation as leaves mature.

The objective of the present study was to confirm the resistance of B.9 rootstock to fire blight as a form of ARR. Rootstock suckers were assessed to determine their ability to overcome B.9 resistance. Rootstock suckers are primary shoots that arise directly from rootstock tissue and are a known avenue for bacterial infection of the rootstock. If B.9 tissue displays ARR it is possible suckers may still serve as mode of infection for B.9 rootstock tissue in the field. This work is necessary to provide growers with a sound recommendation of rootstock susceptibility in orchard plantings and confirm a novel source of rootstock resistance to fire blight.

Materials and Methods

Bacterial Strains

Fire blight inoculum consisted of *E. amylovora* strain Ea273 (2) grown at 28°C in Luria-Bertani (LB) broth in a rotary shaker for 18 hr.

Susceptibility of tissue in relation to the graft union

Bare rooted, 6.35 mm, M.9 and B.9 rootstocks were obtained from Treco Nursery, Woodburn, OR. Rootstocks were grafted in four scion/rootstock combinations using whip and tongue grafts (9). Each rootstock was grafted to both the opposite (B.9/M.9 and M.9/B.9) and identical genotype (B.9/B.9 and M.9/M.9) to determine the effect that wound position, in relation to the graft union, has on lesion development. Grafted plants, trained to a single shoot, were grown in 15 cm pots using soilless-potting mix under greenhouse conditions with a 16 hr photo cycle. Mineral nutrition was supplied in the form of Osmocote® Classic (The Scotts Company LLC, Marysville OH) at planting and additional nitrogen was applied as

Miracle-Gro® (The Scotts Company LLC) at the labeled rate. Each grafted plant represented a single unit of replication; not all graft combinations were available for all replicates. Using a No.10 scalpel, incisions 2 cm in length were made in the bark tissue of actively growing plants, either 3 cm above or 3 cm below the graft union. Incisions were inoculated with 10 µl of *E. amylovora* strain Ea273 ($1x10^7$ cfu/ml) in potassium phosphate buffer (PPB) (0.05M). Control plants were mock inoculated with PPB (0.05M). Grafted plants were maintained until lesions ceased progressing ca. 60 days after inoculation. The initial experiment was conducted in 2006 and repeated in 2007; inoculations occurred on 21 June and 7 Feb. and were evaluated on 21 Aug. and 3 March, respectively. Tissue susceptibility, recorded as disease incidence, was based on the development of a typical fire blight lesion and ooze production at the wound site. The effect of graft combination, rootstock cultivar, and wound position on tissue susceptibility was determined using logistic regression.

Effect of tissue age on fire blight resistance

Bare rooted, 6.35 mm, M.9 and B.9 rootstocks were obtained from Treco Nursery. Non-grafted rootstocks, trained to a single shoot, were grown in 15 cm pots using soilless-potting mix under greenhouse conditions on a 16 hr photo cycle. Mineral nutrition was supplied in the form of Osmocote® Classic (The Scotts Company LLC) at planting and additional nitrogen was applied as Miracle-Gro® (The Scotts Company LLC) at the labeled rate. New shoot growth, referred to as 1^{st} year growth or green tissue, and 2^{nd} year growth or woody tissue, were inoculated to determine the effect of tissue age on B.9 resistance to fire blight. Inoculations were conducted as previously described. Incisions, 2 cm in length, were made using a No.10 scalpel, in the green or woody stem tissue of actively growing plants. Incisions were inoculated with 10 µl of *E. amylovora* strain Ea273 ($1x10^7$ cfu/ml) in PPB

(0.05M). Control plants were mock inoculated with PPB (0.05M). Each plant represented a single unit of replication; not all cultivars were available for all replicates. Tissue susceptibility, recorded as disease incidence, was based on the development of a typical fire blight lesion and ooze production at the wound site. The effect of cultivar and tissue age on tissue susceptibility was determined using logistic regression.

Rootstock Sucker Inoculation Experiment

Plant material consisted of five-year-old orchard trees planted at uniform tree spacing (1 x 3 m) and trained to a trellis. Roots were pruned in April 2006 using a double shank 'zone-builder' along one side of the root zone, approximately 0.6 m from the main trunk at a depth of 38.1 to 45.72 cm, to promote rootstock sucker production. The experimental planting included four scion cultivars, 'Gala', 'Jonagold', 'Gingergold', and 'Red Yorking', planted in combination with two B.9 rootstock clones, B.9-NE (Janssen Bros. Nursery, The Netherlands) and B.9-OR (Treco Nursery), and the susceptible rootstock M.9. In previous orchard trials B.9 clones have demonstrated identical fire blight resistance, but since B.9-NE produces significantly more rootstock suckers than B.9-OR; both clones were included for comparison (18). Ten trees per rootstock were inoculated; not all scion rootstock combinations were available for all replicates. In 2006, one vigorously growing rootstock sucker per tree was inoculated by transversely bisecting the two youngest leaves with scissors dipped in a suspension of *E. amylovora* strain Ea273 ($1x10^7$cfu/ml) in PPB (0.05M) (13,14). Control plants were mock inoculated with PPB (0.05M). Once lesions ceased progressing, lesion length was recorded as a percent of the current season's growth, described as percent infection, and used as a measure of susceptibility (14). The development of typical rootstock blight

symptoms, particularly ooze being emitted from the rootstock, was assessed on 17 July and on 31 Aug. At the end of the experiment, trees with suckers completely blighted were excavated and bacterial progression from the rootstock sucker into the main body of the rootstock was visually assessed. Percent lesion length was analyzed using a single factor ANOVA to assess the effect that scion and rootstock had on mean lesion severity. Rootstock blight that arose from sucker inoculation was assessed using logistic regression based on the presence or absence of diagnostic rootstock blight symptoms.

Results

Susceptibility of tissue in relation to the graft union

The reciprocal grafting of two dwarfing rootstock cultivars resulted in an overall reduction in plant vigor due to the cumulative effect of both genotypes (22). Reduced vigor combined with the stress associated with grafting led to substantial plant death reducing the number of available replicates. Despite initial impediments the reciprocal grafting experiment was repeated with analogous results and data were pooled for final analysis.

Sunken black lesions appeared 30 days after inoculation and continued to develop up to 60 days after inoculation, with ooze produced in severe infections. No lesions or other disease symptoms were observed in the mock-inoculated controls (data not shown). The four plant combinations grafted in this study, M.9/M.9, M.9/B.9, B.9/B.9, and B.9/M.9, did not have a significant effect on lesion development in either tissue type or wound position. Data were therefore combined based on wound position (Figure 3.1). Since residual deviance was higher than expected for binomial distributions, the lesion incidence was modeled with quasi-likelihood functions (Table 3.1). There was a significant effect of rootstock cultivar

on the development of fire blight lesions (P = 0.01). The effect of rootstock on disease incidence is mainly influenced by M.9 tissue, which was significantly more susceptible than B.9, regardless of wound position. Wound position was not significant with regard to lesion development. M.9 did display a significant increase in susceptibility when inoculated above the graft union (P = 0.08), but this increase was only seen in M.9. B.9 tissue displayed the same level of susceptibility when inoculated above or below the graph union (Figure 3.1).

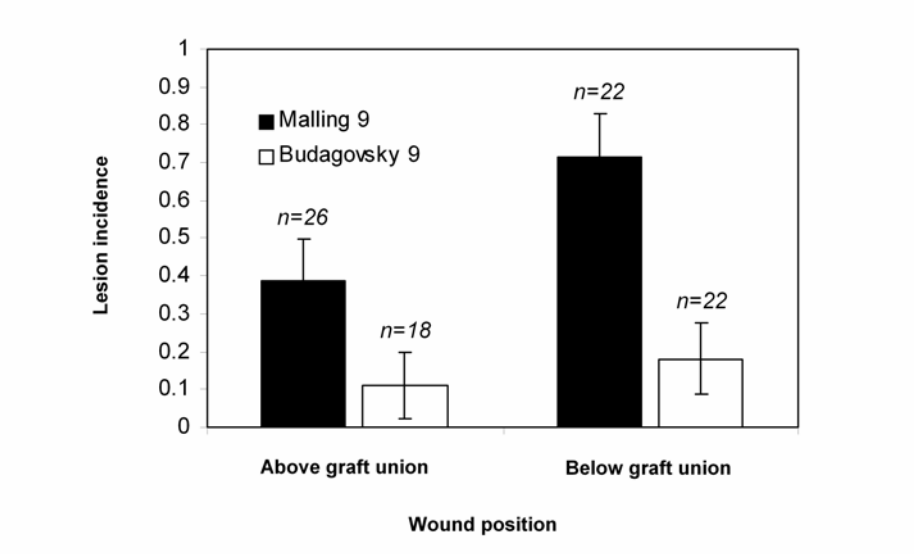

Figure 3.1 Effect of wound position and tissue type on lesion development in four graft combinations, combined by tissue type: Malling 9 (M.9), solid bars, and Budagovsky 9 (B.9), open bars. M.9 had significantly higher incidence of lesion development than B.9, regardless of wound position. Wound position, above and below the graft union, did not significantly affect lesion incidence in B.9 tissue.

Table 3.1. Analysis of deviance for pooled reciprocal graft data. Effect of tissue type, M.9 or B.9, and wound position, above or below the graft union, on lesion incidence.

	Df	Deviance	Residual Df	Residual Deviance	*F*	Pr > *F*
NULL	15	39.76				
Tissue type	1	14.54	14.00	25.22	10.54	0.01*
Wound position	1	5.08	13.00	20.14	3.69	0.08
Tissue type X Wound position	1	0.50	12.00	19.63	0.36	0.56

*significant at P = 0.01

Effect of tissue age on fire blight resistance

Symptoms were first observed in green tissue 5 to 7 days after inoculation. Susceptible reactions included a necrotic zone around the inoculation site and ooze emitted from the growing stem. After two to three weeks, symptomatic green shoots developed severe fire blight infections with lesions encompassing the entire growing shoot. In woody tissue, which is inherently more resistant, visible symptoms of infection took longer to manifest. Lesions first appeared in woody tissue 30 days after inoculation and continued to develop up to 60 days after inoculation. Symptoms appeared as sunken black lesions adjacent to the inoculation site. No symptoms were observed in mock-inoculated controls of either tissue type (data not shown). The experiment was repeated under the same conditions with analogous results and data were pooled for final analysis.

Analysis of deviance revealed that tissue type (P = 0.02), green or woody, and the interaction of tissue type and rootstock cultivar (P = 0.05) each had a significant effect on the development of fire blight lesions (Table 3.2). M.9, a highly susceptible

rootstock, developed lesions in a large proportion of plants when inoculated as both green (0.79) and woody tissue (0.67) (Figure 3.2). Although lesion incidence was lower in woody tissue the observed variation was not significantly different between tissue types. B.9 was comparable to M.9 rootstock with regard to susceptibility of green tissue. Both rootstock cultivars had almost an equal proportion of symptomatic plants, 0.78, and 0.79 respectively, but when inoculated as woody tissue, B.9 rootstock failed to develop any fire blight lesions after 60 days.

Table 3.2. Effect of tissue age and rootstock cultivar on lesion development.

			Residual	Residual		
	Df	Deviance	Df	Deviance	*F*	Pr >*F*
NULL	7	45.10				
Rootstock	1	7.45	6	37.65	5.14	0.09
Tissue type	1	18.82	5	18.83	13.00	0.02**
Rootstock X Tissue type	1	10.75	4	8.09	7.42	0.05*

** significant at P = 0.02

* significant at P = 0.05

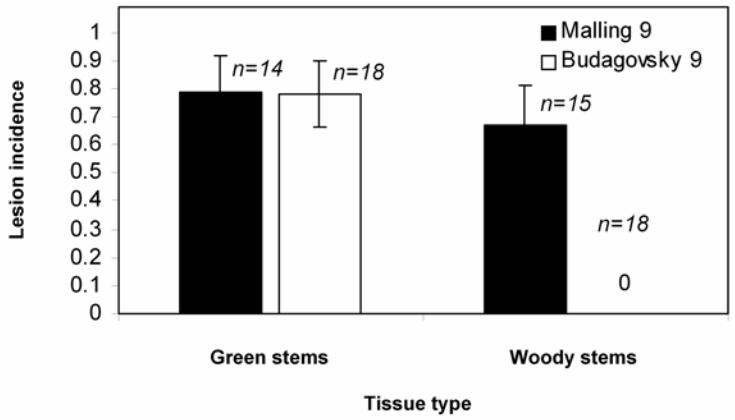

Figure 3.2 The effect of tissue age on lesion development in B.9 (open bars) and M.9 (solid bars).

Rootstock Sucker Inoculation Experiment

Symptoms rapidly developed on rootstock suckers, appearing as necrotic zones along the midvein of inoculated leaves. Lesions continued to progress along the growing shoot, and rootstocks were assessed at regular intervals for the development of rootstock blight. Rootstock blight symptoms were first observed on 17 July as oozing rootstock cankers. No sucker infection or rootstock cankers were observed in mock-inoculated controls (data not shown). Since scion cultivar did not influence the severity of rootstock sucker infection, measured as percent infection, findings were grouped by rootstock cultivar. Mean percent infection, was also independent of rootstock cultivar (Table 3.3). There was slight evidence of a rootstock effect ($P = 0.09$) on lesion severity, which could be attributed to a minor increase in total lesion length for the susceptible rootstock M.9 (Figure 3.3). Regardless, all three rootstock cultivars, B.9-OR, B.9-NE, and M9, exhibited severe rootstock sucker infection, providing ample opportunity for migrating bacteria to gain entry into the rootstock.

Table 3.3. Effect of rootstock and scion cultivars on mean rootstock sucker infection, measured as lesion length.

	Df	Sum of squares	Mean sums of squares	*F*	Pr >*F*
Scion	3	0.54	0.18	0.92	0.45
Rootstock	2	1.03	0.51	2.64	0.09
Scion X Rootstock	3	0.10	0.03	0.17	0.91
Residuals	21	4.08	0.19		

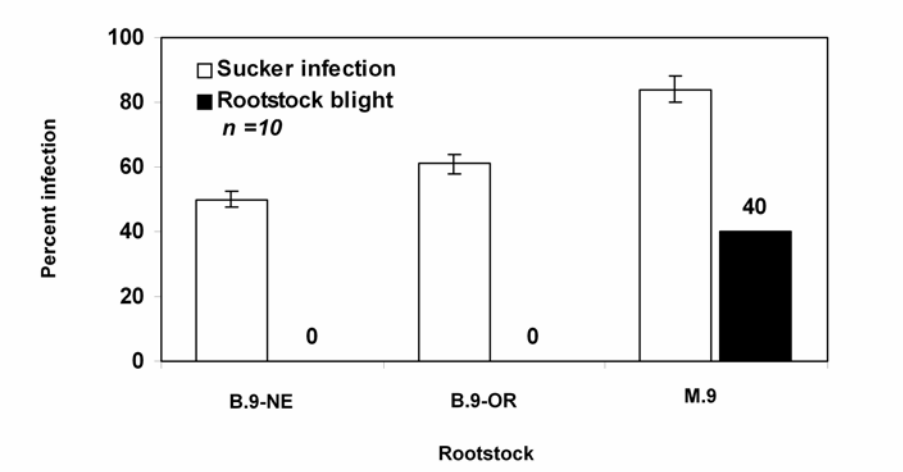

Figure 3.3 Assessing probability of rootstock suckers serving as an avenue of fire blight infection. Open bars represent percent shoot blight of suckers inoculated with *E. amylovora.* Solid bars represent the corresponding level of rootstock blight attributed to sucker infection.

In July and August of 2006 several trees on M.9 rootstock developed rootstock blight cankers. Symptomatic trees were excavated, and necrotic lesions could be visually tracked from inoculated rootstock suckers, through the lateral roots, and into the main body of the rootstock. M.9 rootstocks, which lacked visible rootstock blight symptoms but exhibited severe (100%) rootstock sucker infection, were also excavated. In this subset of M.9 rootstocks, lesion margins were indiscrete indicating active fire blight infections within the conjoined root system (Figure3.4). Throughout the 2006 growing season trees planted with B.9 rootstock remained healthy even when B.9 rootstock suckers exhibited high amounts of infection. Asymptomatic B.9 rootstocks, with severe rootstock sucker infection, were also excavated to assess the degree of lesion penetration into the root system. In B.9 tissue, lesions margins were discrete, and lesions ceased progressing precisely at the junction of the young rootstock sucker to the lateral root system (Figure 3.4). Rootstock cultivar was the only significant factor affecting the development of rootstock blight (Table 3.4).

Table 3.4. Effect of rootstock and scion cultivars on the incidence of rootstock blight.

	Df	Deviance	Residual Df	Residual Deviance	P >|Chi|
NULL	8	11.42			
Rootstock	2	10.10	6	1.32	0.01 *
Scion	3	1.32	3	<0.0001	0.72

* significant at P = 0.1

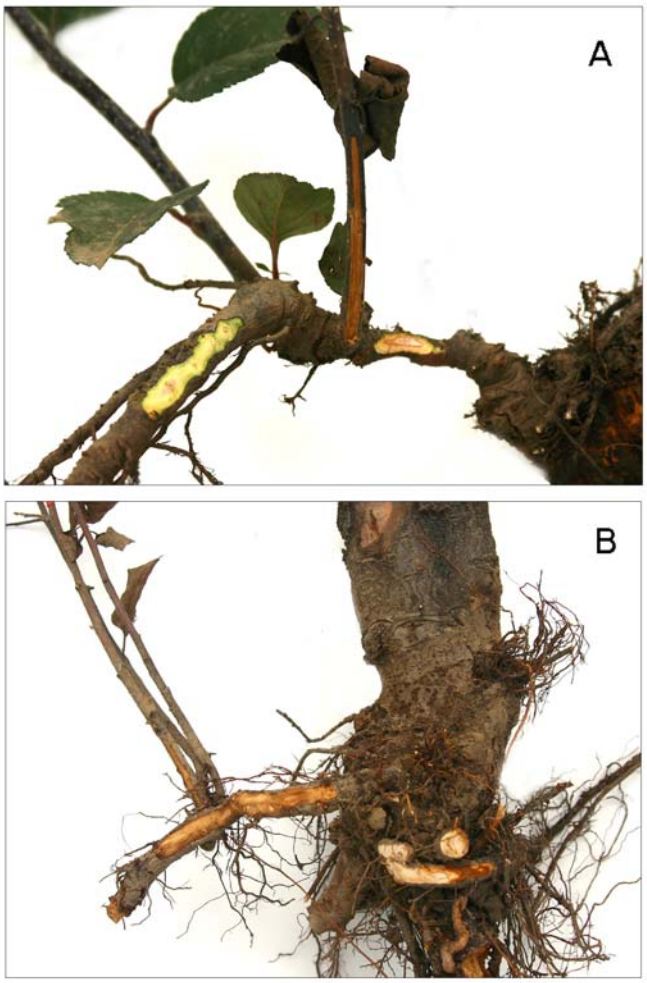

Figure 3.4 Fire blight infection of **A.** B.9, and **B.** M.9, rootstock suckers. **A**. B.9 rootstock sucker with severe infection, indicated by necrotic zone, ending in a discrete lesion margin. Healthy tissue can be seen surrounding the infected rootstock sucker. **B**. M.9 rootstock exhibiting typical rootstock blight symptoms including water soaking and production of bacterial ooze. A necrotic zone was observed progressing from the infected rootstock sucker through the lateral root system into the main body of the rootstock.

Discussion

Results support previous work by Russo et al. (18) verifying a high level of resistance in B.9 tissue to rootstock blight development, in both grafted and non-grafted rootstocks. The lack of a significant effect of wound position in the reciprocal grafting experiment definitively confirms that grafting is not a deciding factor in B.9 resistance. Instead results clearly identify an age related response, which results in the almost complete gain of resistance, as B.9 tissue transitions from green tissue to woody tissue. Developmental changes have previously been linked to ARR by Rusterucci et al. (19) who discovered a similar gain of resistance associated with the transition to flowering in Arabidopsis. Further evidence supporting ARR was observed when verifying the susceptibility of B.9 and M.9 rootstock suckers. In B.9 tissue, disease progression was inhibited at the junction of green shoot tissue, represented as newly emerging rootstock suckers, and mature tissue, or the lateral root tissue from which the rootstock suckers developed. Disease inhibition was evident by the segregation of infected tissue by the host plant, observed as a discrete lesion margin.

It has been widely reported that rootstock suckers serve as an avenue of infection for *E. amylovora*, resulting in the development of rootstock blight (12,13,15,24). However no research has been conducted to validate this theory. Both Norelli et al. (15) and Momol et al. (12) recorded the presence or absence of infected rootstock suckers associated with rootstock blight infections but neither demonstrated a positive association in disease development. This study was the first to conclusively prove that fire blight infection of rootstock suckers can lead to the development of rootstock blight; observed in M.9 tissue as a continuous necrotic lesion from the infected rootstock sucker into the rootstock. Further work is necessary to determine if the discrete lesion margin observed in B.9 tissue prevents bacterial migration into the

rootstock, or if an unidentified factor is involved with disease inhibition in mature rootstock sucker tissue.

B.9 resistance is similar to previously described adult plant or age related resistance (ARR), due to the complete reversal of the susceptible phenotype and the durability of resistance. Age related resistance has been well characterized in several plant species, but predominantly exists as a classic gene for gene interaction (16). Presently no gene-for-gene interactions have been described between *E. amylovora* and any of its rosaceous hosts; therefore it is impossible to assume a similar mechanism is responsible for this unique form of disease resistance. Quantitative trait loci (QTL), associated with fire blight resistance, have been previously described in *Malus* spp.; however, QTL resistance is continually expressed and is neither regulated by tissue age or stage of tissue development (5).

Only two perennial crop species, apple and grape, have well-characterized forms of ARR (1,8,11). In grapes ARR, referred to as ontogenic resistance, has been described in developing berries causing tissue specific resistance, much like B.9, in which only woody tissues are resistant. Likewise, as apple leaves mature, they exhibit a tissue-specific resistance to apple scab by preventing fungal sporulation, thereby reducing disease proliferation. Although highly effective, this type of ARR differs from B.9 resistance to fire blight in that senescing apple leaves lose the ability to inhibit fungal growth in a complete reversal of ARR (11). Instead B.9 resistance appears to be an irreversible phenotypic change that persists over the life of the plant.

Functional resistance in a breeding program is of paramount importance and generally refers to a durable, overall reduction in disease severity. Rarely is this seen as complete disease resistance, although complete resistance to fire blight does exist (10,15,18). ARR would not be a useful trait for apple scion, or fruiting, cultivars which seasonally produce a large amount of susceptible tissue in the form of blossoms

and succulent shoots. Apple rootstocks, however, produce limited susceptible tissue above the soil line and therefore do not require the same level of disease resistance as scion cultivars. Since the majority of apple trees are grafted prior to planting, all rootstock tissue exposed to fire blight infection would be past the age of susceptibility.

Fire bight resistance is routinely determined through the inoculation of newly emerged leaves on young shoots and evaluation of lesion severity. Leaf inoculation is the standard method of screening for fire blight resistance in breeding programs. Although highly efficient this procedure has overlooked more subtle forms of disease resistance, which have the potential to enhance breeding efforts. The existence of a definable phenotype in B.9 resistance also enables complete characterization of apple germplasm for ARR. Using marker-assisted selection for ARR, breeders could preserve selections with promising horticultural traits that might otherwise have been removed due to fire blight sensitivity of green tissue. Selecting for ARR is not likely to replace the standard method of resistance screening, which is cost effective and efficient, however investigation into novel forms of disease resistance is essential for cultivar improvement and future breeding efforts.

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CHAPTER 4

CONCLUSIONS AND PERSPECTIVES ON ROOTSTOCK BLIGHT MANAGEMENT

Rootstock blight, the rootstock phase of fire blight caused by *Erwinia amylovora,* is a significant threat to dwarf apple orchards. High-density systems, which provide a necessary competitive advantage in apple production, rely heavily on the fire blight susceptible dwarfing rootstock M.9. The combination of susceptible scion and rootstock cultivars with increased planting densities has exacerbated damages associated with rootstock blight.

Rootstock blight is a relatively new manifestation of fire blight and has not been studied to the extent that the blossom and shoot blight phases have. To date little is known about why rootstock blight occurs other than that certain rootstock cultivars are highly susceptible and that disease outbreaks are more severe in warmer seasons. Basic scientific study is impeded by the perennial nature of apple, the cost of maintaining 2 to 10 year old trees of susceptible cultivars, and the fatal nature of the disease itself. Essential information is missing for the rootstock blight disease cycle, including the basic environmental conditions favorable to disease development. Quantitative data showing how varying levels of scion infection affect disease incidence as well as information regarding the minimum bacterial population necessary for symptom development would greatly increase our understanding.

Recommendations for the control of rootstock blight are limited. The planting of fire blight resistant rootstock cultivars is the only proven method to prevent losses associated with rootstock blight. The development of apple rootstocks with sustained disease resistance to fire blight has been a goal for a few apple rootstock-breeding

programs. Unfortunately conventional breeding systems require significant amounts of time to combine high levels of disease resistance into a horticulturally desirable rootstock. It is only now that breeding selections from several internationally recognized programs have reached the final stages of organized field trials and commercial release.

Among existing and novel rootstock cultivars several rootstock selections have been identified, with the assistance of Terence Robinson, Gennaro Fazio, and Jason Osborne, which possess both sustained disease resistance and orchard productivity equal to or exceeding that of M.9. Superior yield efficiency of select resistant cultivars, including Geneva® 41 and Geneva® 935, make them competitive with M.9 clones in addition to their elevated disease resistance ratings. Promotion of resistant rootstock cultivars instead of M.9, or other fire blight susceptible rootstocks (e.g. M.26), could essentially eliminate rootstock blight as an economically relevant phase of fire blight. Unfortunately many of the promising resistant rootstocks evaluated in this study have not been released for commercial sale or they are in the early stages of production and distribution is limited. Further testing of new selections and an increase in commercial level propagation is necessary to meet current and future demands. Available rootstocks with proven fire blight resistance, such as B.9 and Geneva® 16, can be utilized to temporarily satisfy the need for resistant rootstocks while newer cultivars are being propagated.

There are no proven control options available for the management of rootstock blight in established orchards planted with susceptible rootstock cultivars. Current recommendations are limited to the immediate removal of fire blight infections in the scion, thereby decreasing bacterial migration into the rootstock. Although recommended, it is unclear if this strategy is effective in all scion rootstock combinations. Orchard trials indicate that less susceptible scion cultivars may reduce

the incidence of rootstock blight regardless of rootstock cultivar. However it is unclear if the observed reduction in disease incidence is due to a decrease in bacterial migration into the rootstock, or to the influence of scion on rootstock susceptibility. These results contrast with those from the bacterial migration study in which scion did not influence rootstock blight development. In this experiment it should be noted that the four scion cultivars evaluated were highly susceptible to fire blight infection, which may have lessened the influence of scion on bacterial migration. To date limited research has been conducted on the effect of cultivar susceptibility on rate of bacterial movement and the incidence of rootstock blight. Previous work has demonstrated that *E. amylovora* migrates at different rates in the cultivars Golden Delicious and Empire, but how this affects rootstock bight development is unclear. A comprehensive experiment evaluating bacterial movement in several scion cultivars at specific time points after infection could illuminate which scion cultivars would benefit from summer pruning and the timing necessary for pruning to be effective. If scion does affect bacterial movement into the rootstock, pruning could be delayed in certain cultivars until the end of the growing season, minimizing labor costs.

General understanding of the genetic basis of fire blight resistance in *Malus* spp. is limited. Fire blight resistance has recently been associated with several QTLs, identified from a fully resistant fruiting cultivar. Although important, QTLs represent broad undefined regions where genes responsible for fire blight resistance are located, limiting their application. Furthermore, additional QTLs may be found in a wider selection of resistant *Malus* spp. The unique method of resistance observed in B.9 rootstocks brings into focus the number of resistance genotypes that potentially exist. Most research on fire blight resistance has focused on high value fruiting cultivars and not on rootstock genotypes, which can benefit form certain forms of resistance, such as age related resistance, which may not be applicable to scion cultivars.

Based on our current knowledge B.9 rootstock is unique in displaying age related resistance (ARR). It is likely, however, that other rootstock cultivars, which have initially appeared susceptible to *E. amylovora* in breeding evaluations, may also display an age related resistance phenotype. By screening *Malus* germplasm collections for ARR more candidates for this type of resistance may be identified, facilitating the genetic mapping of ARR in apple. Determining the biochemical and molecular mechanisms behind B.9 resistance would also promote a more comprehensive understanding of fire blight resistance. Investigation into potential mechanisms of ARR in perennial crops is limited, however annual crops have been studied extensively and provide insight into potential areas of research. Biochemical changes in plant tissue, such as the accumulation of salicylic acid or anthocyanins, may play a role in bacterial inhibition. These compounds, once identified, could be used to improve the resistance in other cultivars. Another approach, which could drastically increase our knowledge concerning fire blight resistance, is to focus efforts on understanding the genetic component of fire blight resistance. By analyzing gene transcription over time in B.9 tissue, using microarray technology or subtractive hybridization, it would be possible to identify the genetic foundation for this form of disease resistance.

Although our collective knowledge on rootstock blight is incomplete there are exciting new avenues of research to be explored. New information on bacterial communication and movement coupled with advances in molecular and biochemical techniques permit almost unlimited exploration into the rootstock phase of *E. amylovora*. Currently rootstock blight is controllable through the use of resistant rootstocks, but continued planting of these rootstocks must continue for the benefit of resistance to be realized. Advancements in breeding using novel sources of resistance will ensure the continued development of resistant cultivars reducing losses from all forms of fire blight.

CHAPTER 5

ISOLATION OF STREPTOMYCIN-RESISTANT STRAINS OF *ERWINIA AMYLOVORA* IN NEW YORK

Abstract

Streptomycin is currently the only antibiotic registered for the control of fire blight, a devastating disease of apple (*Malus*), pear (*Pyrus*), and other rosaceous plants caused by the bacterium *Erwinia amylovora*. Resistance of *E. amylovora* to streptomycin was first identified in California pear orchards in 1971 and is currently endemic throughout the mid and western United States. New York remains the only major US apple-growing region without streptomycin-resistant strains of *E. amylovora*. In 2002, during a routine survey for streptomycin resistance, isolates from two neighboring orchards in Wayne County, NY were found to be highly resistant to streptomycin at 100 μg/ml. This constitutes the first authenticated report of streptomycin resistance in New York State. All infected trees were shipped at the same time from a single nursery in Michigan. Resistance was caused by the acquisition of the *strA-strB* gene pair, inserted into the ubiquitous non-transmissible *E. amylovora* plasmid pEa29. Previously, streptomycin-resistant *E. amylovora* populations from Michigan have been described with a similar mechanism of resistance although the *strA-strB* genes are not unique to Michigan. These findings illustrate how unintentional movement of nursery material infected with resistant bacteria could undermine efforts to prevent antibiotic resistance development.

Introduction

Fire blight, caused by the Enterobacterium *Erwinia amylovora* [(Burrill) Winslow *et al*.], is a devastating disease of rosaceous plants. Most commonly associated with apple (*Malus* spp.) and pear (*Pyrus* spp.), fire blight affects production of one or both of these fruits in over 40 countries (4). Fire blight occurs in three distinct phases; blossom blight, shoot blight, and rootstock blight. Of these, blossom blight the infection of newly opened blossoms is the most significant, resulting in devastating crop losses (36), while serving as a precursor to the shoot and rootstock phases of the disease, either of which can be fatal to trees (28). Blossom blight is the sole phase of fire blight with a functional prediction system and effective chemical control (4).

Blossom blight is primarily controlled through application of the antibiotic streptomycin, which was introduced as a pesticide for horticultural use in 1955 (24,22). Although streptomycin was highly effective, streptomycin-resistant strains of *E. amylovora* were identified in California pear orchards in 1971 (23), and soon after in Washington and Oregon in 1972 (11). At that time antibiotic applications to control fire blight were excessive, reported to consist of ten to fourteen applications at bloom, providing heavy selection pressure for resistance (31). Resistance has been reported throughout the Western United States as well as in British Columbia (Canada), New Zealand, Israel, and Lebanon (22,32,35).

Two mechanisms of streptomycin resistance have been described in wild *E. amylovora* populations. The most common form of streptomycin resistance occurs through a single base pair mutation of the streptomycin-binding site. Streptomycin binds to protein S12 of the 30S ribosomal subunit, encoded by the *rpsL* gene, thereby preventing protein synthesis (22). Chiou and Jones (10) determined that streptomycin resistance was caused by a single base pair mutation in codon 43 of the *rpsL* gene,

which results in the substitution of the amino acid lysine (AAA) by either arginine (AGA), threonine (ACA) or asparagine (AAT or AAC). Only one of these mutations, the conversion of lysine to arginine, produces a stable mutation without any fitness cost to the bacterium (10). Chromosomal resistance is highly stable and has been shown to persist in the environment. Moller et al. (24) reported streptomycin-resistant populations of *E. amylovora* could still be detected in California pear orchards ten years after the application of streptomycin had ceased.

Streptomycin resistance has also been associated with the acquisition of resistance plasmids. Chiou and Jones (7) established that streptomycin resistance in Michigan fire blight populations was based on the acquisition of the plasmid pEa34, which possesses the *strA-strB* gene cluster. Plasmid pEa34, a conjugative plasmid, likely originated in *Pantoea agglomerans* and was acquired by *E. amylovora* through conjugal gene transfer (7,21). The tandem *strA-strB* genes code for aminoglycoside-3-phosphotransferase and aminoglycoside-6-phosphotransferase, respectively (9). These aminoglycoside-modifying enzymes break down streptomycin, and have previously been described in resistant populations of *Pseudomonas syringae* pv. *papulans*, *P.s.* pv. *syringae*, and *Xanthomonas campestris* pv. *campestris* (17,22,34). Although the *strA* and *strB* genes function independently, both must be present to confer high levels of antibiotic resistance (9). Located within the transposable element Tn5393, *strA-strB* have been found on broad host range plasmids such as pRSF1010 (pEa8.7) (29), or inserted into strain specific plasmids like pEa34 (8). Tn5393, with *strA-strB,* has also been discovered inserted into the non-transmissible *E. amylovora* plasmid pEa29 and into the chromosome (21). Chromosomal insertion of *strA-strB* genes generally results in higher resistance than plasmid insertion (9). These mobilizable resistance genes have previously been identified in common orchard epiphytes in association with fire blight infections (5,6,13,27,33)

Currently New York is the only major apple production region in the United States without endemic populations of streptomycin-resistant *E. amylovora*. Streptomycin is routinely applied in New York orchards and the lack of resistance development after continued antibiotic usage is unprecedented. Resistance surveys conducted by Beer and Norelli (1) and Burr et al. (5,6) failed to identify streptomycin-resistant populations of *E. amylovora* between 1975 and 1992. Burr et al. (5,6) however did confirm the presence of the streptomycin resistance genes, *strA-strB,* in orchard populations of the blister spot pathogen *P. s.* pv. *papulans,* often associated with fire blight infections. Orchard surveys in New York are separated by many years at which time resistant populations could become established, thereby limiting the effectiveness of eradication measures. The objectives of this study were to survey orchards throughout New York for the presence of streptomycin resistance isolates of *E. amylovora* and to characterize resistance if found.

Materials and Methods

Orchard Survey

Active fire blight lesions were collected by regional Cornell Cooperative Extension Associates, and Dr. David A. Rosenberger, of Cornell University's Hudson Valley Laboratory, and processed at the New York State Agricultural Experiment Station in Geneva, New York. Isolation sites were chosen based on past streptomycin use and reports of inconsistency in control of fire blight using streptomycin. Over a four-year period, from 2002-2006, samples were collected from 14 counties throughout New York including 56 individual farm sites.

Isolation of Bacteria and Resistance Screening

Single colony isolates of *E. amylovora* were isolated from infected tissue and tested for antibiotic resistance according to Beer and Norelli (1). Samples were surface sterilized in 0.5% sodium hypochlorite solution for 10 min, and rinsed with sterile distilled water. Outer bark was removed and internal tissue plated on Crosse and Goodman (CG) medium (12) diagnostic for *Erwinia* spp. Single colony isolates were identified as *E. amylovora* by colony morphology, and final confirmation was based on the presence of a 1Kb fragment amplified from the ubiquitous *E. amylovora* plasmid pEa29, modified from Bereswill et al. (2). PCR reactions, 50 µl total volume, were carried out using 2.5 µM primers A and B (2), 12.5 mM $MgCl_2$, 5 µl PCR Reaction Buffer (Promega), 0.1 mM dNTPs, 0.625 units Taq® DNA Polymerase (Promega), and 10 µl of bacterial sample, and PCR products were analyzed by electrophoresis using 1% agarose gels. Three colonies, when possible, were stored from each sample for resistance screening.

To assess level of streptomycin resistance, isolates were grown at 28 ˚C in 5 ml liquid Luria-Bertani (LB) broth, and 100 µl aliquots adjusted to 10^8 cfu/ml, were evenly spread on LB medium. Bacteria were challenged with filter paper disks (Schleicher & Schuell Inc., Keene, NH) impregnated with a 0, 10, 50, or 100 µg/ml solution of streptomycin sulfate and incubated at 28 ˚C. Resistance was determined by the presence of a clear zone of inhibition assessed at 24 and 48 hr. Resistance level was compared against the streptomycin-susceptible strain Ea273 and streptomycin-resistant strain CFBP1376. Three replicates of each single colony isolate were tested and experiments were repeated.

Bacterial Strains and Plasmid Selection

E. amylovora strains, CA11 (8) and BCN77 (21), containing the *strA-strB* gene pair, and strain Ea110 containing plasmid pC9, were provided by George Sundin and Gayle McGhee at Michigan State University (Table 5.1). NY17.1 and NY17.2 are the uncharacterized streptomycin-resistant strains that originated from this study.

Table 5.1. Relevant bacterial strains and plasmids.

Strain, plasmid	Relevant characteristic(s)	Source, reference
E. amylovora		
Ea273	Ubiquitous plasmid, pEa29	S.V. Beer
Ea0380	Ea273 w/ Rf^r, chromosomal	S.V. Beer
CFBP1376	Sm^r, chromosomal	J.P. Paulin
Ea88-100	Sm^r, chromosomal	R. Roberts
CA11	Sm^r, pEa34 carrying TN5393 w/ *strA-strB* genes	(8)
BNC77	Sm^r Chromosomal insertion of Tn5393 w/ *strA-strB* genes	(21)
NY17.1	Sm^r, isolated 11/20/2002	This study
NY17.2	Sm^r, isolated 11/20/2002	This study
pC9	4.4kb pEa29 *Pst*I fragment cloned into pGEM3zft(+)	(20)

Virulence Assays

Bacteria that displayed a significant level of resistance to streptomycin were assessed for virulence using both immature green pear fruit (3) and seeding inoculation tests (30). Immature pear fruit were wounded with toothpicks dipped in

inoculum and incubated overnight at 28˚C for 48 hr. Isolates were recorded as virulent if the inoculation site became necrotic and ooze was exuded from wound. Actively growing McIntosh seedlings, approximately 10 cm in height, were inoculated by transversely bisecting the two youngest leaves with scissors dipped in a suspension of streptomycin-resistant strains NY17.1 and NY17.2 ($1x10^7$ cfu /ml) in 0.5 M potassium phosphate buffer according to Norelli et al. (26). Necrotic lesions accompanied by the production of bacterial ooze confirmed virulence on apple seedlings. Results were compared against the susceptible strain Ea273 and resistant strain CFBP1376.

Sequencing of the Ribosomal Protein S12 (*rpsL*) Gene

An internal region of the ribosomal *rpsL* gene, containing codon 43, was amplified from streptomycin-resistant New York strains NY17.1, and NY17.2, along with streptomycin-resistant strains CA11, Ea88-100, CFBP1376, and a streptomycin-sensitive strain Ea273. Primers were based on the *rpsL* gene sequence from *E. amylovora* (GenBank accession number L 36465) (Table 5.2).

Table 5.2. PCR primers developed in this study.

Gene	Primer	Product size
rpsL	rpsL212-F: 5'-cgtacgcaaagttgcaaaaa-3'	
	rpsL212-R: 5'-ggatcaggatcacggagtgt-3'	212 bp
strA	strA406-F: 5'-tgactggttgcctgtcagag-3'	
	strA406-R: 5'-cggtaagaagtcgggattga-3'	406 bp
strB	strB403-F: 5'-atcgctttgcagctttgttt-3'	
	strB403-R: 5'-cgttgctcctcttctccatc-3'	403 bp

PCR reactions, 50 µl total volume, were carried out using 0.4 µM rspL212F and rpsL212R, 12.5 mM $MgCl_2$, 5 µl PCR Reaction Buffer (Promega), 0.1 mM dNTP, 0.625units Taq® DNA Polymerase (Promega), and 10µl of bacterial sample, and PCR products were analyzed by electrophoresis using 1% agarose gels. PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Samples were sequenced using an Applied Biosystems Automated 3730 DNA Analyzer (Foster City, CA) at the Core Laboratories Center (CLC) (Ithaca, NY).

strA-strB Gene Identification

Internal regions of the *strA* and *strB* genes were amplified from New York strains NY17.1, and NY17.2, along with streptomycin-resistant *E. amylovora* strains CA11, CFBP1376, and streptomycin-sensitive strain Ea273. Primers were based on the *E. amylovora strA* and *strB* gene sequences characterized by Chiou and Jones (8) (GenBank accession number M 96392) (Table 5.2). PCR reactions, 50 µl total volume, were carried out using 0.4 µM of either strA406-f and strA406-r, or strB403-f and strB403-r, 12.5 mM $MgCl_2$, 5 µl PCR Reaction Buffer (Promega), 0.1 mM dNTP, 0.625units Taq® DNA Polymerase (Promega), and 10µl of bacterial sample, and PCR products were analyzed by electrophoresis using 1% agarose gels. PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Samples were sequenced to using an Applied Biosystems Automated 3730 DNA Analyzer (Foster City, CA) at the Core Laboratories Center (CLC) (Ithaca, NY) to verify gene identity.

Plasmid Transfer

Plasmid transfer rates were determined according to Chiou and Jones (7). Recipient bacteria were rifampicin-resistant mutants of *E. amylovora* strain Ea273 identified as Ea0380 (CUPPB0380). Equal concentrations of recipient strain Ea0380

at $1x10^{10}$ cfu/ml were combined with $1x10^{10}$cfu/ml of donor strains NY17.1, NY17.2, CA11, and 273. Mixtures were incubated overnight at 28˚C and dilution plated on LB medium amended with rifampicin, streptomycin, or rifampicin plus streptomycin, at 50 μg /ml. Plates were incubated at 28˚C and resultant colony growth on medium amended with both antibiotics signified bacterial conjugation. All strains utilized in this experiment contain the ubiquitous non-transmissible plasmid pEa29, if the *strA-strB* resistance genes are located on plasmid pEa29 or inserted into the chromosome, no transfer of resistance would be observed.

Plasmid Curing

The ubiquitous non-transmissible plasmid pEa29 was cured from *E. amylovora* strains through incompatibility eviction assay previously described by McGhee and Jones (20). Plasmid curing was performed to determine the effect of pEa29 on streptomycin resistance. Plasmid pC9 (Amp^r) (Table 5.1), containing the pEa29 *ori* region, was electroporated into strains NY17.1, NY17.2, CA11, and Ea273. Bacteria were grown on LB medium amended with ampicillin at 50 μg /ml to select for pC9. Plasmid pEa29 eviction was verified using the pEa29 specific primers A and B (2) compared with *E. amylovora* chromosomal primers AJ245 and AJ246 (16). PCR reactions, 50 μl total volume, were carried out using 0.4 μM of either A and B, AJ245 and AJ246, with 12.5 mM $MgCl_2$, 5 μl PCR Reaction Buffer (Promega), 0.1 mM dNTP, 0.625units Taq® DNA Polymerase (Promega), and 10μl of bacterial sample. Plasmid cured strains were dilution plated on LB medium amended with ampicillin, streptomycin, or ampicillin and streptomycin, at 50 μg /ml.

Results

Resistance Screening and Virulence Assay

In August 2002 streptomycin-resistant isolates were recovered from symptomatic Ida red apple shoots collected from a newly planted orchard in Wayne County. Bacteria were confirmed as *E. amylovora* and were found to be resistant to streptomycin at 100 μg/ml; no clear zones of inhibition were produced in response to streptomycin after 24 or 48 hr. Bacteria were virulent on both immature pear fruit and apple seedlings (data not shown). Resistant isolates were cataloged as *E. amylovora* strain NY17.1. Streptomycin-resistant strains of *E. amylovora* were also recovered from a nearby Ida red orchard in May 2003 (NY17.2). Investigation revealed the Ida red trees planted at both orchards sites originated from the same nursery shipment. Resistance was confirmed and both orchard sites and trees were removed in the winter of 2003-2004. Between 2003 and 2006 no new streptomycin-resistant isolates were identified in New York.

Sequencing of the Ribosomal Protein S12 (*rpsL*) Gene

Comparison of the 212 bp sequences amplified from the *rpsL* gene verified a highly conserved region across all six strains of *E. amylovora*. Two single base pair mutations were observed in the streptomycin-resistant strains CFBP1376 and Ea88-100 (Table 5.3). CFBP1376 was shown to have a deleterious mutation, lysine to threonine, while Ea88-100 strain was shown to have the persistent mutation, lysine to arginine. NY strains NY17.1 and NY17.2 along with CA11 were comparable to the susceptible strain Ea273 at codon 43 of the *rspL* gene coding for the wild type amino acid lysine (AAA).

Table 5.3. Sequence comparison of *rpsL* gene from streptomycin sensitive and resistant strains.

Strain	Sm[r]	Sequence
Ea273	-	TGTGTACACGACTACCCCT**AAA**AAACCGAACTCCGCA
		TGTGTACACGACTACCCCT**AAA**AAACCGAACTCCGCA
CFBP1376[z]	+	TGTGTACACGACTACCCCT**ACA**AAACCGAACTCCGCA
		TGTGTACACGACTACCCCT**ACA**AAACCGAACTCCGCA
CA11	+	TGTGTACACGACTACCCCT**AAA**AAACCGAACTCCGCA
		TGTGTACACGACTACCCCT**AAA**AAACCGAACTCCGCA
Ea88-100[y]	+	TGTGTACACGACTACCCCT**AGA**AAACCGAACTCCGCA
		TGTGTACACGACTACCCCT**AGA**AAACCGAACTCCGCA
NY17.1	+	TGTGTACACGACTACCCCT**AAA**AAACCGAACTCCGCA
		TGTGTACACGACTACCCCT**AAA**AAACCGAACTCCGCA
NY17.2	+	TGTGTACACGACTACCCCT**AAA**AAACCGAACTCCGCA
		TGTGTACACGACTACCCCT**AAA**AAACCGAACTCCGCA

[z]Persistent mutation, lysine (AAA) to arginine (AGA), produces stable streptomycin resistance

[y]Deleterious mutation, lysine (AAA) to threonine (ACA), produces unstable streptomycin resistance, which retards colony growth

strA-strB Gene Identification

StrA and *strB* gene primers amplified 406 and 403 bp regions of the *strA* and *strB* genes from both New York streptomycin-resistant strains. Band size was consistent with CA11 and which is positive for the *strA-strB* gene cluster (Figure 5.1). No amplification occurred in strains with chromosomal resistance conferred by

mutation of the *rpsL* gene or in the wild type streptomycin-sensitive strain. Sequence results confirmed the identity and conserved nature of the *strA* and *strB* genes previously described from *E. amylovora*.

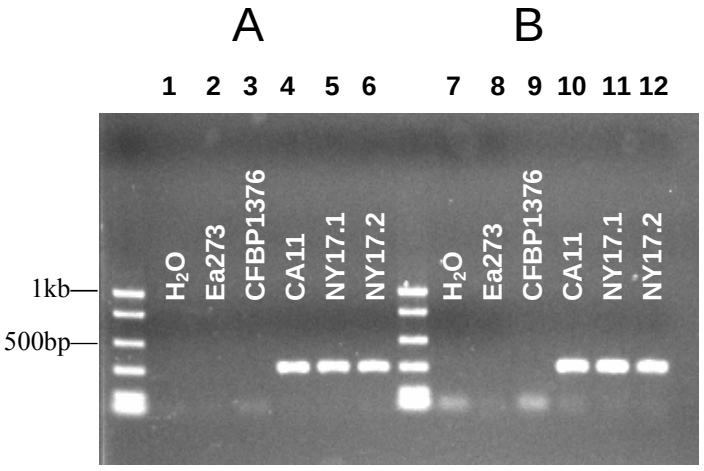

Figure 5.1 Agarose gel depicting *E. amylovora* strains that amplified with primers based on the 406 and 403 bp internal fragments of the A. *strA* and B. *strB* genes, respectively. Lane 1&7. H_2O, 2&8. strain Ea273, 3&9. CFBP1376, 4&10. CA11, 5&11. NY17.1, 6&12. NY17.2.

Plasmid Transfer

Plasmid transfer of streptomycin resistance was observed when CA11 was utilized as the donor stain but not with the wild type strain Ea273. CA11 contains the *strA-strB* gene pair on conjugative plasmid pEa34, previously characterized by Chiou and Jones (7) (Table 5.4). Growth of CA11 on media containing streptomycin exceeded growth on medium harboring both antibiotics, but frequency of plasmid transfer remained substantial. Growth of the recipient strain Ea0380 remained constant in all matings and did not contribute to differences in plasmid transfer. No

transfer of resistance was observed between Ea0380 and either NY isolate NY17.1 or NY 17.2. No spontaneous mutations conferring either streptomycin or rifampicin resistance were observed during the course of the experiment. Experiments were repeated with similar results.

Table 5.4. Resistance profile of strain Ea0380 (Rf^r) after bacterial conjugation with streptomycin-resistant strains CFBP1376, CA11, NY17.1, and NY17.2.

		Colony development (cfu/ml)[y]		
				Tranconjugants[z]
Donor strain	Recipient strain	Sm	Rf	Sm w/ Rf
CFBP1376	Ea0380	5.0×10^{7}[x]	3.0×10^{7}	0
CA11	Ea0380	7.0×10^{7}	3.8×10^{6}	1.6×10^{6}
NY17.1	Ea0380	1.6×10^{8}	1.0×10^{7}	0
NY17.2	Ea0380	2.6×10^{7}	1.3×10^{7}	0

[z]Tranconjugant growth signifies transfer of streptomycin resistance plasmid

[y]Bacterial growth measured as cfu/ml on media supplemented with either streptomycin (Sm) rifampicin (Rf) or a combination of both at 50 μg/ml

[x]Colony counts based on averages of 2 replicated plates each w/ 3 colony counts Experiment repeated with analogous results

Plasmid Curing

Plasmid pEa29 was evicted from strains Ea273, NY17.1, and NY17.2 through the introduction of plasmid pC9. Plasmid eviction was verified by the absence of a 1Kb fragment from pEa29 as determined by PCR (data not shown). Complete eviction of pEa29 was not achieved for streptomycin-resistant strain CA11, even in the presence of pC9; verified by resistance to ampicillin at 50 μg/ml (Table 5.5). Based on the nature of streptomycin resistance in CA11 (9,10) results were not influenced by incomplete eviction of pEa29. Results clearly demonstrated strains NY17.1 and NY17.2 lost the ability to grow on streptomycin-amended medium after eviction of plasmid pEa29 (Table 5.5). No spontaneous mutations conferring either streptomycin or ampicillin resistance were observed during the course of the experiment. Experiments were repeated with similar results.

Table 5.5. Resistance profile of strains cured of plasmid pEa29 through incompatibility eviction assay.

	Colony development (cfu/ml)			
Strain[z]	LB	Amp[y]	Sm	Amp+Sm
273 (w/ pC9)	8.3×10^{10}	6.83×10^{10}	0	0
CA11 (w/ pC9)[y]	1.9×10^{8}	1.27×10^{8}	1.17×10^{8}	3.67×10^{7}
NY17.1 (w/ pC9)	8.0×10^{9}	9.83×10^{8}	0	0
NY17.2 (w/ pC9)	7.33×10^{9}	3.03×10^{9}	0	0

[z]designates strains cured of plasmid pEa29 by incompatibility eviction assay

[y]Ampicillin (Amp) resistance signifies acquisition of the selective plasmid pC9

[z]CA11 demonstrated incomplete eviction of pEa29, growth of bacteria in culture was slowed

Discussion

In 2002 and 2003 resistant strains of *E. amylovora* were isolated from two neighboring orchards in Wayne County. New York isolates were found to be highly resistant to streptomycin without suffering a discernible reduction in virulence. These results constitute the first authenticated report of streptomycin-resistant *E. amylovora* in New York. In 2003 both orchards were removed in an effort to eradicate trees harboring streptomycin-resistant bacteria. Beyond the two initial plantings, no streptomycin-resistant fire blight has been identified in New York since 2003. Early identification and prompt orchard eradiation can be attributed in halting the spread of resistance and forestalling the loss of streptomycin for future control of fire blight.

Since the discovery of streptomycin resistance in California in 1971 antibiotic usage in apple and pear regions has been reduced dramatically. Reduction in the number of antibiotic applications has been proposed as the main impediment to antibiotic resistance development (22,24). Streptomycin usage in New York State however has historically been modest compared to usage in California and to a lesser extent in Michigan, where resistance developed in the early 1970' and 1990's respectively. Moller et al. (24) suggested that with continued reliance on streptomycin, resistance development is inevitable due to constant mutation and selection in bacterial populations. Although reduction in antibiotic usage is necessary to maintain the effectiveness of streptomycin, there are other means by which resistant bacteria become established in new areas.

Over the past 200 years fire blight, originally identified in the Hudson Valley, New York, has been unintentionally disseminated throughout the world via the movement of infected plant material (4). Presently bacterial movement still poses a great risk for countries without endemic fire blight. Potential impact on apple production is magnified by the probability of importing *E. amylovora* predisposed to

streptomycin resistance. It is widely believed that streptomycin resistant fire blight identified in Lebanon in 2000 originated in neighboring Israel. The lack of previous antibiotic use in Lebanon, reducing selection pressure for the development of resistance, further supports this theory (22).

With regard to the streptomycin-resistant New York isolates, evidence supports the theory that trees were infected with *E. amylovora* resistant to streptomycin prior to planting. Resistant isolates from Wayne County were identified in orchards that had been planted the previous year and had yet to flower. Fire blight cankers in these plantings were considerable despite the young age of the planting and limited exposure to fire blight, which indicated prior infection. Furthermore only one of the plantings had been treated with streptomycin prior to detection of resistant bacteria. Planting material in both orchards originated from the same nursery located in southwestern Michigan where resistant *E. amylovora* has been described previously (7,8,9,10). Trees were sold following a fire blight epidemic in 2000 that destroyed much of the apple production in SW Michigan, resulting in several million dollars worth of damage and tremendous tree loss (19). The strongest evidence supporting the movement of infected nursery material is the presence of the *strA-strB* gene cluster, found to be the cause of resistance in New York. This type of resistance occurs associated with fire blight only in Michigan save for a single Californian isolate, which contains a unique resistance plasmid (29), not observed in the New York isolates.

Although evidence strongly suggests resistant strains were imported on infected nursery stock, it is difficult to prove conclusively. With the exception of *Rubus* strains, *E. amylovora* are genetically homogeneous with few identifying characteristics (25). Jock et al. (14) using PFGE analysis could discern distinct patterns relating to origin in European *Malus E. amylovora* strains, but similar

differentiation of North American strains was not observed (15). The presence of the *strA-strB* gene cluster, although significant, is inconclusive as a marker for bacterial origin. The *strA-strB* genes are common antibiotic resistance genes and have been well characterized in New York populations of epiphytic bacteria that exist in close proximity to *E. amylovora* (13,33).

Movement of plant material is a common practice in the apple industry. Many of the prominent nurseries are currently located on the West Coast only marginally removed from areas with streptomycin resistance. These findings although inconclusive do address the potential for infected nursery material to serve as a vehicle to spread resistant bacterial populations.

Alternate materials for control of fire blight have been investigated, however nothing has proven to be as effective or as durable as streptomycin. In California and Michigan oxytetracyline, has been approved on a limited basis where resistant bacteria have been identified. Oxytetracycline, however, is not as effective as streptomycin on antibiotic sensitive strains and only outperforms streptomycin in areas where resistance occurs (22). In Israel, oxolinic acid is used to control fire blight but resistant strains were identified only one year after commercial release (18). Concern over the spread of antibiotic resistance to human pathogens makes registration of new antibiotics for agricultural use very difficult (22). Streptomycin is the most effective antibiotic for use on apple and is likely to remain as such; therefore it is imperative to identify cases of antibiotic resistance early before bacterial populations become established.

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