DISEASE MANAGEMENT IN APPLES USING TRUNK INJECTION DELIVERY OF PLANT PROTECTIVE COMPOUNDS

By

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ABSTRACT

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The two most important pathogens of apple *Erwinia amylovora* (fire blight) and *Venturia inaequalis* (apple scab) require pesticide sprays for control. This leads to accumulating side effects such as disease resistance, contamination of environment, elevated fungicide residues in fruit, and increased health risks to consumers and workers. While sprays are effective for disease control, need for increasing the sustainability of apple production by reducing pesticide use in the environment incited our research on delivering pesticides via trunk injection. This method delivers the compound into the canopy via tree xylem and could increase the efficiency in disease control. To find out how, where and when injected compounds distribute in the apple tree, thus affecting the efficiency in pest control, we injected imidacloprid through 1, 2, 4, or 8 injection ports per tree. By quantifying leaf residues we demonstrated variable spatial distribution of imidacloprid in the canopy. Spatial uniformity of distribution increased with more injection ports and 4 ports provided uniform distribution. To demonstrate the efficiency of injected compounds in fire blight and apple scab control we injected apple trees with antibiotics, plant resistance inducers, and fungicides. Antibiotics, potassium phosphites (PJ) and acibenzolar-S-methyl (ASM) provided weak control of blossom and shoot blight while oxytetracycline was the most efficient. ASM and PJ significantly expressed PR-1, 2, and 8 protein genes showing resistance activation in apple leaves (SAR) which suppressed the pathogen. Four injections of PJ in spring controlled leaf apple scab for 2 seasons, similar to 2 seasons of standard sprays. To

optimize injections for apple scab control we evaluated 1-2 and 4 cross-seasonal and 1-2 seasonal injections of PJ and fungicides. PJ provided better scab control than propiconazole, cyprodinil and difenoconazole and showed better or equal and more persistent scab control with fewer injections than sprays. Control varied among canopy organs due to different transpiration, with best scab control on shoots, fruit, and then spurs. Good scab control is provided by 2-3 spring injections. Residues of synthetic fungicides in fruit were always below the residue tolerances. Fall injection did not improve apple scab control. To get temporally uniform imidacloprid distribution in the crown, best results were achieved by injection dose delivery at 4 times, 14 days apart. Injection method comparison showed that drill-based injection of the liquid imidacloprid formulation provided the highest residue concentration in the canopy when compared to other injection methods. Comparison of 7 trunk injection devices showed that drill-based devices did not provide higher residue concentration of cyprodinil and difenoconazole in apple leaf canopy when compared to needle-insertion device Bite, while Wedgle was similar. All the injection devices allowed similar apple scab control with fungicides. When monitoring the rate of trunk injection port healing in apple trees, we found that port closure with callus lasted for 1-1.3 and >2 years depending on the port size and type. Port closure was faster on the ports with smaller diameters. Around all injection port types, bark cracking due to frost events was higher in vertical direction of the trunk. The visible port depth declined faster on port from 11/64” drill bit and on lenticular injection port from double-edge blade, versus the port from 3/8” drill bit. When the port from 3/8” drill bit was sealed with an Arborplug, visible and covered port depths significantly increased in time due to callus formation on the top and laterally, around the plug. Overall, trunk injection of injection formulated pesticides could be a viable option for disease control in apples with minimal impact of injection ports on the tree.

To my beloved wife Dana, my parents Ivana and Goran and brother Žarko.
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KEY TO SYMBOLS AND ABBREVIATIONS

a. i. - active ingredient

ASM - acibenzolar-S-methyl

BABA - DL-ß-aminobutyric acid

CRD - completely randomized design

cv. - cultivar

DAI - days after injection

DAFI / DASI - days after the first injection / days after the second injection

DBH - tree trunk diameter at breast height

dbi - days before inoculation

DCC – difenoconazole and cyprodinil combination

DFH - tree trunk diameter at one foot height (30.48 cm)

DHFH - tree trunk diameter at half foot height (15.24 cm)

DPI - days post inoculation

MRL - maximum residue limit(s)

PCA - prohexadione-carboxylic acid

PR - pathogenesis related (proteins)

RCBD - randomized complete block design

SA - salicylic acid

SAR - systemic acquired resistance

VPD - vapor pressure deficit

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Apple production and its significance

According to Food and Agriculture Organization of the United Nations (FAO) the total apple production in the world in 2011 amounted to 75,635,283 metric tons (FAOSTAT 2013). The largest producers of apple are shown in Table 1.

Table 1. The largest apple producers in the world.

Country	metric tons
World	
China	35,987,221
USA	4,272,840
India	2,891,000
Europe	
Poland	2,493,080
Italy	2,411,200
France	1,858,880
Asia	
Turkey	2,680,080
Iran	1,651,840
The rest	
Brazil	1,330,000
Russia	1,200,000
Chile	1,169,090
Argentina	1,115,950

The production of apples in USA with 4,272,840 metric tons in 2011 brings value of $1,807,043,000 (FAOSTAT 2013). According to USDA National Agricultural Statistics Service (NASS), Michigan, New York and Pennsylvania in 2007 had around 100,000 acres of bearing apple trees (www.nass.usda.gov). According to Michigan Apple Committee, in Michigan the

third largest apple-producing state in the USA after Washington and New York, the average apple harvest amounts to 375,574 metric tons (www.michiganapples.com). According to NASS, harvest in Michigan in 2011 was 446,788 metric tons with the total crop value of $194.7 million (www.nass.usda.gov). The same source states that in 2012, due to unusually warm weather in March and then severe frosts which followed, apple production in Michigan was only 52,163 metric tons. According to estimates for 2013 it is expected that around 810,800 metric tons of apples will be harvested in Michigan (www.michiganapples.com). The same source states that in Michigan there is around 36.500 ha of apples which generates an average economic contribution to the state of $700-$900 million annually. The most frequently planted apple cultivars in Michigan are 'Red Delicious', 'Golden Delicious' and 'Gala' (http://www.applejournal.com). Around 40% of apple crop produced in Michigan is consumed fresh while the remaining part is processed into fresh-cult slices, cider and applesauce.

By the dietary importance for human health, US Department of Agriculture and US Department of Health and Human Services have placed fruits in Food Plate to a large portion of 20% (http://www.choosemyplate.gov). Fruit crops offer health benefit in prevention of myriad diseases provided by the antioxidants, fiber, and vitamins crucially necessary in everyday human diet (Wu et al. 2004). Further, since apple contains no fat and has myriad compounds providing significant antioxidant activity, it is an ideal food commodity which has been shown to preventively reduce health risks from more than several clinical conditions (Liu 2003; Boyer & Liu 2004).

Fire blight Erwinia amylovora and its significance

Fire blight caused by bacterium *Erwinia amylovora* (Burrill) Winslow et al., is the most destructive prokaryotic disease of rosaceous plants (Bonn & Van der Zwet 2000; Norelli et al. 2003). Fire blight losses in the world are tremendous. Severe fire blight outbreaks were reported in Italy where 500,000 trees were destroyed in Emilia-Romagnia region (Calzolari et al. 1998). In Spain it has been reported that fire blight could cause losses of €2,3 billion if quarantine plant protective measures are not timely implemented to stop this disease (Unió de Pagesos 2013). Hunderds of thousands of dollars in losses are further extensively reported in New Zealand, Egypt, Hungary, Macedonia, Turkey, Slovenia, Serbia and many other countries in the world (Jones & Sutton 1984; Panić & Arsenijević 1992; Mitrev 1995; Pejchinovski 1995; Bonn & Van der Zwet 2000; Vanneste 2000; Bubán et al. 2001; Günen et al. 2004; Van der Zwet 2004; Balaž et al. 2009; Végh 2012; Balaž et al. 2013).

In the USA, fire blight losses and control costs per year are estimated to be more than $100 million (Norelli et al. 2003). In Michigan, fire blight led to economic losses of $42 million in 2000 which resulted from removal of 350,000 to 450,000 apple trees, while previous outbreak in 1991 led to economic losses of $3.8 million (Longstroth 2001; Aldwinckle et al. 2004; Douglas 2006). In Washington and northern Oregon, economic losses on pomaceous fruits were estimated to be over $68 million (Stockwell et al. 2002).

The fire blight bacterium overwinters within hold-over cankers remaining from the previous growing season infections of woody tissues on branches (Beer & Norelli 1977; Biggs et al. 2008). In the spring, with favorable environmental conditions, primary inoculum occurs in the form of bacterial exudate on the canker surface and enables pathogen spreading to the blossoms

by rain splash combined with wind and by insects (Thomson 2000; Van Der Zwet & Keil 1979; Steiner 1989; Lightner & Steiner 1992). Infected blossoms produce secondary inoculum which allows further blossom and fruitlet infections and subsequent infections of intensively growing shoots (Beer 1979; Keil et al. 1966). Primary inoculum can also originate from shoots emerging from buds close to overwintering cankers (Biggs et al. 2008). Bloom and then active shoot growth are the most susceptible stages of apple development to fire blight. Fire blight severity varies between years and depends on the abundance of overwintering inoculum, host and cultivar susceptibility, crop management system and environmental conditions (Van Der Zwet & Keil 1979).

Fire blight control on apple, pear and quince is a very difficult task. Successful management is dependent on use of cultural practices such as use of resistant or tolerant cultivars, mechanical removal of all symptoms and preventive copper and antibiotic sprays (Norelli et al. 2003). The two major difficulties in fire blight management are that currently there are no synthetic compounds with fully systemic properties available to improve fire blight protection programs and there are no acceptable resistant cultivars of apple, pear and quince (Adaskaveg et al. 2011; Balaž et al. 2013). Another management difficulty is the establishement of *E. amylovora* resistance to antibiotics and and tolerance to copper (Chiou & Jones 1991; 1995; Loper et al. 1991; Ordax et al. 2006). Pathogen resistance to antibiotics hampers their future use in agriculture due to fear that antibiotics for animal growth regulation and protection and plant protection contribute to development of antibiotic resistance in human pathogens (McManus et al. 2002). Potential transfer of antibiotic resistance genes from plant to human pathogenic bacteria via conjugative plasimds with transposable elements could have repercussions on human health (Sundin et al. 1995; Sundin & Bender 1996).

To find alternative management methods, a vast front of scientific research in the past two decades has focused on the investigating the use of bio-control agents and systemic acquired resistance (SAR) activators in fire blight control (Johnson et al. 2000; Maxson-Stein et al. 2002; Norelli et al. 2003). For now, the results show limited efficiency inferior to antibiotics and need for frequent reapplication of these new biological agents and compounds. As an outcome these new agents are being recommended solely as a supplement to antibiotic-based programs, to aid in delaying the occurrence of fire blight pathogen resistance.

Conventional management of *E. amylovora* relies upon preventive topical sprays of bactericides (Norelli et al. 2003). This provides direct contact of the material with the pathogen, before or immediately after the pathogen reaches the apple flowers or shoots. Successful fire blight management is dependent on controlling the epiphytic pathogen populations before they enter into the plant and initiate infection. Since most of the time *E. amylovora* is harboured inside diseased tissues it is a difficult and highly restrictive target for bactericides, both from a temporal and spatial perspective. Foliar sprays more than 24 hours after a fire blight event are unlikely to prevent infection. Many fire blight forecast models like MARYBLYT and COUGARBLIGHT have been developed in past four decades and are used in different parts of the world to predict disease occurrence (Billing 1989; 2007; Steiner 1989; Lightner & Steiner 1992; Smith 1995; 1998; Smith & Pusey 2010). These forecast models provide an opportunity for time-precise delivery of protection, while avoiding unnecessary antibiotic sprays against fire blight. On the other hand, poor, non-uniform canopy coverage, resulting from inefficient topical application can also result in failure of bactericide to prevent infections. It has been reported before that air-blast sprayers are inefficient in topical compound delivery with pesticide solution losses into the environment of up to 44-71% (Steiner 1969). Wasted bactericides during

unnecessary applications or spatially imprecise delivery are not only a problem for controlling the target disease, but compound drift can promote the development of resistance in plant pathogenic bacteria in the environment. Research has shown that when non-target bacterial populations are exposed to pesticides in the agro-ecosystem, already resistant populations can be selected by the applied antimicrobial activity of a pesticide and furhter transfer these genes to the other sensitive bacteria and plant pathogens such as *Erwinia amylovora* (Chiou & Jones 1993; Sundin et al. 1995).

Even with the best spray coverage, the activity of some of the topically applied bactericides, plant resistance activators (PRA) and biological agents are negatively affected by weather conditions (rainfall, sunlight, temperature), specific properties of phylloplane, and a limited rate of absorption and subsequent movement in the plant (Windels et al. 1985; Scholz & Reinhard 1999; Percival 2001; Gozzo 2003; Adaskaveg et al. 2010; 2011; Cabrefiga et al. 2011). These difficulties bring into question the means by which materials for fire blight control are delivered and support investigation of alternative solutions such as chemotherapy or *in planta* delivery of plant protective agents (McIntyre et al. 1979; Mclntyre & Lacy 1979; Brundtland 1987; Hiruki 1988; Cooley et al. 1992; McManus et al. 2002; Spitko 2008; Düker & Kubiak 2011a). Recent research on trunk injection or trunk drenches with PRA-s and bactericides for control of fire blight and other bacterial and phytoplasmal diseases has shown promising results in disease reduction (Hiruki 1988; Raju & Nyland 1988; Spitko 2008; Düker & Kubiak 2011a; Graham & Myers 2013). Trunk injection could be a valuable alternative approach for delivery of plant protective compounds in tree-based agriculture but to become economically viable for cultivated tree protection it requires further advancement in the area of used injection technology. Since it is based on harnessing the vascular transport capacity of a tree to distribute

the compound into the canopy, trunk injection could minimize or eliminate aforementioned negative side-effects of bactericide application and potentially enhance their activity due to direct delivery into the plant (Heaton & Dullahide 1990; Wicks & Hall 1990; Guest et al. 1995). Since the accumulation of the injected compounds in fruit is an important consideration in agriculture, recent research addressing injection of insecticides in avocado thrips control and apple insect control shows that their residues depended on active ingredient and were either not detected in fruit, some were detected but at concentrations far below the current MRL set by EPA, while some rarely exceeded the MRL and reqired preharvest limits (Byrne et al. 2012; Byrne et al. 2014; Wise et al. 2014).

However, so far it is not known whether trunk injection of PRA-s and bactericides can achieve commercially acceptable control of *E. amylovora* on apples and how many injections per season are needed for this control. Further, it is not known whether trunk injection can enhance the activity PRA-s and bactericides due to *in planta* delivery (Heaton & Dullahide 1990; Wicks & Hall 1990; Guest et al. 1995). At last, it is not known how well trunk-injected bactericides and SAR-activators distribute and accumulate in the apple canopy and why.

Apple scab Venturia inaequalis and its significance

The apple scab fungus *Venturia inaequalis* (Cooke) Winter is widely known as the second most significant and economically challenging pathogen for control in conventional apple production. It causes tremendous losses in apple quality and yield every year and in many countries (Jeger 1981; Holb et al. 2003; M. Bengtsson et al. 2006). The apple scab infections do not kill the tree but result in leaf and fruit loss with severe defoliation of susceptible cultivars

sometimes in mid-summer (Jamar 2011). If no apple scab control is used over the years, defoliation can chronically weaken the tree, reduce its fertility and increase the incidence of frost damage on branches during the winter (Jones & Sutton 1984; Jones & Sutton 1996; Ivanović & Ivanović 2001). The immediate economic loss is due to fruit drop and reduction of fruit quality. The USDA's National Agricultural Pesticide Impact Assessment Program estimated that 100% of eastern apple orchards are infected with apple scab and that without fungicide treatments yield losses would be as high as 90% (Lewis & Hickey 1972; Hickey 1991). Yield and quality losses in the Netherlands caused by apple scab are estimated to be around 80% if no control measures were taken (Holb et al. 2003). Besides direct loss in fruit, apple scab can severely weaken the viability of trees for next season since infections reduce photosynthesis, wood growth is limited and fruit bud initiation is prevented or reduced (Jamar 2011). Apple scab can also continue developing in storage and allow other post-harvest pathogens to establish on fruit (Tomerlin & Jones 1983).

During the saprophytic stage of life cycle, *V. inaequalis* overwinters in previously infected fallen apple leaves on the orchard floor (Hirst & Stedman 1962; Jones & Sutton 1984; 1996; MacHardy 1996; Ohlendorf 1999; Ivanović & Ivanović 2001). Shortly after leaf drop in fall, fungal mycelium grows into the dead leaf tissue where sexual reproduction occurs and immature pseudothecia are formed. During the winter and in the spring of the following season, embedded pseudothecia gradually mature and finally differentiate asci. Usually at bud break, ascospores form in asci and each pseudothecia protrudes onto the leaf surface with an ostiole. During wet periods mature ascospores discharge from asci through the ostiole. Caught by the air currents, ascospores are carried onto the newly developing green tissues of apple trees. They germinate only in water droplets and initiate primary infections on green tissues, thus

establishing parasitic stage of fungal life cycle. Compact mycelium develops subcuticularly and afterwards forms conidia which rupture the cuticle. With rain and dew, exposed conidia are detached and mostly spread within a tree. They initiate secondary scab infections. *V. inaequalis* is a polycyclic pathogen and in favorable conditions can have even more than 20 generations of conidia.

In regions with humid climate apple scab control can require up to 14-22 topical spray treatments of fungicides, usually every 7-14 days (Van der Scheer 1992; Berrie & Xu 2003; Cuthbertson & Murchie 2003; Holb et al. 2003). In Michigan, heavy apple scab infections require apple scab control every year (Ehret et al. 2010). The effective use of fungicides to manage apple scab depends on application prior to release of fungal ascospores as primary infection inoculum and on uniform coverage of foliage and fruit (Sutton 1996; McArtney & Obermiller 2008). Further, time-precise application and preventive coverage are crucial in reducing mutations that confer fungicide resistance (Ma & Michailides 2005).

However, extensive fungicide applications lead to three major negative side-effects such as (1) off-target environmental exposure due to drift losses (Steiner 1969; Johansen 1977; Reichard et al. 1979; Pimentel & Levitan 1986; Johansen & Mayer 1990; Pimentel et al. 1992; Pimentel & Lehman 1993; Pimentel 1995; 2005; Ecobichon 1999; Hamilton et al. 2004; Zhu et al. 2006; Pettis et al. 2013), (2) health risks due to pesticide residues in food products which are the main focus of public concern from 1960's (Ragsdale & Sisler 1994; Sutton 1996; Berrie & Xu 2003; Pimentel 2005), and (3) fast development of pesticide resistance in pathogens due to high selection pressure by fungicides (Köller et al. 1997; Stević et al. 2010; Chapman et al. 2011; Lesniak et al. 2011). Finally, conventional pest management usually takes 23-30% or more of the annual costs in fruit production, thus significantly influencing the fruit price and marketable

yield (Perry 1998; Berrie & Xu 2003). All this points out that conventional fruit production has the lowest level of sustainability due to negative side effects of pesticide application (Reganold et al. 2001).

Due to public awareness and concern regarding pesticide use in agriculture, research in many branches of agriculture and science led to development of Integrated Pest Management (IPM), sustainable food production, and organic agriculture (Altieri 1995; Pretty 1995; Kogan 1998; Raynolds 2000; Roling & Wagemakers 2000; Brandt & Mølgaard 2001; Khatounian 2002; Bengtsson et al. 2005; Ehler 2006; Badgley et al. 2007; Willer & Yussefi 2012; Conway & Barbier 2013). All these programs and new concepts were developed to minimize the negative impacts of pesticide application through reduced or no use of synthetic pesticides and invention of eco-friendly plant protective replacements. In spite of their development, organic and IPM certified fruit productions are still extremely low in percentage of dedicated cultivable land and are limited to drier climates with fewer disease and pest problems (Garcia 2002; Willer & Yussefi 2012). Currently, only 0.9% of the agricultural world land is managed by the principles of organic production (Willer & Yussefi 2012). In USA, USDA's Economic Research Service estimated that IPM at the most basic level was used on approximately 50% of cropland in 1994 and it was planned to increase this ratio to 75% until 2000 (Jacobsen 1996; Jacobsen 1997). However, this goal remained largely unachieved in both USA and Western Europe due to high complexity and time demanding use of the natural pest control concepts (pesticides seem cheaper and easier insurance that crops will not be lost), the weak integration of pest management disciplines at the university level, and the need for more knowledge before this can truly be achieved (Ehler & Bottrell 2000; Ehler 2006).

Nevertheless, both organic and IPM fruit productions today are far from matching the conventional tree-based agriculture as a major source of fruit for the world (Tinker 1997; Willer & Yussefi 2012). It has been noted that lack of funding for IPM research, development and implementation, from the perspective of plant pathogen control, is responsible for the scarcity of IPM programs for the majority of the U.S. crop acreage (Jacobsen 1997).

The key obstacles for increasing the sustainability of fruit production are lack of market accepted disease resistant cultivars and the inability of current topical plant protection approaches and linked technologies to be more target-precise and significantly reduce off-target drift-driven pesticide losses (Pimentel et al. 1992; Pimentel & Lehman 1993; Pimentel 1995; Reganold et al. 2001). Researchers from various branches of applied and basic science are aware of this obstacle since new genetic and engineering technologies that could yiled disease resistant cultivars and avoid pesticide drift, with parallel development of novel pesticide formulations that suit the latter need, are being investigated and invented (Navarro et al. 1992; Holownicki et al. 2000; Torii 2000; Duqiang 2001; Doccola et al. 2003; 2007; Takai et al. 2003; 2004; Düker et al. 2006; Llorens et al. 2010; Düker & Kubiak 2011a; Shang, Liao, et al. 2011; 2011; Doccola & Wild 2012; Montecchio 2011; 2013).

One of the earliest developed branches of target-precise pest control research is trunk injection and infusion of pesticides for plant protection in landscape tree care and urban forestry. Many invasive species of insects and pathogens, such as emerald ash borer (*Agrilus planipennis* Fairmaire) and Dutch elm disease (*Ophiostoma ulmi* (Buisman) Melin & Nannf.), which decimated some of the most abundant native tree species in USA and Europe, resulted in development of research and commercialization of pesticide trunk injection. Trunk injection is an alternative approach for delivery of plant protective compounds to trees. It is based on

harnessing the vascular transport capacity of a tree, which allows active ingredient translocation and subsequent distribution in the tree canopy, where the plant protection effect is expected. It has first been used in urban forestry since topical spray application of pesticides was not possible due to large tree sizes or due to the vicinity of urban areas (Gibbs & Dickinson 1975; Smalley 1977; Guillot & Bory 1997; Doccola et al. 2003; 2007; 2008; 2012; Cregg et al. 2005; McCullough et al. 2006; Tanis et al. 2007; Mota-Sanchez et al. 2009; Smitley et al. 2010; Doccola & Wild 2012; Haugen & Stennes 1999). Today, trunk injection plant protection is used extensively in urban forestry since it is the most efficient way to protect trees from pests.

One specific branch of this research, which is still at its infancy, is control of fruit tree pathogens using trunk injection of pesticides. Previous results on trunk injection of fungicides on apples and other fruit species have shown promising results in disease reduction and good potential for transferring trunk injection concept into tree-based agriculture, but have not addressed some of the key obstacles to make this possible sooner (Pinkas et al. 1973; Shabi et al. 1974; Clifford et al. 1987; Schutte et al. 1988; Long et al. 1989; Guest et al. 1995; Percival & Boyle 2005; VanWoerkom 2012). For example, from the phytopathological perspective, it is still not known how many injections per season are needed to achieve commercially acceptable control of *V. inaequalis*. Further, it is not known whether trunk injection can enhance the activity of trunk injected fungicides and biopesticides due to *in planta* delivery and why (Heaton & Dullahide 1990; Wicks & Hall 1990; Guest et al. 1995). Also, it is not known how well other trunk-injected fungicides for apple scab control distribute and accumulate in the apple canopy and whether this affects their activity. At last, it is not known what are key chemical properties of injected compound that influence translocation and accumulation in the crown and how can they be manipulated.

Brief history and introduction to trunk injection in plant protection

Fruit tree injection as a concept as well as technology in agriculture started developing as early as in the 12th century. The first written record of tree injection for the purpose of enhancing flower and fruit properties were discussed by Ibn al-Awwam in 1864 in the book *"Le livre de l'agriculture (Kitab-al-Felahah)"* where he cited Nabataean agriculturalist Hadj de Granade who in 1158 described tree injection methods for imparting special scents, flavors and medical qualities to the ripening fruits (revisited by Roach 1938). Thus it is likely that this is also the first written record of tree injection in general. Leonardo da Vinci in the 15th century also described the technique for tree injection and carried out experiments which proved delivery of poisonous compounds from the trunk into the fruits (revisited by Stoddard & Dimond 1949). He accurately noted that the injection should be made in a large bored hole and when the sap is rising in the trees (revisited by Roach 1938; Stoddard & Dimond 1949).

The first report on tree injections for the purpose of plant protection and control of insects, was presented anonymously in *"The Orchard and Garden"* magazine in 1602 in London (revisited by Roach, 1938, in *Injection for the Diagnosis and Cure of Physiological Disorders of fruit trees*). The pioneer to bring the idea of tree injection for control of fungal diseases was Shezyrez in 1894 (revisited by Rumbold, 1920, in *The Injection of Chemicals into Chestnut Trees*). He was the first who injected copper sulfate in grapevines most probably for control of downy mildew (Rumbold 1920b; Roach 1934; 1938). An engineer Berget followed since he was reported to experiment on injections of salts to fertilize vines and protect them from fungal diseases (Roach 1938). In 1903 Mokrzecki publishes *"Report of the Governmental Entomologist of Tavricherkago Zemstvo for 1902-3"*, as the first record of apparently successful curative

control of gummosis disease on apple and pear by tree injection of 1% salicylic acid (discussed by Rumbold 1920b; Roach 1938). Rumbold (1920) continued experiments with sallycilic acid injections on chestnut for the purpose of control of *Endothia parasitica* (Murr.) but the results were variable and unreliable (Rumbold 1920a). The report by Mokrzecki was also the the first report on use of salicylic acid as a plant protective compound which today is known to be associated in signalling or resistance activation in plants in general (Gaffney et al. 1993).

As with any revolutionary idea in applied or basic science, it was also the case with tree injection of pesticides and fertilizers that brought a lot of controversy and dispute within the scientific communities of 19th and 20th centuries. The reason for this was lack of research and knowledge. In a wider sense, *in planta* delivery of chemical compounds that can benefit the plant was considered as an analog concept to infusion delivery of nutrients and medicaments in clinical medicine. However, this concept in plant health is much more complex due to the fact that there is limited information on what are the key properties of compatible formulation for injection of a compound and how these properties can be manipulated and adjusted to provide undisturbed plant functioning, while administering protection or nutrition (Pinkas et al. 1973; Shabi et al. 1974; Campana et al. 1979; Nair 1979; Nair et al. 1981; Perry et al. 1991; Duqiang 2001; Takai et al. 2003; Doccola et al. 2012; Doccola & Wild 2012). The second complexity associated with creation of trunk injection ports, which allow access to the tissues during injection, is that the mode ot tissue healing in higher plants after injuries is conceptually different than in animals (Shigo & Marx 1977; Shigo 1978; Shigo & Service 1979; Shigo 1981; Shigo 1984; Shigo 1985; Perry et al. 1991). Healing is defined as a restoration or regenerative process through which injured and infected tissues are repaired or replaced in the same spatial position (Shigo 1985). In trees this process does not happen in its fundamental sense. Quite

contrary, wounded structures in trees only become sealed off i.e. walled from the healthy, living tissues and this is designated as compartmentalization (Shigo 1985). This process of isolating the injury prevents further tissue damage (Shigo & Marx 1977). Therefore, it can be said that trees do not heal since they do not replace the injured tissues. In fact, after sealing off, trees form new tissues in new positions, and usually over the injured i.e sealed-off part. On the trunk this process of new formation of functional tissues, above the compartmentalized ones, is facilitated by the secondary meristem called cambium. Cambium forms callus from which new functional tissues are differentiated above the injured sections. Even though tree wound healing is fairly understood and was well discussed in relation to trunk injection, research attempting to determine what are the key traits of trunk injection technology that can minimize trunk wounding but still allow efficient compound delivery to trees, is limited (Düker et al. 2006; Montecchio 2013). The simplified perception of *in planta* delivery of compounds to higher plants and the lack of knowledge about compatible compound formulations and tree wound healing have made early trunk injection research difficult and inconclusive.

Early trunk injection experiments during the 19th century were severely hampered by the limited knowledge in basic sciences available at that time. In the 20th century, with the development and advancement in sciences, more modern trunk injection research arose in botany, plant physiology, agriculture, horticulture, and forestry (Rumbold 1920b; Roach 1938; Harries 1965; Pinkas et al. 1973; Shabi et al. 1974; Gibbs & Dickinson 1975; Clifford et al. 1977; 1987; Sachs et al. 1977; Kielbaso 1979; Campana et al. 1979; McCoy & Donselman 1979; Robbins 1981; Mclntyre & Lacy 1979; Barney et al. 1984; 1985; Raese et al. 1986; Fuchs 1988; Schutte et al. 1988; Long et al. 1989; Miller 1991; Navarro et al. 1992; Cooley et al. 1992; Fernández-Escobar et al. 1993; Guest et al. 1995; Tattar et al. 1998; Haugen & Stennes 1999).

Many of these research references were first of its kind and had a specific problem such as nutrient deficiency, plant disease, or tissue functionality that needed to be tackled or elucidated. Trunk injection was considered as a valuable approach or a method for investigation. However, still a little was known about processes governing the injected compounds in their activity, and each and every reference fairly pointed out on one or more specific issues that needed to be addressed in basic research to follow. For example, in plant physiology the process of water and sap movement in trees was largely unexplained. Late in the 19th century and early in the 20th, out of several theories, cohesion-tension theory of water movement by Dixon-Joly and Askenasy (1894, 1895, 1914, 1924) arose as the most accepted in the science community (Zimmermann 1963; Tyree 1997). On the other hand, involvement of trunk injection with dyes helped in discovering the variable patterns of sap flow in trees associated with different types of xylem tissues in different species (Kozlowski & Winget 1963).

Nevertheless, during 19th and 20th centuries, the reason for development and use of trunk injection for research and plant protection was series of invasive or new pathogens and insects which occurred in landscapes and orchards where sprays could not be applied to cover effectively due to large tree sizes or were ineffective due to pathogen nature to reside in xylem or phloem, respectively (Kielbaso 1979; Jones & Rosenberger 1979; McIntyre et al. 1979; McCoy & Donselman 1979; Long et al. 1989; Cooley et al. 1992; Fernandez-Escobar et al. 1999; Cregg et al. 2005; McCullough et al. 2006; J.J. Doccola et al. 2007; 2008; 2012; Eisenback 2008; Mota-Sanchez et al. 2009; Smitley et al. 2010; Ploetz et al. 2011; Ahmed et al. 2010; Gibbs & Dickinson 1975).

For example, some of those diseases were/are: Dutch elm disease (*Ophiostoma ulmi* (Buisman) Melin & Nannf., *Ophiostoma novo-ulmi*), lethal yellowing of coconut palms,

(‘*Candidatus* Phytoplasma palmae’), pear decline (‘*Candidatus* Phytoplasma pyri’), peach X-Disease (‘*Candidatus* Phytoplasma pruni’), oak decline (*Phytophthora cinnamomi* Rands), apple collar and crown rot (*Phytophthora cactorum* (Lebert & Cohn) J. Schröt., 1886) and laurel wilt of avocado (*Raffaelea lauricola* T.C. Harr. Fraedrich & Aghayeva). The most recent problem of invasive nature in USA is Huanglongbing disease or citrus greening (‘*Candidatus* Liberibacter asiaticus’, ‘*Candidatus* ‘Liberibacter africanus’). Some of the invasive insects were/are: emerald ash borer (*Agrilus planipennis* Fairmaire), hemlock woolly adelgid (*Adelges tsugae* Annand), Asian longhorned beetle (*Anoplophora glabripennis* Motschulsky, 1853), and many others.

Based on research involved in controlling these pests, it has been slowly discovered that the effectiveness of injected protective compounds in tree canopy depends on several key groups of factors. These can be broadly classified as biological, chemical, ecological, and technological.

Biological factors influencing injected compound

The main biological factors which revolve around the plant physiology encompass tree species, size, health, cultivar, grafting rootstock, daily water consumption, xylem type, time of injection and other trunk tissue properties (Rudinsky & Vité 1959; Kozlowski & Winget 1963; Kozlowski et al. 1967; Owston et al. 1972; Owston et al. 1972; Waisel et al. 1972; Kozlowski 1976; Landsberg et al. 1976; Davies & Lakso 1979; Čermák et al. 1984; Brough et al. 1986; Green & Clothier 1988; Liu et al. 1993; Kramer & Boyer 1995; Dawson & Pate 1996; Mendel 1998; Green et al. 2003; McCulloh et al. 2003; Nicolas et al. 2005; Čermák et al. 2007; Owens & Moore 2007; Holbrook & Zwieniecki 2008; Pallardy & Kozlowski 2008; Beck 2010).

Based on the extensive research in arboriculture, forestry and fruit industries, different species and cultivars significantly vary in their ability to translocate and distribute injected compounds (Sachs et al. 1977; Santamour Jr 1986). Differences are biochemical, cellular, anatomical and morphological (Perry et al. 1991). Due to differences in vascular tissue anatomy different tree species differ in the patterns of compound translocation and spatial accumulation into the canopy after trunk injection (Orians et al. 2004; Orians 2005; Zanne et al. 2006; Pallardy & Kozlowski 2008). Specific differences in xylem anatomy will be discussed later. Further, different tree species have variable tree sizes which indicate that after injection, the compound could spend more time in translocating to the canopy of tall forest trees versus the small size fruit trees. With the increasing height of trees more resistance points for sap and injected compound flow, exist since every point of branching is a resistance point in flow. Also, with the increase in size, overall trunk volume increases and the injected dose per tree needs to be adjusted, most likely increased, so that it can account for this large volume, reach the canopy and substantially distribute in it. Compound dosing in landscape tree care is determined according to the trunk diameter at breast height (DBH). At last, it is known that potential energy of water (kPa) in plant depends also from gravity force or gravitational potential which becomes more important factor i.e. component of overall water potential in plant when the tree size increases. In regards to health tree status, infected or damaged trees most likely have slower sap flow and radial trunk growth thus slowing the uptake and the effect of the injected compound.

Variable rootstocks with different water uptake properties might be an important consideration when trunk injection is investigated on fruit trees in agriculture. It is possible that subtle cultivar/rootstock combination differences can govern the uptake of injected plant protective compounds. Most likely apple trees grafted on more vigorous rootstocks can have

somewhat higher and faster water uptake rate per day, in comparison to the trees grafted on less vigorous rootstocks. This could influence slower uptake and the lower amount of compound being translocated into the canopy on less vigorous rootstocks, and faster uptake and the higher amount of compound being translocated into the canopy on more vigorous rootstocks.

Transport of injected compound through the xylem and distribution within the canopy is dependent on daily water uptake typical for a tree species. Water uptake is driven by the rate of daily transpiration (Davies & Lakso 1979; Cohen et al. 1981; Brough et al. 1986). Depending on age, health and weather conditions, some forest tree species absorb and translocate between 100-200 gallons of water and in extreme cases even 300 gallons of water per day (Kozlowski 1976; Owens & Moore 2007; Holbrook & Zwieniecki 2008). On the other hand, mature apple tree depending on weather conditions and age uses only around 15-50 gallons of water (Vossen & Silver 2000). This implies that in injection of fruit trees more water should be amended to the injected compound to decrease its viscosity and ease the transport through the xylem and distribution and accumulation in the canopy.

In hardwoods, xylem wood consists of tracheids, vessels, fibers and rays. Main two xylem types in hardwoods are ring porous xylem with the largest vessels in the early wood (*Quercus, Ulmus,* and *Fraxinus*) and diffuse porous xylem with vessels of similar size distributed evenly throughout the growth layer (*Betula, Acer, Populus, Malus*) (Beck 2010; Orians et al. 2004; Orians 2005; Zanne et al. 2006; Pallardy & Kozlowski 2008). In softwoods (conifers), xylem wood consists primarily of tracheids, rays and resin canals (Pallardy & Kozlowski 2008; Beck 2010).

In the case of ring porous and diffuse porous xylem types, additional xylem elements i.e. vessels with large diameters allow less resistance in movement of the injected compound and its

faster translocation into the canopy. Diffuse porous xylem type is also characterized by the presence of vessels and tracheids, however vessels are not as extensive in diameter as in the ring porous trees but are still bigger in diameter compared to the tracheids. Therefore vessels allow good transport properties for the injected compounds since there are more vessels dispersed throughout one season growth ring than in the case of ring porous xylem type. At last, the number of active layers of xylem tissue below the bark dictates the speed of injected compound movement. Within sapwood the water conductance varies. The most active conducting sections are the current year growth ring and in certrain species 2-3 nearby, former year growth rings. In order to maximize compound uptake, this needs to be taken into consideration when injections are conducted. This specifically relates to the depth of trunk injection port or injection element setting in the xylem which should result in good alignement of the most conductive growth rings with the delivery openings of the injection device.

In the case of conifers with densely packed tracheid cells which have small diameter, movement of an injected compound is exposed to more resistance points in comparison to the large vessels present in the ring porous and diffuse xylem types of hardwoods. Further, conifers also have present resin canals in the xylem tissue and in case of any trunk wounding, including infliction of trunk injection ports, very soon after these injuries the wounds become filled with resin and stop the conductivity of the injection port for further use. The resin filling is considered a defense system which offers tree trunk protection from tracheid embolism, water accumulation, microbial infections and injuries from insects and vertebrates (Beck 2010). Thus resin can reduce the conductivity of conifer xylem for injected compounds.

It has also been discovered that depending on xylem type there is a certain level or sectoriality in movement and distribution of injected compound. Namely, in ash trees with ring

porous xylem, xylem architecture patterns are sectored 'zigzag' and influence sectored flow distribution of injected imidacloprid thus governing variable compound distribution and variable control of insect pests (Tanis et al. 2012). The patterns of movement of injected dyes have also shown differential spiral patterns of ascent through the xylem (Kozlowski & Winget 1963; Kozlowski et al. 1967). In xylem of pines, distribution of injected emamectin benzoate was also sectored (Takai et al. 2004). On the contrary, diffuse porous xylem type in hardwood tree species (*Betula, Acer, Liriodendron*), has a very low level of compound flow sectoriality (more integrated compound flow). Xylem properties such as high density of vessels, increased vessel-to-vessel contact, higher lateral pitting of vessel element walls, larger pit size and high density of intervessel pits, all contribute greatly to better radial and integrated vertical distribution of compounds in the tree crown (Orians et al. 2004; Orians 2005; Zanne et al. 2006).

Time of the year influences translocation and distribution of trunk injected compounds in trees. For example, trunk injection of fungicide imazalil in apple trees provided different levels of translocation and distribution at different times of injection: after harvest, before bud burst, and during flowering (Clifford et al. 1987). Post-harvest injection of imazalil showed most extensive movement of fungicide, both up and down the trunk and into the branches. In contrary, injection before bud break showed limited movement in the trunk, and imazalil was detected in branches only at the end of the season. Injection at flowering led to substantial amounts of imazalil in the apple branches and twigs.

Fall pear trunk injections of thiabendazol and methyl 2-benzimidazole-carbamate, shortly before leaf fall, resulted in fast distribution of compounds into branches and leaves and at the beginning of next season translocation continued in new green canopy growth (Shabi et al. 1974). When these fungicides were injected during winter, primary translocation was poor and

secondary was limited. In the same study, spring injections, as expected, gave considerable primary distribution, however with delayed deposition in developing leaves.

All in all, this implies that most of the compound uptake after injection occurrs during the most intensive transpiration in spring and summer which is facilitated by the new green growth i.e. the canopy. In apple trees, transpirtation gradually declines towards the end of the season (Dragoni et al. 2005). More research on apple injection time schedules, multiple season injections, different doses, and their efficiencies is needed.

The main biological factors related to pest are pest species, time of occurrence, infestation pressure and the plant organ they attack.

Control of wilt diseases on trees such as laurel wilt of avocado (*R. lauricola*), Dutch elm disease (*O. ulmi*), oak decline (*P. cinnamomi*), seems somewhat easier due to confinement of the pathogen to the trunk and branch tissues typical for this fungi lifestyles. On the other hand control of foliar and fruit limited diseases such as apple scab (*V. inaequalis*) and, for an initial infection period of time, fire blight (*E. amylovora*), seems much harder. This is primarily due to the fact that injected compounds reach systemic pathogens probably much sooner and at higher concentrations in the xylem than in the case of pathogens on leaves as an end point.

Compound translocation after trunk injection is a time consuming process which then demands careful choosing of time(s) of injection(s) so that the highest compound accumulation in the tree canopy can be secured at the time of the highest pest pressure in the season. Further, if the infestation pressure is variable from year to year, single or multiple injections per season might be required to deal with such pests successfully.

Chemical factors influencing injected compound

The major chemical factors associated with the injected compound and which influence its effectiveness in the tree canopy include water solubility, carbon adsorption coefficient (Koc), molecular weight, formulation, dose per tree and solution concentration, pH in water and time of degradation (Norris 1967; Smalley 1977; Kondo 1978; Campana et al. 1979; Kielbaso 1979; McCoy & Donselman 1979; Mclntyre & Lacy 1979; Nair 1979; Nair et al. 1981; Hiruki 1988; Raju & Nyland 1988; Miller 1991; Cox et al. 1997; Doccola et al. 2003; 2007; 2012; Byrne et al. 2012; Doccola & Wild 2012; Montecchio 2013).

Organic Carbon-Water Partitioning Coefficient (ml/g or µg/g) or carbon adsorption coefficient (Koc) of the a. i. and formulation components expresses the level of adhesion of the a. i. to the carbon rich compounds within certain environment such as soil or xylem (Doccola et al. 2012; Doccola & Wild 2012). It is defined as the ratio of the mass of a chemical that is adsorbed in a certain environment per unit mass of organic carbon in that environment per the equilibrium chemical concentration in solution. Compounds with high Koc values bind strongly to the organic compounds in soil, sap and xylem structure and hamper the movement of the injected compound into the canopy. This makes compound accumulation in tree crown very slow or negligent, thus leading to poor compound distribution and low level of pest control. Compounds with low or moderate Koc values act completely the opposite and this property is desirable for the injected compounds.

Molecular weight represents a mass of a molecule. It is highly likely that pesticide a. i.-s with lower molecular weight have smaller molecule diameters in angstroms (Å) or a smaller effective molecular radius which is defined as the size that molecule displays in a solution. Thus, pesticide a. i.-s with smaller molecule diameters than the perforated end walls of vessels and pitted end walls of tracheids can transport substantially through the vessel elements and

tracheids. If the molecule diameters are larger than these diameters, less or no compound is transported through the xylem since the molecules are kept in the cells due to clogging of these openings. In apple xylem, average diameter of vessels lumen is between 17-48 μm (Wheeler et al. 2005). For example, the molecule of insecticide imidacloprid has a 199.15 $Å^3$ volume or 0.19915 nm^3 (1.9915×10^{-10} μm^3). However, molecular weight and diameter as properties are highly coupled with the other chemical and physical properties of a molecule discussed further.

Water solubility is the ability to dissolve certain amount of a compound in certain amount of water but that can form homogeneous solution. High water solubility is absolutely crucial for easier and faster uptake and translocation of the injected compound and its formulation into the tree canopy (Pinkas et al. 1973; Shabi et al. 1974; Marsh 1977; Smalley 1977; Nair 1979; Nair et al. 1981; Hiruki 1988; Tattar et al. 1998; Young 2002; Percival & Boyle 2005). This allows higher accumulation of the compound in tree crown and secures its high efficiency against pests. Designing new compound formulations which allow higher water solubility and lower Koc of the a. i.-s, is of the prime importance for trunk injection as plant protection approach (J.J. Doccola et al. 2007; 2008; Doccola & Wild 2012; Montecchio 2013).

Closely related property to solubility is pH of the solution and the formulation designed for trunk injection (Pinkas et al. 1973; Shabi et al. 1974; Smalley 1977; Montecchio 2013). pH is defined as a measure of acidity of basicity of an aqueous solution. When in xylem, systemic plant protective compounds can flow readily with the sap flow if the sap pH is slightly acidic or neutral. If the injected compound solution is alkaline (pH 7.5-7.9) the basic component will adsorb to the xylem walls which have the negative charges (Marsh 1977; McCoy & Williams 1982). Within the tree and during the season, sap pH varies and fluctuates, respectively, and the range of pH values differs across the tree species (Schill et al. 1996; Thomas & Eamus 2002;

Alves et al. 2004). After trunk injection of 'Golden Delicious' apple trees with thiabendazole solution at pH 3.4, the pH was buffered and increased to 6.7, which is similar to the pH of xylem sap (Pinkas et al. 1973). The same and other authors state that the solubility of most fungicides is low and that they must be solubilized in a non-phytotoxic medium before injection (Campana et al. 1979). Trunk-injected thiabendazole accumulates mainly around the injection ports and it is hypothesized that it precipitates due to rise in pH level (Pinkas et al. 1973). Further, it has been noted that systemic compounds such as benzimidazoles can only be water soluble at a low pH and that when in xylem, compound solubility can undergo considerable change since host tissue contains sap with pH ranging from 5.0-7.0 (Smalley 1977; Campana et al. 1979). Therefore, the lack of effect and distribution of injected compound can be due to unsuitable pH of the injected water solution. The incompatible pH forces a. i. to bind to xylem walls and precipitate due to solution pH change triggered through buffering effect of the sap (Campana et al. 1979). According to this source, as a result of precipitation, compound distributes non-uniformly in the tree canopy.

At last, dose response studies with different concentrations of a. i. in water, are highly needed for injected plant protective compounds and are regularly conducted before the pesticide is allowed for use and production. This is due to the fact that effective concentrations of various pesticides for good pest control are largely unknown in different tree-pathogen systems (Eisenback 2008). Further, it has been shown through research that high doses of pesticides per tree and especially at a low a. i. concentrations in water, cause phytotoxicity effects on tree (Long et al. 1989; Guest et al. 1995; Dias 2012). It is known that more water amended with the injected pesticide enables increased dilution of the compound and eases its translocation into the apple canopy. Therefore, translocation and accumulation of the injected compound is faster.

When in plant, different pesticides are subject to plant metabolic processes of degradation (oxidation, hydrolysis) and conjugation and can remain in active molecular state for different amounts of time (Lindquist 1965). Some trunk injected compounds, such as imidacloprid, after degradation remain active due to myriad of active metabolites stable in time (Nauen, Koob, et al. 1998; 1998; 1999; Mota-Sanchez et al. 2009; Tanis et al. 2012). The extent and timeline of degradation of each compound is variable. For example, apple tree injected fungicide uniconazole showed that its concentration decreased 49% over 4 months (Sterrett 1990). Trunk injection of 7 systemic fungicides showed apple scab control over 2 consecutive growing seasons (Percival & Boyle 2005). The fungicidal effect of fosetyl-Al in control of *P. cactorum* on apple lasted for 15 months (Long et al. 1989). In trunk injection studies with imidacloprid and other insecticides in apple trees, after initial increase in concentration of leaf residues with time there is steep decrease of parent compound content (VanWoerkom 2012). This indicates that for each different compound length of their activity and the level of their concentration change after injection needs to be determined in time. Using this information one can be able to determine season-long efficient doses and multiple season injection time schedules that could provide efficient pest control.

Technological factors influencing injected compound

The main technological factors hypothesized to affect the activity of injected compound in the canopy are tree injection device, position and number of trunk injection ports per tree, size and type of injection port, and volume of injected solution (Campana et al. 1979; Neely 1979; 1988; Tattar et al. 1998; Sánchez-Zamora & Fernández-Escobar 2000; 2004; Young 2002;

Costonis 1980; 1981; 2003; 2007; 2011; Shigo 1978; Wasniewski, Chaney & Holt 1993; Smith & Lewis 2005; Shortle et al. 2010; Doccola & Wild 2012).

According to the targeted trunk tissue, trunk injection devices can be classified as xylem- and cambium-targeting trunk injection devices. Majority of devices deliver injected compounds to the xylem. The injection device can be active and passive based on whether it relies on use of external pressure (compressed air, CO_2) to inject the compound into the trunk or not, respectively. Active devices usually use pressures between 6-110 psi and can be classified to the devices using high and low pressures. The passive injection devices rely on the negative pressure uptake generated by the upward speed of sap flow in tree xylem and this process is often mentioned as infusion instead of injection (Montecchio 2013). Further, according to the size of injection port, injection devices can be for micro-injection (diameter 4.76 mm or less, depth of 19.05 mm or less), and macro-injection (diam. 9.53 or greater, depth 2.54 mm or more) (Costonis 1981). However, this is a subject of wide discussion since several other sources consider 9.53 mm ports also for micro-injection (Young 2002; Doccola et al. 2003; Spitko 2008). Finally, based on the type of injection port, devices can be needle insertion-based, which create lenticular injection port and drill-based which create cylindrical injection port. In the case of latter, part of the wood tissue is removed and the injection port can be sealed permanently with a specialized plug or temporary with a feeder tube, while administering the injection solution. In the case of needle insertion-based injection devices only a small portion of wood bark tissue can be removed. In both device groups, injection ports are perpendicular to the trunk axis or rarely at an angle of 45º, with a port opening at a higher position on bark versus the port bottom in the xylem. These two main groups of injection devices are currently the most used in landscape tree care and the majority of these devices use external pressure to aid the delivery of injected

solution. There is very limited number of research references that compare trunk injection technologies in their performance and features, and especially in temporal and spatial distribution profiles of the injected compound that they provide in the canopy (Fuchs 1988; Düker et al. 2006).

There is no comparative research which shows how different types and sizes of injection ports influence the distribution and performance of trunk injected compounds in tree canopy. For example, the most important comparison would be between different diameters of drilled injections ports and a comparison to and between the lenticular shaped injection ports of different sizes. The important question behind these comparisons would be does removal of trunk tissue when ports are drilled significantly contributes or not to better delivery of the injected pesticides. Further, these comparisons would also provide a possibility to evaluate what are the advantages and disadvantages of lenticular shaped ports which do not have removed trunk tissue in the process of injection.

It has been postulated that position and number of trunk injection ports per tree influences the spatial and temporal uniformity of compound distribution within the tree crown, thus dictating the efficiency of trunk-injected compounds in pest control (J.J. Doccola et al. 2007). However, no direct dependence of pesticide compound residues detected in tree canopy after trunk injection with variable number of injection ports was shown after variable disease control results.

Currently, majority of injection tools except High Volume Macro-Infusion^{TM} Kit and Low Volume Macro-Infusion Kit^{TM} (Rainbow Treecare Co., Minnetonka, MN) allow injection of very low solution volumes per tree, ranging from 0.1-1200 ml. When more water is added to the injected pesticides, thus increasing the volume of the total injected solution, it can be

expected that higher dilution of the compound will be achieved. This in turn most likely causes easier and faster pesticide translocation and accumulation of the higher concentrations in the tree crown. Therefore, better distribution and efficiency of trunk-injected pesticides can be expected in the canopy of urban trees. No comparative studies in performance have been done between the trunk injection systems which can provide high and low total solution volumes.

Ecological factors influencing injected compound

The main ecological factors known to indirectly affect the activity of injected compound in the canopy by influencing the tree physiology are water potential, vapor pressure deficit (VPD), relative air humidity and temperature.

Water potential is defined as the potential energy of water per unit volume in reference conditions (kPa, MPa). This parameter quantifies the tendency of water to move from one area of plant to another. Besides the dependence of water potential in plant from gravity (gravitational potential), mechanical pressure (pressure potential), and surface tension (matric potential), water movement is largely dependent on the amount of solutes in it (osmotic potential) (Zimmerman & Brown 1971; Larcher 2003). The more solutes are dissolved in water the lower potential energy status of water is present. Pure water has maximum of 0 kPa water potential and this is the highest energy status of water without pressure. Water moves from the place of higher to the place of lower i.e. more negative water potential or from the place with less solutes in water to the place with more solutes in water (Zimmerman & Brown 1971; Larcher 2003). Along with the upward sap transport in xylem, trunk-injected compound lowers the water potential in sap,

around the place of injection, and is diluted with the sap with higher water potential. This and the xylem grain structure drive and shape the radial compound distribution in the trunk.

In general, water potential in the water column within the tree xylem, which stretches continually from soil, to roots, to trunk, to branches, to leaves and to air, is deployed as water potential gradient that becomes more and more negative as it goes up towards the leaves and then air (Zimmerman & Brown 1971; Larcher 2003). In the air water vapor potential is usually the lowest in this gradient, especially when the temperature is high and relative air humidity is low (up to -125kPa). This difference between the water potentials in leaves and air drives the evaporation of water from the plant. This process is known as transpiration and it occurs through the stomata on green tissues (Zimmerman & Brown 1971; Larcher 2003). The evaporative demand is imposed to the plant by the Vapor Pressure Defficite in the air (VPD). VPD is defined as the difference between the amount of water vapor which air could hold on a certain temperature and the actual amount of water vapor which air currently holds. Therefore, transpiration creates negative pressure i.e. upward pulling force in the water column in xylem due to VPD which drives the transpiration (Zimmerman & Brown 1971; Larcher 2003). In the water column, the polar molecules of water are bound by the adhesive forces which maintain the water column during transpiration. After trunk injection, transpiration is the main driver of compound transport to the canopy with the xylem sap column. The rate of transpiration is one of the factors which shape the rate of compound translocation and the level of its accumulation in the tree canopy.

VPD is highly dependent on temperature. At higher temperatures air expands and this increases VPD and lowers the relative air humidity. Since air expands it can hold more water vapor and the bigger the VPD is the higher is the transpiration from the plant (Zimmerman &

Brown 1971; Larcher 2003). Relative humidity is defined as the amount of water vapor which air currently holds relative to the maximum water vapor which air could hold at a given temperature**.**

Thus, for fast translocation and good distribution of trunk-injected pesticide into the canopy, ideal conditions would be midium to high temperatures, low relative air humidity (high VPD), sunny and windy weather and substantial water supply in the soil. At these conditions all the stomata in tree canopy are opened and transpiration is at full capacity. In regards to the plant, ideal conditions for trunk injection are large leaf area i.e. crown size and good plant health.

Hypothesized factors influencing injected compound

The major factors hypothesized to severely influence the efficiency of trunk-injected pesticides in tree canopy are the spatial and temporal uniformity of compound distribution within the crown (Norris 1967; Smalley 1977; Wilson 1979; Cowles et al. 2006; J.J. Doccola et al. 2007; Mota-Sanchez et al. 2009; Dilling et al. 2010; Tanis et al. 2012). In previous trunk injection studies, on few fruit tree species, variable pest control results in the canopy were only indirectly associated with different properties of translocation and spatial distribution of injected compounds in the tree crown (Pinkas et al. 1973; Shabi et al. 1974; Clifford et al. 1977; Pilbeam 2003; Percival & Boyle 2005; Spitko 2008; Ahmed et al. 2010; Byrne et al. 2012). Few studies showed only temporal distribution of injected compounds in the fruit tree canopy (Schutte et al. 1988; Tattar et al. 1998). Little is known about the distribution, accumulation and persistence of injected compounds in trees and how pesticide efficacy varies depending on these factors (Metcalf 1966; Mota-Sanchez et al. 2009). Investigation of compound distribution after trunk injection would serve as basis for establishing treatment rates, injection port density and timing,

and determining risks for fruit consumers and non-target organisms (Norris 1967; Eisenback 2008). How, where and when trunk-injected compounds distribute in the tree canopy are key missing components in explaining the compound efficiency in the crown of fruit trees.

Secondly, it has been reported that currently used trunk injection tools are not able to provide slow i.e. controlled release of injected compound in time. In trunk injection it is needed to release compound in a controlled manner so that it distributes temporally uniform in crown and secure protection of a tree at a correct dose and at a needed length of time. Previous research has shown that temporal distribution of injected compounds in the canopy is not uniform and that the compound dose is at some times oversupplied and at others undersupplied (Shabi et al. 1974; Clifford et al. 1987; Schutte et al. 1988; Tattar et al. 1998; VanWoerkom 2012).

Further, there is very limited number of research references that compare main trunk injection technologies in temporal distribution of injected compounds in the tree canopy (Fuchs 1988; Düker et al. 2006). Temporal residue profile is the most useful distribution parameter which could indicate whether trunk injection tool is successful or not in its purpose of allowing the injected compound to provide efficient pest control (Schutte et al. 1988; Tattar et al. 1998).

Finally, it is not known whenther tree wounding with injection ports leads to economically important damage to trees and impairment of tree longevity and fertility. Historically, this is widely discussed topic in landscape tree care industry and is the most frequently hypothesized obstacle for potential implementation of trunk injection in tree-based agriculture (Shigo et al. 1977; Shigo & Marx 1977; Shigo & Service 1979; Costonis 1980; Shigo 1981; 1978; 1984; 1985; Santamour Jr 1984; 1986; Neely 1988; 1979; Perry et al. 1991; McGillivary et al. 1993; Wasniewski, Chaney & Holt 1993; Smith & Lewis 2005; Shortle et al. 2010; Doccola et al. 2011). However, one of the first parameters which could indicate potential

of trunk injection wounds for significant tree damage is the time needed for different types of injection ports to heal. Few studies investigated some aspects of injection port wound healing on forest tree species, apple and peach trees and grapevines (Neely 1979; 1988; Costonis 1980; Wasniewski, Chaney & Holt 1993; Percival & Boyle 2005; Düker et al. 2006; Doccola et al. 2011; Smith & Lewis 2005; Cooley et al. 1992; Shigo et al. 1977; Shigo & Marx 1977). None of these studies showed the rate of wound closure on fruit trees in time. Real impact of trunk injection wounding is largely obscure from the perspective of agricultural trees.

Control of fire blight (Erwinia amylovora (Burrill) Winslow et al.) on apple using topical and trunk injection treatments of alternative plant protective compounds

Acibenzolar-S-methyl

Belonging to a novel class of plant resistance activators, acibenzolar-S-methyl (ASM) as a functional analogue of salicylic acid is a well investigated compound which activates Sistemic Acquired Resistance (SAR). It is also known as benzothiadiazole derivative or BTH from benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester. ASM showed good efficiency in the control of various plant pathogens and has been commercialized under trade names Actigard® in USA and Bion® in Europe.

The primary mode of action of this compound is the activation of defense gene expression resulting in accumulation of defense related enzymes and pathogenesis related proteins (PR) (Brisset et al. 2000; Maxson-Stein et al. 2002; Sparla et al. 2004; Hassan & Buchenauer 2007). ASM mimics the role of salicylic acid (SA) and mediates activation of the

salicylic acid signaling pathway which leads to activagtion of plant defense responses among which are produced enzymes that can to degrade the parts of bacterial cell walls (Shimono et al. 2007; Kessmann et al. 1994; 1996; Thomson, Brisset, et al. 1998; 1998; Oostendorp et al. 2001). Thus, as SA, the increaseingly accumulated ASM in the cytoplasm interacts with NPR1 protein, a negative regulator of plant defense responses (Gozzo & Faoro 2013). NPR1 oligomer is disassembled and migrates in nucleus where as monomer interacts with TGA transcription factors which leads to the expression of PR-1 defense gene and subsequent reaction cascades which lead to SAR activation (Durrant & Dong 2004; Kesarwani et al. 2007). Further investigation showed that SA/ASM interaction with the NPR1 also includes its paralogues and SA receptors NPR2, NPR3 and NPR4 which interact with NPR1 and depending on SA levels degrade NPR1 or not (Gozzo & Faoro 2013). Overall, SAR activation is SA and NPR1 dependent and SA acts as a signal for NPR1 activation.

Gene expression studies performed on apple seedlings treated with ASM, showed elevated expression of PR-1, PR-2 (β-1,3-glucanase), PR-8 (class III chitinase), and PR-10 proteins (Ziadi et al. 2001; Maxson-Stein et al. 2002). However, PR protein gene expression analysis in shoots of mature apple trees and 2-year old pear trees, followed by treatment with ASM, showed that there was no significant induction of PR-1, PR-1a, PR-2, PR-5, and PR-8 genes (Bonasera et al. 2006; Sparla et al. 2004). In summary both research groups conclude that: a) PR-1 protein expression in pear trees was constitutive and not influenced by ASM treatment or inoculation with *Erwinia amylovora*; b) in apple plants differing results could occur due to different developmental stage of treated tissues; and c) the induction of PR genes in apple as a result of SAR activators remains under question. It has been found though that PR-2, PR-5, and PR-8 genes were upregulated in mature apple trees in response to inoculation with *E. amylovora*

(Bonasera et al. 2006). In addition, the protection against pathogen *E. amylovora* in apple seedlings was associated with the activity and accumulation of two defense-related enzymes, peroxidases and β-1,3-glucanases (PR-2) (Brisset et al. 2000; Hassan & Buchenauer 2007).

Control of *E. amylovora*, with topical ASM treatments provided variable effects in apple and pear and their different organs affected. Among other alternative bio-control methods and compounds for fire blight management, ASM has a significance place as an SAR activators (Zeller 2006).

When ASM (75 and 150 mg /L) was applied topically, in regimes of 2-3 times before, during and after bloom, on few different apple cultivars in orchard with natural levels of inoculum, significant control effects of fire blight were recorded (Thomson, Gouk, et al. 1998). However this control was not as effective as antibiotic streptomycin.

When shoots of highly susceptible apple rootstock M26 were sprayed under greenhouse conditions with 0.012% solution of Bion, two days before scissor inoculation of young leaves with *E. amylovora* (5×10^6 CFU/ml), fire blight symptom development and bacterial concentration in the leaves significantly decreased for 70 and 60%, respectively (Zeller & Zeller 1998). The same authors report that bacterial pathogen was not directly inhibited by ASM when *in vitro* agar-diffusion assays were conducted. However, in the same study, treatments with Bion water solutions of 0.06 and 0.012%, by dipping unripe pear slices, showed that after inoculation with the bacteria (1×10^8 CFU/ml) ASM significantly inhibited production of bacterial exudate as up to 63%.

When ASM as Bion solutions (0.024 and 0.012%), was applied at full bloom in apple orchard of cv. ‘James Grieve’, *Malus domestica* Borkhausen, 7 days before the inoculation with

pathogen (5×10^7 CFU/ml), a significant control of 56-68% of blossom blight was recorded (Zeller & Zeller 1998).

When the effects of ASM were evaluated in shoot blight control on apple cv. 'Gala' and cv. 'Idared', *Malus domestica* Borkhausen, in greenhouse conditions, significant reduction was achieved but differed depending on the cultivar (Basak et al. 2001). When high concentration inoculum of *E. amylovora* (10^7 and 10^8 CFU/ml) was used in the same study, ASM was not able to provide disease protection on pear fruitlets of cv. 'Conference', *Pyrus communis* L. Further, ASM efficiency screening experiments in control of fire blight on M26 apple rootstock plants (Baysal & Zeller 2001; 2004) provided similar significant results.

In preliminary studies on apple cv. 'Jonathan', *Malus domestica* Borkhausen, weekly ASM applications (56.7g/100 gal.) starting on 30 April provided fire blight control of 81.1% compared with 97.6% with streptomycin (226.8g/100 gal.) under severe natural infections occurring in petal fall (Maxson & Jones 1999). The same authors report that bi-weekly ASM applications, starting from the same date, at the same dose, provided only 58% of control under severe natural infections.

In two consecutive years of testing on naturally infected apple trees of cv. 'Jonathan' and cv. 'Fuji', *Malus domestica* Borkhausen, weekly ASM application (75 mg/L) provided significant control of fire blight by exhibiting fewer infected strikes per tree compared to bi-weekly spraying interval and untreated control (Maxson-Stein et al. 2002). In the same study weekly interval of application, three times before and three times after the inoculation of cv. 'Jonathan' (1×10^7 CFU/ml), significantly reduced percentage of shoots infected with fire blight just in the first year of trials. Progression of the necrosis on inoculated shoots, in both years, was

significantly less in the trees treated weekly with ASM than in the control. However, bi-weekly application schedule did not cause reduction of necrosis progression, similarly to the control (Maxson-Stein et al. 2002). Further, the same authors showed that necrosis extension and canker formation on perennial tissues was increasingly reduced with the increase of ASM rate weekly applied and ranging from 0-300 mg/L (R^2=93).

Topical treatment with ASM (100 or 200 mg/L) on cv. 'Golden Delicious', *Malus domestica* Borkhausen, provided significant control of shoot and blossom fire blight on artificially inoculated scions (1×10^9 CFU/ml), and mature trees ($1\times10^{7\text{-}8}$ CFU/ml), respectively (Brisset et al. 2000). The control was satisfactory ranging from 50% on trees with infected blossoms to 69% on seedlings with infected shoots, regardless of delays between inoculation and application of ASM (2-10 days). On six-weeks old apple seedlings of the same cv., ASM (0.1 mg/ml) applied two days before inoculation with *E. amylovora* (1×10^8 CFU/ml) reduced fire blight severity measured as browning discoloration index (BDI) for 51.9%, and stem bending index (SBI) for 66.7% (Hassan & Buchenauer 2007). In the same study untreated inoculated seedlings had BDI of 95.8% and SBI of 95%, and substantial bacterial populations of 7.2×10^7 CFU/g, while in the treated seedlings the population levels were significantly reduced to 2.7×10^5 CFU/g.

In another trial on cv. 'Golden Delicious', foliar treatments with ASM alone or in combination with BABA (DL-ß-aminobutyric acid) were applied at different number of days before inoculation (Hassan & Buchenauer 2007). When used at relatively high rates, amino-acid BABA has been reported to provide broad-spectrum activation of systemic resistance in various crops targeting fungi and plant pathogenic bacteria (Oostendorp et al. 2001; Jakab et al. 2001).

Furthermore, even a curative effect of BABA has also been reported (Tosi et al. 1998; Cohen 1996; 2002). The mechanism of action behind BABA's influence of plant pathogens has not been completely elucidated yet (Oostendorp et al. 2001; Ton & Mauch-Mani 2004). Based on previous research it has been found that BABA activated a spectrum of defense mechanisms encompassing production of peroxides, phenolics, callose, lignin, PR-proteins, and most importantly, that this activated response depends highly on the plant-pathogen system (Cohen 2002). In studying BABA activated resistance on *Arabidopsis* against necrotrophic fungal pathogens *Alternaria brassicicola* and *Plectosphaerella cucumerina* if has been found that this resistance is based on primed callose accumulation, which is under the control of ABA-dependent defense pathway (Ton & Mauch-Mani 2004). The effects of BABA and other structurally similar amino-acids with topical treatments for control of *E. amylovora* and leaf injecting treatments for control of *V. inaequalis* were investigated before and the most promising ones will be further discussed (Cohen 2002; Hassan & Buchenauer 2007; Baysal et al. 2006; MacLennan et al. 1963).

In greenhouse trial on apple seedlings of cv. 'Golden Delicious', foliar treatments with BABA (DL-ß-aminobutyric acid, 1.0 mg/ml) were applied alone or in combination with ASM (0.1 mg/ml), at different number of days before inoculation (dbi). Further, BABA was applied in succession with ASM and vice versa, such as that one of the compounds was applied 4 dbi and then the other compound was applied two days later. Inoculation with *E. amylovora* was performed 4 days after the first or the only treatment and bacterial suspension in sterile water containing 1×10^{8} CFU/ml was used. Control of fire blight was expressed as browning discoloration index (BDI, %) and stem bending index (SBI, %).

When BABA was applied alone at 4 dbi, BDI was reduced by 44.1%, and SBI was reduced by 46.7%, compared to the inoculated untreated seedlings with BDI of 95.8% and SBI of 95% (Hassan & Buchenauer 2007). In this trial BABA followed by ASM, and vice versa, applied sequentially at 4 dbi and then at 2 dbi, severely reduced disease symptoms by 94.1% (BDI) and by 95.5% (SBI). Interestingly, when combination of both compounds was applied simultaneously at 2 dbi disease severity was reduced less effectively and seedlings showed significantly higher BDI of 42% and SBI of 28% when compared to the same indices of sequential 4 and 2 dbi applications of these compounds (Hassan & Buchenauer 2007).

The same authors also evaluated both SAR activators for *in vitro* inhibition of *E. amylovora* and found that BABA (0.5 and 1.0 mg/ml) and ASM (0.1 and 0.2 mg/ml) did not inhibit bacterial growth. However, when the effect of both compounds was evaluated in different arena, i.e. in apple seedling leaves, spray treatment with BABA at 4 dbi followed with ASM at 2 dbi led to reduction in bacterial populations with 2.7×10^5 when compared to the untreated inoculated control with 7.2×10^7 CFU/g of leaf tissue (Hassan & Buchenauer 2007).

In all above BABA (1.0 mg/ml) and ASM (0.1 mg/ml) treatments with sole compound, succession of two compounds, and their combination, free and total salicylic acid and peroxidase activity were determined in seedling leaves at 0, 2, 4, 6, and 8 days after the last treatment. Sole BABA or ASM treatments or sequential treatment with BABA at 4 dbi and then ASM at 2 dbi, caused significant increase of peroxidase activity in leaves of non-inoculated seedlings when compared to water treated control (Hassan & Buchenauer 2007). The increase of peroxidase activity in non-inoculated seedlings was reached slower in BABA than in ASM treatment and the maximum for BABA was at 6 days after the application. In contrary rapid increase of peroxidase activity in the ASM treatment led to detection of the highest levels at 2 and 4 days after the

application. BABA treatment followed by ASM showed the highest peroxidase activity at 4 days after the application for BABA and 2 days after the application for ASM (Hassan & Buchenauer 2007). Thus the higher peroxidase activity was detected in leaves treated with ASM alone and when BABA was followed by ASM. The peroxidase activity in all of the treatments decreased in relatively similar patterns until the end of experiment. Water treated control remained relatively low through all detection time points.

In the inoculated seedlings, on the other hand, water treated control showed elevation in peroxidase activity at 2 days after the application and remained almost constant until the end of experiment. In BABA alone treatment peroxidase activity continued increasing after the inoculation and kept on progressing slowly until the end of experiment. This was different in comparison to the decrease tendency after the maximum at 6 days from application in non-inoculated seedlings and towards the end of experiment. Finally, in ASM alone, and BABA followed by ASM, treatments increase in peroxidase activity was tremendous and rapid, leading to elevated maximums reached at 4 days after the treatment or 2 days after the inoculation (Hassan & Buchenauer 2007). After this elevated maximums were reached peroxidase activity remained relatively stable until the end of the experiment. Thus, peroxidase accumulated much more in the infected leaf tissues treated with ASM and sequential treatment of BABA and then ASM, then in the BABA alone treatment (Hassan & Buchenauer 2007).

In the same study accumulation of free salicylic acid (SA) was the lowest in the leaves of non-inoculated control seedlings and in ASM treatment. Control and ASM treatment did not differ and both remained at similar levels through 6, 8, and 10 days after the application. Thus ASM did not cause accumulation of free SA. In BABA only treated seedlings content of SA acid was higher compared to the control, accumulating very early at 4 days after the treatment and

staying at a very similar level until the end of experiment (Hassan & Buchenauer 2007). In BABA followed by ASM treatment, and BABA only treatment, levels of free SA were significantly higher compared to ASM followed by BABA treatment. When a combination of ASM and BABA was applied simultaneously, slight increase in content of free SA was detected at 2 and 4, while it the decrease was detected at 6 and 8 days after the treatment (Hassan & Buchenauer 2007). The same authors concluded that BABA alone or in sequential treatment followed by ASM led to high accumulation of free SA in apple seedling leaves.

In all the treatments total content of SA was overall 10 times more elevated than the free SA content. The patterns of higher and lower total SA content among treatments were relatively similar to the patterns of higher and lower free SA content. One of the differences was in the increasing trend of total SA in BABA treatment from 0-10 days after application which was opposite to the same treatment in free SA content (Hassan & Buchenauer 2007). The second difference was in the two sequential treatments with ASM and BABA, or vice versa, where the total SA content was only slightly lower compared to BABA treatment alone. In a combination of ASM and BABA, applied simultaneously, total SA content remained relatively stable until 8 days after application but after the increase between 0 and 2 days after application (Hassan & Buchenauer 2007). The same authors conclude that ASM application alone did not allow increase in total SA in infected apple seedlings leaves, and that BABA alone or in sequential treatment followed by ASM, led to the increase in total SA content.

Overall, two sequential treatments with ASM and BABA, and BABA treatment alone, showed to accumulate the highest levels of both total and free SA content in apple seedling leaves (Hassan & Buchenauer 2007).

Topical application of ASM seems to be a valuable treatment that can aid the regular control programs for fire blight on pomaceous fruit trees, and possibly offer replacement to highly efficient antibiotics. Streptomycin, kasugamycin, and other antibiotics are well known and used in control of fire blight. However, they are often subject of controversy due to the worldwide public fear of resistance in human, animal, and plant pathogens and are banned or highly restricted for use in agriculture of vast majority of countries (Vanneste 2000; Vanneste et al. 2008). However, in all the spray trials antibiotics always show superior activity and higher efficiency in comparison to ASM or other SAR activators. This fact is still luring fruit growers in certain countries to illegally apprehend and use veterinary streptomycin products or banned stock supplies of agricultural antibiotics to combat fire blight. The use of ASM shows promising potential, but due to limited effectiveness recorded on pear has mainly reserved its place in apple production (Thomson, Gouk, et al. 1998).

Mono- and di-potassium salts of phosphorous acid and prohexadione-calcium

Alkali metal salts of phosphorous acid (H_3PO_3), also known as phosphites, are exquisitely interesting eco-friendly plant protectants and nutritional compounds since now it is know that previously controversial complex mode of activity of these compounds is in fact dual and is based on (a) direct toxic or inhibiting effect on fungal, pseudo-fungal, and bacterial plant pathogens, and (b) indirect effects including rapid and strong activation of plant defense mechanisms (Jones 2010; Smillie et al. 1989; Grant et al. 1990; Guest & Bompeix 1990; Guest & Grant 1991; Jackson et al. 2000; Landschoot & Cook 2007; Brunings et al. 2010; Deliopoulos et al. 2010). Two of the most known and registered active ingredients in fungicides are potassium di-hydrogen phosphite (KH_2PO_3) and di-potassium hydrogen phosphite (K_2HPO_3).

Besides direct and indirect effects on plant-pathogen system, phosphites are also known to cause negative side-effect of phytotoxicity on the plants and this usually happens if doses applied topically are higher than 5 g/L or 36 kg/ha (Hardy et al. 2001; Barrett et al. 2003). Agricultural use of phosphites has been extensively investigated primarily due to their effects on plant pathogens and not for the purposes of plant nutrition (Deliopoulos et al. 2010). It is the conclusion of previous experiments on disease control and reduction of plant susceptibility that the complex mechanisms of preventive activity provided by phosphites limits resistance development in plant pathogens (Grant et al. 1990; Deliopoulos et al. 2010). Overall, phosphites have not been clearly shown to activate SAR through upregulating the PR-protein genes, and by now are mostly considered candidate SAR activators.

Prohexadione-calcium (27.5%, Apogee® BASF Corp., Research Triangle Park, NC) is registered as a plant growth regulator and expresses its effect on apple shoots by reducing their vegetative growth. Its mode of action is inhibition of the biosynthesis of growth active gibberellins (GA) and this leads to reduction of longitudinal shoot growth (Evans et al. 1999). The shoot growth reduction on apple trees has been shown to control fire blight disease causes by *E. amylovora*. Recent research results on apple cv. 'Gala', *Malus domestica* Borkhausen, have led to hypothesis that Apogee is enabling decrease in fire blight shoot infection through altering the tree physiology which results in cell wall thickening in the cortical shoot parenchyma, and creation of physical barrier that is able to stop the infection and systemic spread of fire blight bacteria (McGrath et al. 2009). Different sources reported Apogee as an SAR activator or similar to SAR activators in its effect (Momol et al. 1998; Bubán et al. 2004; W. Rademacher 2004; Spinelli et al. 2007). It was also reported that besides acting as a growth regulator, prohexadione-calcium influences flavonoid metabolism and casues accumulation of 3-deoxycatechin

luteoliflavan in shoots of pomaceous fruit trees (Spinelli et al. 2005). However, the same authors report that luteoliflavan was not active against the bacterium *in vitro*, but that under the same conditions its highly reactive and unstable precursor luteoforol showed very effective bactericidal activity on *E. amylovora*. They also report that prohexadione-carboxylic acid inhibits an enzyme in flavonoid synthesis and as a result various 3-deoxyflavanoids, particularly luteoforol, were synthesized. Even though the levels of synthesized luteoforol were not determined, the results led to conclusion that prohexadione-calcium induced synthesis of luteoforol and that this phytoalexin represents the principle mode of prohexadione-calcium activity (Spinelli et al. 2005). Answering the question whether prohexadione-calcium (200 ppm) can cause elevated PR protein expression, as a hallmark of SAR activation, recent results in attempt of controlling apple scab fungus *V. inaequalis* on seedlings of cv. 'Golden Delicious' in green-house conditions, showed that mRNA's of all PR-1, PR-2, PR-5, PR-8 proteins were upregulated in both, seedlings treated only with prohexadione-calcium and seedlings treated with this compound and then challenged by inoculation with apple scab fungus (Bini et al. 2008). Indirect advantage in use of prohexadione-calcium in plant protection is that treated trees have a more opened canopy, thus enabling better spray coverage of other protective compounds (Wilhelm Rademacher 2004).

The trials investigating fire blight control effects with phosphite products are very rare. In one of the reports aimed towards control of blossom blight authors tested registered systemic fungicide Agri-Fos 3.35L® (mono- and di-potassium salts of phosphorous acid 45.8%, Agrichem Manufacturing Industries PTY., Ltd., Loganholme, Queensland, Australia) applied as a non-conventional trunk application and compared it with the effects of foliar application of antibiotics (Yoder et al. 2007). Trunk bark treatments on 26-year-old trees of apple cv. 'Idared',

Malus domestica Borkhausen, in this study were applied at half inch green and pink growth stages, while the foliar treatments were applied at early to mid-bloom. All the bark treatments were applied to the trunk by covering the trunk area from the soil level up to 91.44 cm, and to the 30.48 cm of bark on any scaffold branch on the trunk below the trunk height limit of 91.44 cm. Agrifos was used alone or in combination with nonionic wetting agent and bark penetrating surfactant Pentra-Bark® (mixture of alkylphenol ethoxylate, polysiloxane polyether copolymer and propylene glycol 99.8%, Quest Products Corp., Louisburg, KS). This product is intended for improving penetration through bark of water based basal applications.

The first of the trunk bark treatments was applied later at the pink stage on 20 April and consisted of Agrifos at 1.89 L/3.78 L of water and 85 g of Pentrabark (I). On 30 March, at quarter-half inch green stage following treatments were applied: (II) 85 g Pentrabark /3.78 L of water; (III) 1.89 L Agrifos/3.78 L of water; (IV) 0.95 L Agrifos + 85 g Pentrabark /3.78 L of water; (V) 1.89 L Agrifos + 85 g of Pentrabark /3.78 L of water; (VI) 0.95 L Agrifos + 85 g Pentrabark /3.78 L of water followed by 3 foliar treatments of Agrimycin® 17, each 113.4 g/378 L water (Nufarm Limited, Melbourne, Australia); (VII) 1.89 L Agrifos + 85 g Pentrabark /3.78 L of water followed by 3 foliar treatments of Agrimycin, each 113.4 g/378 L water. Accompanying three foliar antibiotic treatments were applied in the morning of 23 April (king bloom), 25 April (full bloom), and 2 May (late bloom). Defoaming product Fighter-F (dimethylpolysiloxane, polypropylene glycol, and methylated silicone 10%, Loveland Products, Inc., Greeley, CO) was first added to water and then amended with all trunk treatments at a rate of 14.79 ml/3.78 L water (Yoder et al. 2007). In all the trunk treatments followed with foliar antibiotic spray treatments, surfactant Regulaid at a rate of 473.2 ml/3.78 L was amended with solution for foliar application.

Total of four selected branches per replicate tree, that carried 25-40 blossom clusters, were spray inoculated with *E. amylovora* (1×10^6 CFU/ml). Inoculations were conducted only in the evenings of 23 and 25 April as a follow up to the morning antibiotic spray treatments. Percent of infected clusters was calculated based on cluster number counts on 21 April, before inoculation, and on the infected cluster number counts on 8, 9 and 14 May encompassing all treatment replicates (Yoder et al. 2007).Weather conditions were favorable for disease development and according to *MaryBlyt* fire blight forecast system natural infection periods occurred on 24 and 25, 27 April, and on 1-2 May.

Under these treatment and weather conditions all the trunk bark treatments, excluding the ones followed up with antibiotic spray, provided slight but statistically significant level of fire blight suppression (Yoder et al. 2007). The results for each respective trunk only treatment expressed in percent of infected clusters were as follows: (I) 61.1%, (II) 64.7%, (III) 71.2%, (IV) 70.2%, and (V) 65.6%. The trunk treatments followed with the antibiotic sprays gave better control levels of 24.6% (VI), and 19.7% (VII). All of these treatments were significantly different from untreated control with 85.4% of infected clusters. However, when Agrifos rate defining treatments only, were compared between themselves there was no significant difference in control of fire blight. The earliest application of Agrifos at pink growth stage, with 61.1% disease level, provided numerically the lowest but not significantly different fire blight control in comparison to the same treatment at half inch green growth stage with 65.6% of infected clusters (Yoder et al. 2007).

Unexpectedly, trunk treatments of Agrifos with 0.95 and 1.89 L/3.78 L water followed by three Agrimycin spray applications led to statistically higher disease levels of 24.6 and 19.7%

infected clusters when compared to 10.4% in the same dose Agrimycin foliar applied treatment only (Yoder et al. 2007).

In another experiment with foliar application of Agrifos, antibiotics, and other known and candidate SAR activators, blossom blight control was evaluated on 36-year-old trees of apple cv. 'Golden Delicious' and cv. 'Rome Beauty', *Malus domestica* Borkhausen (Yoder et al. 2008). SAR activators were applied two times at pink growth stage, on 16 April, and then two weeks later on 30 April as post bloom, while antibiotic control treatments were applied at bloom only. Antibiotic treatments were applied in the morning on 23 April at full bloom on cv. 'Golden Delicious' and at early bloom on cv. 'Rome Beauty'. The treatments applied with a single-nozzle handgun sprayer, at 400 psi, were as follows: 1. Untreated control; 2. Agrifos at 0.95 L/0.4 ha; 3. Prophyt at 0.95 L/0.405 ha (potassium phosphite 54.5%, Luxembourg-Pamol Inc., Memphis, TN); 3. Topaz® at 0.95 L/0.405 ha (mono- and di-potassium salts of phosphorous acid 53%, Agriliance LLC., St. Paul, MN); 4. Agrimycin high at 226.8 g/0.4 ha; 5. Agrimycin low at 113.4 g/0.4 ha; 6. Kasumin® 2L at 1.89 L/0.4 ha) (Arysta LifeScience North America, LLC., Cary, NC); and 7. Flameout™ at 0.45 kg/0.405 ha (oxytetracycline hydrochloride 18.3%, Cerexagri Inc., King of Prussia, PA) (Yoder et al. 2008).

Spray inoculation of flowers with *E. amylovora* suspension (1×10^6 CFU/ml) was performed in two occasions on 23 April in the evening, and on 24 April in the morning, both in two by two replicates manner out of total four tree replicates per treatment. Weather conditions were very favorable for fire blight development according to *MaryBlyt* forecast system. Besides inoculation imposed disease pressure, natural infection periods took place close to full bloom stage on 25-26 April, and again at late bloom on 3 and 6 May. Blossom blight was rated on 15

May and 23 June and on 15 May percent of infected clusters was calculated based on flower cluster counts on the day of the first inoculation. However, on 23 June infected flower cluster leaves counts were rated only.

The two cultivars' untreated controls yielded in somewhat different disease levels due to cultivar differences in the onset and the advancement of bloom stage. With the same day of inoculation cv. 'Rome Beauty', which was at early bloom, yielded in lower blossom blight of 47% on 15 May, whereas on the same rating date in cv. 'Golden Delicious' disease advanced to 70% of blossom blight. In cv. 'Golden Delicious' on 15 May statistically significant disease suppression was recorded only in the treatments with antibiotics (27-49%). The phosphite products did not provide significant control of blossom blight compared to the untreated control and yielded with disease levels of 63% in Agrifos, 61% in Prophyt, and 68% in Topaz. In cv. 'Rome Beauty' on 15 May there was no significant effect of any of the treatments and compared to the untreated control phosphite products yielded with disease levels of 37% in Agrifos, 39% in Prophyt, and 32% in Topaz (Yoder et al. 2008).

On 23 June flower cluster leaves disease levels in untreated controls were 58% in cv. 'Golden Delicious' and 25% in cv. 'Rome Beauty'. In cv. 'Golden Delicious' significant control was only achieved with the application of antibiotic oxytetracycline (26%). When compared to the untreated control in cv. 'Golden Delicious', phosphite products yielded with disease levels of 56% in Agrifos, 52% in Prophyt, and 55% in Topaz. In cv. 'Rome Beauty', *Malus domestica* Borkhausen, on the same date significant effect compared to the untreated control was recorded in Agrifos with only 10% of disease level, and Kasumin with 9%. Disease levels in Prophyt and Topaz treatments were 16 and 17%, respectively (Yoder et al. 2008).

Alternative approaches to control fire blight with phosphites and prohexadione-calcium were also attempted through their trunk injection (Spitko 2008). Injectable formulation ArborFos^{TM} (mono- and di-potassium salts of phosphorous acid at 45.8%, Mauget Inc., Arcadia, CA), and a foliar formulation Apogee were evaluated in shoot blight control.

In the first trial with trunk injected Arborfos, applied through micro-injection capsules at a dose of 7.5 ml per 2.54 cm of apple trunk diameter at breast height (DBH), and with Apogee formulated for microinjection at concentration of 125 and 250 ppm in total capsule volume of 6 ml per 2.54 or 15.24 cm DBH, objective was to evaluate the effect of these products on shoot blight phase of fire blight pathogen *E. amylovora* (Spitko 2008). Further, the goal was to assess the effect of Apogee as a growth regulator on apple shoot growth and expansion and potentially record any symptoms of phytotoxicity that the products could cause. Trees of highly fire blight susceptible apple cv. 'Paula Red', *Malus domestica* Borkhausen, on M7 rootstock, were trunk-injected in spring on 30 May when foliage in apple canopy had completely expanded. Capsules have emptied within 1 h after installation in predrilled injection delivery port, according to manufacturer instructions, and removed from the trunk after 24 h. Total of 10 intensively growing apple shoots per tree were scissor inoculated on 5 June after dipping into fire blight bacteria suspension of 1×10^{8} CFU/ml, i.e. six days after trunk injection (Spitko 2008). Disease rating at weekly intervals was conducted using Hickey's disease rating system (1998) and expressed as fire blight incidence, and lasted until disease progression and spreading within the season ceased. On 5 separate healthy shoots per each tree shoot growth was measured at bi-weekly intervals and phytotoxicity evaluations were conducted 10 days after trunk injection.

Disease successfully established on shoots 15 days after inoculation and was repeatedly rated for the next 30 days. On 20 June untreated control yielded disease index of 2.97 which was

significantly higher compared to the injected Arborfos which provided disease suppression with index of just 1.30 (Spitko 2008). The effect of Apogee with concentrations of 125 ppm/2.54 cm DBH and of 125 ppm/15.24 cm DBH yielded in disease indices of 1.56 and 1.85, respectively, and was not significantly different from both the untreated control and Arborfos. Strikingly, higher Apogee concentrations of 250 ppm/2.54 cm DBH and of 250 ppm/15.24 cm DBH yielded in numerically higher disease indices of 2.11 and 2.03, respectively, and were not significantly different from the untreated control and Arborfos as well. All Apogee treatments were statistically similar between eachother. On 10 July disease indices in all the treatments increased. However, Arborfos still remained significantly more efficient in fire blight control by providing index of 2.10 compared to 4.94 in untreated control. The effect of Apogee with concentrations of 125 ppm/2.54 and 15.24 cm DBH, and of 250 ppm/2.54 and 15.24 cm DBH cm, yielded in disease indices of 2.78, 3.05, 3.56, and 3.73, respectively, and again was not significantly different from both the untreated control and Arborfos (Spitko 2008). Higher Apogee concentrations were still with numerically higher disease indices compared to the lower Apogee concentrations. Finally, based on the results recorded on 24 July the disease progress completely ceased and the disease indices remained the same as on 10 July. Therefore, Arborfos was able to reduce shoot blight incidence for 67% and Apogee was ineffective (Spitko 2008). In all the treatments there was no phytotoxic effects caused by trunk-injected compounds.

The effect of Apogee on shoot growth was not detected since there were no significant differences after total of four shoot length measurements were taken pre-injection and post-injection (Spitko 2008). Based on absence of the effect on shoot growth authors emphasized that there is a strong indication that prohexadione-calcium probably did not translocate through the tree tissues after it was trunk-injected and thus was not able to distribute in the apple tree crown.

Regalis® (prohexadione-calcium 10%, BASF Crop Protection, Germany) has been previously evaluated on 6 apple cultivars on M9 rootstock using two doses of 150 and 100 mg/ml in an aqueous solution with 600L/ha. Dash™ HC has been added to this solution at 0.5L in 600L/ha (37.5% fatty acid esters+22.5% alkoxylated alcohols-phosphate esters, BASF Crop Protection, Germany) (Bubán et al. 2004). Higher concentration was applied on 19 April and lower on 28 May. The six apple cultivars were 'Elstar', 'Freedom', Jonagold Decosta', 'Jonica', 'King Jonagold' and 'Sampion'. Shoot inoculations were conducted two weeks after the treatments with scissors dipped into *E. amylovora* suspension (10^7CFU/ml) and by cutting the 2-3 youngest leaf blades per shoot tip. Inoculations were conducted only on container-grown apple trees of cv. 'Freedom' and cv. 'Idared' and the shoot blight control experiment was conducted three times in two years. By measuring the length of shoots three times in the season, in all 6 apple cultivars Regalis significantly reduced shoot growth. This was primarily due to shortening of the lengths of internodes and not due to decline in their number (Bubán et al. 2004). 'Elstar' shoots responded most strongly and 'Freedom' shoots responded the most weakly to Regalis treatments.

Shoot blight control was better on 'Idared' than on 'Freedom' since the length of necrotic lesions was consistently and significantly smaller on 'Idared' (Bubán et al. 2004). In both years, all spray treatments with Regalis at 13, 14 or 15 days before inoculation showed significant reduction of shoot blight severity at 7, 14 and 21 days after the inoculation (Bubán et al. 2004).

In another experiment on apple cultivars 'Rome Beauty', on MM106 rootstock, 'Golden Delicious', on M7 rootstock, and 'Law Rome', on MM111 rootstock, Apogee applied topically twice at bloom showed significant reduction of shoot blight severity when inoculations were

conducted 10 days after the second treatment (1×10^5 or 1×10^8 CFU/ml) (Yoder et al. 1999). On 'Golden Delicious' Apogee was applied at 250 or 375 mg/L, on 'Rome Beauty' Apogee was applied 375 mg/L, and on 'Law Rome' Apogee was applied at 125 or 250 mg/L. Each sprayed volume was amended with Regulaid adjuvant at 0.03 or 0.125% (Yoder et al. 1999). Similar reported effects were discussed and summarized for all pomaceous fruits (Wilhelm Rademacher 2004).

Prohexadione-carboxylic acid

Besides above described attempts in target precise and environmentally safer fire blight control, one other study boldly investigated the effect of trunk-injected free acid of prohexadione-calcium (Apogee) or prohexadione-carboxylic acid in control of blossom infections by *E. amylovora* (Düker & Kubiak 2011a).

Prohexadione carboxylic acid (PCA) was trunk-injected for fire blight control on 2-year-old apple trees of cv. 'Weisser Klarapfel', *Malus domestica* Borkhausen, under greenhouse conditions, and on four-year-old apple trees of cv. 'Gala Must', *Malus domestica* Borkhausen, in field conditions, respectively (Düker & Kubiak 2011a). Using one ChemJet Tree Injector® injector per plant (Chemjet Trading Pty Ltd., Bongaree, Queensland, Australia), trees of cv. 'Weisser Klarapfel' were trunk-injected with 40 mg of PCA per tree by delivering 20 ml of solution with concentration of 2000 mg/L of water. Trees of cv. 'Gala Must' were trunk-injected with 10, 20, 30 and 40 mg of PCA per tree by delivering 20 ml of PCA solutions with concentrations of 500, 1000, 1500 and 2000 mg/L of water, respectively (Düker & Kubiak 2011a). Injected 20 ml of water served as a control while trees sprayed with streptomycin (1600

mg/L), four hours prior to inoculation, served as a standard treatment. When the trees were almost in full bloom spray inoculation with *E. amylovora* (10^6 CFU/ml) was conducted eight and 30 days after trunk injection of cv. 'Weisser Klarapfel' and cv. 'Gala Must', respectively.

Blossom blight percentage, rated seven and 29 days after inoculation, showed that in cv. 'Weisser Klarapfel' PCA provided significant fire blight control with disease level of 7.9%, in comparison to water control which yielded 24%, while it was not significantly different from streptomycin with disease level of 2% (Düker & Kubiak 2011a).

On cv. 'Gala Must', injected with doses of 10, 20, 30 and 40 mg of PCA per tree, blossom blight percentages of 8.0, 6.8, 4.1, and 6.9%, respectively, showed significant control of fire blight in comparison to water control with disease percentage of 21.6%. However, including streptomycin with blossom blight of 5.2%, there were no significant differences in disease control between different PCA doses (Düker & Kubiak 2011a).

In both cultivars the effects of trunk-injected PCA on shoot stunting, an expected Apogee effect was detected but not measured. In general, shoot stunting was found not to reduce in intensity with declining dose gradient from 40 to 10 mg of PCA per tree, and these growth conditions led to significant fruit yield losses (Düker & Kubiak 2011a).

Monitored PCA residue contents in the harvested fruits of cv. 'Gala Must' in the treatments with 30 and 40 mg, showed that compound levels after trunk injection were 0.0032 and 0.0017 ppm, respectively, which in both cases is significantly and several-fold lower compared to the maximum allowed residue levels of 0.05 ppm prescribed by the European Union (EU) pesticide regulations (Düker & Kubiak 2011a).

The authors discuss that previous field trials with topically applied prohexadione-calcium (Bazzi et al. 2003), registered in EU as Regalis® (prohexadione-calcium 10%, BASF Crop

Protection, Germany), showed insufficient effectiveness in blossom blight control in pears. It is implied that the gained effects of PCA in control of blossom blight through trunk injection could serve as a potential alternative to the topical application of streptomycin as a standard treatment for blossom blight control in apples. Further, due to very low residue levels of PCA in the fruits, it is concluded that use of trunk-injected PCA under field conditions could be possible from the perspective of public health. The effect of shoot stunting detected on the injected trees rose a question whether there could be a possibility for reduction of undesired influence of PCA on the longitudinal shoot growth, while maintaining the effect of control of blossom blight infections (Düker & Kubiak 2011a).

The future in dealing with a devastating pathogen such as *E. amylovora* might lie in approaches like integrated disease or pest management (IDM, IPM) and trunk injection of plant protection compounds. IPM is based on the concept of harnessing the incremental effects of various plant protection practices combined to achieve the ultimate goal of substantial disease control. For instance, application of eco-friendly SAR activators, or biological control agents, coupled with extremely reduced use of antibiotics, and supported with prompt mechanical removal of initial fire blight symptoms by pruning, might yield in satisfying effect but somewhat higher costs. Further, trunk injection approach in delivering conventional and bio-pesticides, as well as antibiotics, through tree's xylem tissue imposes itself as a very serious alternative to topical application. This is because there are indications that *in planta* effect of SAR activators increases considerably after trunk injection, and due to the fact that this concept allows target precise disease control with relatively low volumes and no losses in deployed active ingredient (Wise et al. unpublished). For example, trunk injection of protective compounds at the end of the growing season, when *E. amylovora* forms a carry-over wood cankers as the main sources of

inoculum in the next season, could allow remedy for the plants and provide curative effect against fire blight. The potential for curative control of '*Candidatus* Liberibacter asiaticus' which causes citrus greening has been proven by *in planta* antibiotic delivery (Zhang et al. 2011). However, besides numerous advantages of this method, several obstacles require more investigation, demand refinement in technology, and crave for addressing the issue of application costs in fruit growing.

Control of apple scab (Venturia inaequalis (Cooke) Winter) using topical and trunk injection treatments of alternative plant protective compounds and fungicides

Acibenzolar-S-methyl

The use of ASM in control of apple scab, caused by fungal pathogen *V. inaequalis*, is also under investigation. However, the literature sources are considerably more scarce compared to the research on SAR activators in control of fire blight (*E. amylovora*). Therefore, research results on use of ASM in control of *V. nashicola* Tanaka & Yamamoto, causing agent of scab on Japanese pear, *Pyrus pyrifolia* (Burm.) Nak., are further presented.

Spray application of ASM on apple seedlings 2 days before inoculation with *V. inaequalis* showed significant reduction of the scab severity index from 3.0 in control, to 0.4-1.1 in ASM (Bengtsson et al. 2008). Further, the same source demonstrates the reduction of conidia produced from 6.1×10^6 conidia on the untreated control to 0.1×10^6 conidia per plant on the ASM treated plants. In comparison to fungicide sulphur, *in vitro* assays showed that ASM had no significant effect in inhibition of conidial germination in apple scab fungus (Bengtsson et al.

2008). However, observing pre-penetration events of *V. inaequalis* on the leaves, the same authors report that ASM significantly reduced conidial germination from 58-64% in the untreated control to 45-50.5%, and that it also reduced the percentage of conidia that form appressoria from 80-94.5% in control to 41.6-86%. In the same study, observations of penetration events showed reductions in the percentage of conidia achieving penetration. For instance, in leaves pre-treated with ASM this percentage was reduced to 19.8% at 1 day post inoculation (DPI), 49.5% (3 DAI), and 62.5% (5 DPI), from 35%, 80.5%, and 87.5% in the untreated control, respectively.

Overall, observations of post-penetration events showed that ASM pre-treatments significantly reduce percentage of germinated conidia forming primary stromata, at 3 and 5 DPI, and conidia forming runner hyphae and secondary stromata, at 5 DPI (Bengtsson et al. 2008).

Gene expression relative quantification, encompassing PR1 and PR8 genes, showed significant up-regulation of PR1 gene in ASM treated seedlings compared to water treated, non-infected control, 5 DPI. PR8 gene was up-regulated by ASM treatment at 2 and 5 DPI, and by the inoculation in water treated seedlings, 2 DPI (Bengtsson et al. 2008). All in all, under controlled conditions apple scab can be significantly controlled by ASM (Bengtsson et al., 2006a,b, 2008).

Research on mechanisms and control of *V. nashicola*, causing agent of Japanese pear scab and very close relative of apple scab fungus, showed that under severe disease pressure two applications with ASM (100 μg/ml), performed 7 and 3 days before inoculation with pathogen, lad to considerable reduction of disease leaf percentage for 41.7% (21 DPI) and 29.9% (30 DPI), and of disease severity for 40.7% (21 DPI) and 47.5% (30 DPI), when compared to water treated

control (Faize et al. 2004). Similar results have also been reported before in control of *V. nashicola* (Ishii et al. 1999; Ishii et al. 2001).

More recent research, including ultrastructural investigation of *V. nashicola* rase 1 infection behavior after application of ASM, confirmed that the growth of fungus was suppressed when the preventive leaf treatments were applied on susceptible Japanese pear cv. 'Kousui' (Jiang et al. 2008). In this research, with the objective to determine the mechanism of scab activated resistance by ASM, the parameters of conidial germination, appressorial formation, and penetration were observed. The difference in infection behavior, compared to control leaves treated with water, was observed only during penetration events which took place in epidermal pectin layers and middle lamellae (Jiang et al. 2008). These authors found low frequency of subcuticular hyphae in the leaves pretreated with ASM, compared to water. Further, more hyphae had been found to collapse in ASM-pretreated leaves and their cell walls divided into many amorphous fibrous pieces. Findings of morpho-metrical analysis on hyphae, in the same study, suggested that in comparison to water treatment, the production or activity of pectin degrading enzyme from hyphae was inhibited by ASM treatment. These results lead to a conclusion that the ASM-activates SAR to Japanese pear scab pathogen can probably be associated with cell-wall degrading enzymes originating from the host itself (Jiang et al. 2008).

The infamous *Venturia* spp. pathogens causing leaf, fruit, and shoot scab diseases of apple and Japanese pear show valuable responses to treatments with ASM. Topical treatments with this compound have promising effects and could aid to the control of scab diseases of pomaceous fruit trees. Even though in all the spray trials fungicides always show superior activity and higher efficiency in scab control when compared to ASM or other SAR activator, the question whether ASM could be a fair alternative to highly efficient fungicides used in

production still points on need for more research. The directions of further research should be investigation of the agents that could be added to enhance the SAR activity of ASM, and in-depth investigation of underlying mechanistic and genetic background of ASM-activated resistance. Successful control of scab diseases with SAR activators could be possible especially since there is evidence that SAR activator such as mono- and di-potassium salts of phosphorous acid (phosphites) applied via trunk injection can provide substantial apple scab reduction (VanWoerkom 2012).

Mono- and di-potassium salts of phosphorous acid (phosphites)

There are very few recent literature sources that investigated effects of phosphites in control of the two most important apple diseases apple scab (*V. inaequalis*) and fire blight (*E. amylovora*). In one study with several PRA-s for control of apple scab, potassium phosphite formulated as Phoenix (300 g phosphorous acid per liter water, Orion, Future Technology Ltd., UK) was evaluated when applied topically one or multiple times at a rate of 10 ml/L of water at four key apple growth stages or their combinations: bud break, green cluster, 90% petal fall, and early fruitlet (Percival et al. 2009). Leaf scab severity index was used in disease rating and in two consecutive years of trials, when damaging outbreaks of apple scab were recorded, control trees of cv. 'Golden Delicious' had 4.8 and 5.0 disease severity indices, respectively (leaf scab severity index: scale 0 = no scab observed; 1 = less than 5% of leaf area affected; 2 = 5%–20% of leaf area affected with some yellowing; 3 = 21%–50% of leaf area affected, significant leaf yellowing; 4 = 51%–80% of leaf area leaves affected, severe leaf yellowing; 5 = 81%–100% of leaf area with complete leaf yellowing).

In the first year of trials, potassium phosphite was effective in control of apple scab on leaves when applied at three or four consecutive growth stages, starting from bud break, with disease indices of 3.6 and 3.0, respectively, and was significantly different from the untreated control with disease index of 4.8. However, in the second year of evaluation, besides significant effectiveness when applied at three and four consecutive growth stages, potassium phosphite also showed significant control of the disease when applied at just two consecutive growth stages, starting from bud break (Percival et al. 2009). The disease indices were 3.8, 3.4, and 4.5, respectively, and the untreated control had disease index of 5.0. When the same topical treatments were evaluated in control of apple scab on the fruit the results showed that in the first year only full schedule application of potassium phosphite in four consecutive growth stages, with disease index of 1.8, was effective and significantly different from untreated control with disease index of 2.3 (Percival et al. 2009). In the second year, besides four growth stages schedule, with 1.4 disease index, the effect of the compound was also significant in three consecutive growth stages schedule with 1.6 disease index, and was different from the untreated control with disease index of 2.5.

Overall when the effects of Phoenix on chlorophyll content were investigated(Percival 2010)(Percival, 2010) it was found that, when both seasons were averaged, thia product improved chlorophyll content for 30-118% in apple (Percival et al. 2009). It was also found that, when both seasons were averaged, the yield of apple fruit in comparison to water treated control was improved for 0-27% (Percival et al. 2009).

Dealing with such a devastating pathogen as *V. inaequalis*, which occurs every year in apple production, is very hard (Sutton 1996). This especially stands from the perspective of organic and integrated pest management (IPM) fruit productions. Further, in conventional apple

production as well, resistance of apple scab pathogen to fungicides magnifies the problem of apple scab control from year to year (Chapman et al. 2011; Lesniak et al. 2011). Various old and novel pathogen control strategies are being developed and tested worldwide. The most known examples are resistance management strategies and IPM approaches, which include interchangeable and careful application of compounds with different modes of action, and combined use of environmentally friendly and conventional protection compounds. However, results of these strategies still show superiority of conventional synthetic fungicides compared to limited or good effects of new alternative compounds. The ultimate solution of having environmentally safe and efficient alternative scab diseases control is still missing and thus requires more research.

In brief, even though SAR activators are very desirable and promising compounds that aid plant protection, their topical deployment rarely provides complete disease control and most of them yield in quite variable efficiency ranging from 20-85% (Walters et al. 2005). There is still much unknown that needs to be revealed in order to successfully develop and improve approaches and techniques that will maximize their efficiency. One of the main directions that needs to be pursued in order to achieve increased efficiency of SAR activators is research that will lead to understanding the influences of environment, genotype, and crop nutrition on their activity (Walters et al. 2005). The same authors also conclude that more work is needed in finding the best way for implementation of plant resistance activation into the disease control strategies and crop management practices, and that SAR activators are not, and should not be deployed solely as “safe fungicides”. It is our opinion that more novel approaches should be utilized in delivering SAR activators to the plants. The boundaries of classical plant protection tendencies should be pushed or broken in order to open more space and readiness to alternatives

such as trunk injection of plant protective compounds. This novel approach harnesses the tree xylem with the goal to precisely deliver the compounds to the pest and requires more attention and research, especially in use of SAR activators.

Fungicides

According to the only recent reference on control of apple scab using trunk injection, single injection of 8 fungicides into apple trees of cv. 'Crown Gold' during May 2002, showed significant protection against apple scab (*V. inaequalis*) on leaves and fruits in two consecutive seasons for 7 fungicides (Percival & Boyle 2005). The injected fungicides which were formulated for spray application were: myclobutanil (20%, Systane) penconazole (10%, Topas), thiabendazole hypophosphite (26.6% Storite Clear), propiconazole (41.8%, Tilt), carbendazim (43%, Tripart Defensor FL), pyrifenox (20%, Dorado) (Percival & Boyle 2005). The injected fungicides which were formulated for injection application were: Aliette® (9.5% fosetyl-AI, Aluminum tris /0-ethyl-phosphonate/) and Systrex® (00.88% triadimefon). All these fungicides except Systane gave significant apple scab control at a rate of 0.25 g of active ingredient (a. i.) per each 2.5 cm of trunk diameter. They were applied using 50 ml microcapsules for injection (Morriston, FL, USA). Specific number of used microcapsules depended on trunk diameter. Usually 1 capsule was used per 15 cm of trunk girth (a measurement of the distance around the trunk of a tree measured perpendicular to the axis of the trunk). In this study no average girth of tree subjects was presented. Capsules were mounted on drilled injection ports (3 mm in diameter) on apple trunk and pressurized to 10 psi for injection. Next visual indexing scale was used as the method for apple scab rating: 0 = no scab observed; 1 = less than 5% of leaves

affected; 2 = 5-20% of leaves affected with some yellowing but no defoliation; 3 = 20-50% of leaves affected, significant defoliation, leaf yellowing; 4 = 50-80% of leaves affected, defoliation, severe yellowing; 5 = 80-100% of foliage affected with 90–100% defoliation (Percival & Boyle 2005). Due to the 30% severity range of the visual severity scale, gained significant apple scab severity control, based on statistical analysis of the indices, is believed not to be accurate enough to provide good insight in true disease impact. This is especially true for apple scab on fruit and authors acknowledge this by stating that even though 7 tested fungicides significantly reduced scab levels on fruit by 23-50%, total scab control was not achieved (Percival & Boyle 2005). Thus, fungicides which gave significant control were probably with considerably high leaf and fruit disease incidences which were very likely much higher than the acceptable commercial standards in apple scab control.

Older research references report that fungicide thiabendazole injected in apple trees of cv. 'Golden delicious', reached the top of the crown and continued accumulating after 42 days providing fungitoxic levels of 18-132 μg/g of fresh weight of the twigs and leaves (Pinkas et al. 1973). Pear trunk injections with thiabendazol and methyl 2-benzimidazole-carbamate, showed that after secondary uptake distribution of both fungicides gave an effect on pear scab *V. pirina* (Pinkas et al. 1973; Shabi et al. 1974). However, no data on disease control are shown since disease pressure was low due to dry spring and weak symptoms only showed in non-injected control trees while no scab was detected on any of the injected trees.

In general, research on pesticide trunk injection for control of apple scab received little attention since not so excellent results have been gained (Percival & Boyle 2005). However, the authors of these studies pointed on possible reasons why poor effects were gained, and among the first ones they discuss pH of the injected solution, solution pH change after injection, and the

water solubility (Pinkas et al. 1973; Shabi et al. 1974; Percival & Boyle 2005). For example, low water solubility of myclobutanil formulated for topical spray application in oil in water emulsion, was most likely the reason for the recorded failure in apple scab control (Percival & Boyle 2005). Further, the authors state that either no control or significant control of apple scab indicated on no translocation or good translocation of the injected fungicides into the apple crown.

Commercial standards in control of fire blight and apple scab with protective compounds

Fire blight (Erwinia amylovora)

Timely and successful control of blossom blight using antibiotic sprays with streptomycin (Agrimycin) and kasugamycin (Kasumin) in commercial apple orchards should usually yield disease incidences between 0.2-3.5% and 1.5-5.6%, respectively (Sundin et al. 2009; McGhee & Sundin 2011). This level of control can be regarded as commercially acceptable fire blight control. Topical treatments with oxytetracycline (Mycoshield®, Nufarm Limited, Melbourne, Australia) showed relatively unsatisfactory control of blossom blight of 29-37% when *E. amylovora* is sensitive to both strepromycin and oxytetracycline (McManus & Jones 1994). This antibiotic showed to be only partially effective (Norelli et al. 2003). Streptomycin kills *E. amylovora*, while oxytetracycline is only bacteriostatic, and when the levels of oxytetracycline decline on plant surfaces since it has much shorter half-life than streptomycin, bacterial populations build-up again (Johnson & Stockwell 1998; McManus et al. 2002). If applications of oxytetracycline are well-timed during bloom blossom blight control can

be done successfully (McManus et al. 2002). Another study showed that oxytetracycline at two bloom sprays reduced the fire blight incidence by an average of 42% compared to water treated control (Stockwell et al. 2007).

If fire blight control with antibiotics is conducted successfully, secondary fire blight infections of shoots should be controlled as well i.e. should not be taking place. However, if the primary inoculum levels in cankers are abundant, or the sprays with bactericides did not provide full coverage of blossoms, thus allowing oases of blossom blight on trees and build-up of secondary inoculum, additional bactericide spray(s) on apple are needed in petal fall so that the intensively growing shoots can be protected. This scenario in disease development is especially possible at the onset of favorable weather conditions for fire blight such as warm, rainy and humid days, and when the critical stage of bloom has not been protected from the infections. To our knowledge, no published references exist on evaluation of trunk injection of antibiotics for fire blight control on apple trees.

Preventive sprays during dormancy and at the silver- or green-tip with copper compounds have purpose to reduce primary inoculum of *E. amylovora* at the beginning of the season (Van Der Zwet & Beer 1995; Koski & Jacobi 2009). Overall, control level when copper products are applied during dormancy or pre-bloom is low, but significant as part of a control program (Smith 2012). Often, treatments with copper based products at 0.2-0.5%, depending on product a. i., conducted in fall and at the beginning of bud break help in prevention of infections (Balaž et al. 2013).

In control of blossom blight on apple cv. 'Idared' on M7 rootstock with copper compounds such as copper sulphate (Phyton® 27AG, copper sulphate pentahydrate 21.27-21.36%, Phyton Corp., New Hope, MN) and copper hydroxide (Mankocide®, copper hydroxide

46.1% + mancozeb 15%, DuPont Chemical industry Co., Wilmington, DE) there was a significant reduction of blossom blight incidence to only 33.1 and 39.2%, respectively, in comparison to water treated control with incidence of 61.4% (Aldwinckle et al. 2002). In this study Phyton an Mankocide were applied at rates of 2 x 128.2 g/50L and 2 x 79.7 g/50L, respectively, with one treatment one day before and the other treatment one day after the inoculation (1×10^7 CFU/ml). Shoot blight incidence in the same study was reduced to 43.4 and 64.3%, respectively, in comparison to water treated control which had incidence of 87.3%.

Other sources report that when copper products are applied multiple times to open flowers, blossom blight reduction varies from 20 to 60% compared to a non-treated inoculated control (Smith 2012). Besides, poor control of fire blight during vegetation, negative effect of copper formulations was fruit russeting which was not significant for copper sulphate pentahydrate. Due to this and other phytotoxicity effects on leaves, copper products are generally not used for control of fire blight during vegetation. However, new formulations of copper such as Cu-peptidate, Cu-EDTA chelate, Cu-amino complexes and Cu-gluconate, with reduced negative side-effects on flowers, leaves and fruit are being investigated and show some promising results in aiding fire blight control (Lešnik et al. 2010; 2011; Smith 2012; Balaž et al. 2013).

Apple scab (Venturia inaequalis)

Humid continental climate in Michigan and other parts of the world sets the apple industry in highly favorable conditions for apple scab infections every season (Cuthbertson & Murchie 2003; Jamar et al. 2007; Jamar 2011). The demand for higher yields and blemish-free

fruit require expensive apple scab protection every year. Depending on precipitation during the year, apple scab control can require 7-25 topical spray treatments of fungicides every 7-14 days (Van der Scheer 1992; Jones & Sutton 1996; Ivanović & Ivanović 2001; Berrie & Xu 2003; Cuthbertson & Murchie 2003; Holb et al. 2003; Percival & Boyle 2005; Ehret et al. 2010).

In brief, the main classes of fungicides used in apple scab control are inorganic fungicides (copper, sulphur), thio-phthalimides (captan, folpet), ethylene-bis-dithiocarbamates (mancozeb, maneb, metiram), dimethyl-dithiocarbamates (ziram, ferbam), anilino-pyrimidines (cyprodinil, pyrimethanil), strobilurins (kresoxim-methyl, trifloxystrobin, azoxystrobin), triazoles (difenoconazole, fenbuconazole), guanidines (dodine), pyridinyl-ethyl-benzamides (penthiopyrad), pyramides (fluopyram), thiophanates (thiophanate-methyl), phosphites (mono- and dibasic salts of phosphorous acid) (Wise et al. 2013).

Depending on the used combination of fungicides with different modes of action, the level of apple scab incidence which can be regarded as commercially acceptable in control in apple orchards is between 0-7% for fruits, 0-3% for spur leaves and 0-8% and 12-17% for shoot leaves, early and late in the summer, respectively (Ehret et al. 2010). Season-long control of apple scab can be achieved when multiple spray applications of newer fungicides, and usually up to three or five times, are interspersed with the applications of classical contact fungicides. For example, newer fungicide combinations such as Luna® Sensation (21.4% fluopyram + 21.4% trifloxystrobin, Bayer Crop Science, Research Triangle Park, NC) and Luna® Tranquility (11.3% fluopyram+33.8% pyrimethanil, Bayer Crop Science, Research Triangle Park, NC) showed to be excellent addition for apple scab control programs when used 3-5 times per season, alone or one after another (Ehret et al. 2010).

CHAPTER 2. SPATIAL AND TEMPORAL DISTRIBUTION OF TRUNK-INJECTED IMIDACLOPRID IN APPLE TREE CANOPY

Abstract

Pesticide use in orchards creates drift-driven pesticide losses which contaminate the environment. Trunk injection of pesticides as a target-precise delivery system could severely reduce pesticide losses. However, pesticide efficiency after trunk injection is associated with their underinvestigated spatial and temporal distribution within the tree crown. This study quantified spatial and temporal distribution of trunk-injected imidacloprid within apple crowns after trunk injection using 1, 2, 4, or 8 injection ports per tree.

Spatial uniformity of imidacloprid distribution in apple crowns significantly increased with more injection ports. Four ports allowed uniform spatial distribution of imidacloprid in the crown. Uniform and non-uniform spatial distributions expressed early and lasted throughout the experiment. Temporal distribution of imidacloprid was significantly non-uniform. Upper and lower crown positions did not significantly differ in compound concentration. Crown concentration patterns indicated that imidacloprid transport in the trunk occurs through radial diffusion and vertical uptake with a spiral pattern.

By showing where and when trunk-injected compound distributes in apple tree canopy this study addresses the key knowledge gap for explaining the compound efficiency in the crown. These findings allow the improvement of target-precise pesticide delivery for more sustainable tree-based agriculture.

Introduction

During the past decades, use of pesticides in tree fruit agriculture has undergone broad changes in pest management approaches and technologies employed to provide environmentally safer, more sustainable and efficient plant protection (Sutton 1996). Alternative approaches such as Integrated Pest Management (IPM), organic and sustainable agriculture have developed. Legislative restrictions by US Environmental Protection Agency (EPA) and European law on Registration, Evaluation and Authorization of Chemicals (REACH) have led to the elimination, restriction or registration limitations for many pesticides (US EPA 1996; Düker et al. 2006) Further, more restrictive standards regarding environmental impacts, worker exposure and dietary risks for use of pesticides have been implemented (Mota-Sanchez et al. 2012).

Along with these changes, new protective compounds, such as US EPA designated Reduced Risk and OP Alternative pesticides received expedited registration for agriculture use. Among them, a class of neonicotinoid insecticides was introduced with expectations of minimal impact on non-target organisms, human health and contamination of the environment (US EPA 1997; Tomizawa & Casida 2005; Yu 2008). Neonicotinoids imidacloprid, clothianidin and thiamethoxam are also systemic acquired resistance (SAR) inducers, aiding control of fungal and bacterial plant pathogens (Francis et al. 2009; Ford et al. 2010; Graham & Myers 2011).

Regardless of implementation of new chemistries and alternative protection approaches, the key obstacle for increasing sustainability of fruit production is inability of current technologies to eliminate or significantly reduce off-target drift-driven pesticide losses to the environment (Steiner 1969; Pimentel et al. 1992; Pimentel & Lehman 1993; Pimentel 1995; Perry 1998; Zhu et al. 2006). Both the frequency of application and topical pesticide delivery are

the key components to this problem. Human and environmental impacts of conventional pesticide application in commercial fruit production leads to numerous long term negative side effects due to pesticide drift losses (Pimentel & Levitan 1986; Pimentel et al. 1992; Pimentel 1995; Perry 1998; Ecobichon 1999; Düker et al. 2006).

One novel approach in tree-based agriculture that could increase sustainability of fruit production through target-precise and efficient pest control is delivery of plant protective compounds into the tree trunk using direct injection. By harnessing the xylem transport capacity of a tree, compounds are translocated into the canopy and distributed throughout the crown where they provide protection against pests (Percival & Boyle 2005; Düker & Kubiak 2011a). Trunk injection for plant protection and nutrition is widely used in landscape tree care because it is often the most effective method of pest control and because large tree sizes, proximity of urban areas, and extensive drift-driven losses, do not allow ground sprayer treatments or aerial application (Guillot & Bory 1997; Hillebrand et al. 1998; Düker et al. 2006). This approach is valuable in control of invasive pest and pathogen species of quarantine importance, such as *Agrilus planipennis* Fairmaire and *Phytophthora ramorum* Werres et al., which endanger native and specimen tree species in collection gardens and arboretums (Tanis et al. 2012; Defra 2010; Wedgwood et al. 2012). However, the pest control and dietary risk standards in commercial fruit tree production are much higher than in landscape tree care, and the ability of trunk injected pesticides to protect tree fruits from damaging pests is not well investigated.

The effectiveness of trunk-injected protective compounds in tree canopies depends on many factors such as tree physiology, environmental conditions, compound properties, and nature of pest or pathogen. The major biological factors are tree species and size, xylem type, health, grafting rootstock, daily water consumption, time of pest occurrence and infestation

pressure, time and frequency of injection, and the vertical position and number of trunk-injection delivery ports (Tattar et al. 1998; Sánchez-Zamora & Fernández-Escobar 2000; 2004; Young 2002; J.J. Doccola et al. 2007; Doccola & Wild 2012). Major factors pertaining to the injected compound include water solubility of active ingredient, carbon adsorption coefficient, compound formulation type, volume or dose per tree, and concentration and uniformity of distribution within the tree canopy (Cox et al. 1997; Doccola et al. 2003; J.J. Doccola et al. 2007; Doccola et al. 2012; Byrne et al. 2012).

The two crucial factors hypothesized to severely influence the efficiency of trunk-injected insecticides in the canopy of urban trees are the spatial and temporal uniformity of compound distribution within the crown (Cowles et al. 2006; J.J. Doccola et al. 2007; Mota-Sanchez et al. 2009; Dilling et al. 2010; Tanis et al. 2012). Several studies on the efficiency of trunk-injected pesticides in the canopy of different fruit tree species, indirectly associated pest control results with different properties of translocation and spatial compound distribution in the tree crown (Pinkas et al. 1973; Shabi et al. 1974; Clifford et al. 1977; Pilbeam 2003; Percival & Boyle 2005; Spitko 2008; Ahmed et al. 2010; Byrne et al. 2012). A few of these studies show only temporal distribution of injected compounds in the fruit tree canopy (Schutte et al. 1988; Tattar et al. 1998). Little is known about the distribution, accumulation and persistence of injected compounds in trees and how the efficacy varies depending on these factors (Mota-Sanchez et al. 2009). Investigation of compound distribution after trunk injection would serve as basis for establishing treatment rates, injection port density and timing, and determining risks for fruit consumers and non-target organisms (Eisenback 2008). How, where and when trunk-injected compounds distribute in the tree canopy are key missing components in explaining compound efficiency in the crown.

Using imidacloprid as a model compound, the first objective of this study was to quantify the spatial and temporal distribution of trunk-injected imidacloprid in the crown of apple trees. The second objective was to determine the spatial and temporal distribution of imidacloprid in apple tree crown after trunk injections of a unique dose through a variable number of injection delivery ports. It was hypothesized that increasing the number of trunk injection delivery ports would provide spatially more uniform distribution of imidacloprid in the apple tree canopy. It was further hypothesized that spatial uniformity after delivery through more injection ports would require longer time to express in the canopy than non-uniform distribution after delivery through fewer injection ports. Using the results, the ultimate goal of this study was to provide insights into the key processes and mechanisms behind the vascular transport of trunk-injected compounds in diffuse-porous xylem of apples.

Materials and methods

Chemical materials

Orchard experiments were conducted during 2011 at Michigan State University's Trevor Nichols Research Center in Fennville, MI (42º 36' 0.94" N, 86º 9' 12.02" W) in continental climate with an average 84 mm and 520 h of rainfall in the last 5 years (www.enviroweather.msu.edu). In 2011, total average rainfall at this location was 85.7 mm with 590 h of rainfall. Mature, 29 year old trees of apple cultivar 'Mac Spur', *Malus domestica* Borkh., were trunk-injected with imidacloprid using a unique dose of 1 g of active ingredient (a. i.) per tree (20 ml of IMA-jet^{TM}, 5% imidacloprid, Arborjet Inc., Woburn, MA) across all

treatments. This dose corresponds to the maximum allowed seasonal rate of imidacloprid used as foliar spray per 0.405 ha with 250 planted apple trees, according to the EPA registration label in USA. The four different treatments imposed on 15 June included delivery through 1, 2, 4, and 8 injection ports per tree, equally spaced along trunk circumference, and positioned on a trunk according to cardinal and intermediate directions (Figure 2). The total dose of 20 ml of IMA-jet was equally divided among the injection ports; i.e. 20 ml of IMA-jet (1 g a. i.) was injected into 1 injection port, 10 ml of IMA-jet (0.5 g a. i.) was injected into two ports, 5 ml of IMA-jet (0.25 g a. i.) was injected into 4 injection ports, and 2.5 ml of IMA-jet (0.125 g a. i.) was injected into 8 injection ports.

Trunk injection

Trunk injections were performed by drilling injection ports (25.4 mm deep × 9.5 mm diameter) into the trunk xylem tissue. Ports were sealed at bark level with Arborplugs® no. 4 (Arborjet Inc., Woburn, MA). Injection of treatment and port-specific compound volumes was performed with Quik-jet® micro-injection system (Arborjet Inc., Woburn, MA) inserted through the one-way valve septum on Arborplugs, into the freshly drilled xylem port. All trunk injection ports were oriented based on cardinal and intermediate directions. For the 1-port injection, trees were injected on the south trunk direction. For the 2-port injection, trees were injected on the north and south direction. For the 4-port injection, injections were oriented in all four cardinal directions, and for the eight port injection, injections were oriented in all eight cardinal and intermediate directions. Ports were positioned approximately 30 cm above the ground surface, and vertically separated by approximately 5 cm between opposing port pairs. At this height

worker access to the trunk is the easiest during injection while still allowing considerable trunk length above the ports for achieving good radial dissipation of the compound in xylem, before reaching the branching points. To reduce the experimental unit variability, before injection apple trees were selected based on uniform canopy (approximately 3.7 m high and 3.5 m wide) and the trunk diameter at 30 cm height (diameter means with standard errors for 1, 2, 4, and 8 port injections were 28.5±0.89 cm, 28.1±0.69, 28.7±0.64 and 28.8±0.73). The experiment was arranged in a completely randomized design (CRD) with 6 replicate trees per treatment.

Leaf sampling and imidacloprid residue profile analysis

In sampling of spatial distribution treatments each apple tree crown was first divided vertically into upper and lower crown positions, and then horizontally by cardinal directions into four quadrants: north, south, east, and west. Thus a total of 8 quadrants were identified, i.e. upper quadrants: NU, SU, EU, WU, and lower quadrants: NL, SL, EL, and WL (Figure 1). Quadrant was used as subject of repeated measurements through time.

Composite leaf samples were taken from each of 8 crown quadrants, within a tree replicate, and each sample consisted of 40 leaves collected throughout the quadrant. Samples were collected three times, at 14, 28, and 42 days after injection (DAI).

Leaf samples were stored in 120 ml glass jars (Qorpak, Division of Berlin Packaging, Bridgeville, PA) with 90 ml per sample of HPLC-grade dichloromethane (DCM, EMD Chemicals Inc., Gibbstown, NJ), and held at 5ºC. To determine imidacloprid residues in the leaves, after adding 1-2 g of sodium chloride crystal (J.T. Baker Chemical Co., Phillipsburg, NJ), each sample was ground in DCM with PTFE pestle for Potter-Elvehjem tissue homogenizer

(Wheaton Science Products, Millville, NJ) and DCM was decanted through 50 g of reagent-grade anhydrous sodium sulfate (EMD Chemicals Inc., Gibbstown, NJ) to remove water. Each sample extract was separated from DCM and dried at 40ºC using rotary evaporator R-114 (Büchi Labortechnik AG, Flawil, Switzerland). Dry extract residue was dissolved in 2 ml of HPLC-grade acetonitrile (EMD Chemicals Inc., Gibbstown, NJ). Dissolved sample residue was collected and passed through HPLC certified 0.45 µm Acrodisc CR 25 mm syringe filter with PTFE membrane (Pall Corp., East Hills, NY) to remove any remaining particulates, and stored at 5ºC in 2 ml HPLC glass vials (Agilent Technologies Inc., Santa Clara, CA) until HPLC analysis. According to previously reported method (Bayer 1998; Wise et al. 2006), samples were analyzed for imidacloprid residue (parent compound) with a Waters 2695 Separator Module HPLC equipped with Waters MicroMass ZQ mass spectrometer detector (Waters, Milford, MA), and Waters X-Bridge C18 reversed phase column (50 × 3.0 mm bore, 3.5 µm particle size, Waters Corp., Milford, MA). The mobile phase, solvent A, was water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid, and was initially held at 80% solvent A and 20% solvent B and followed by a gradient shown in Table 2. Column temperature was 40ºC. Monitored ions for imidacloprid were 175, 209, and 255.9 m/z (Da). The HPLC level of quantification was 0.05 mg kg $^{-1}$ of a. i., and level of detection was 0.01 mg kg $^{-1}$. By using above described extraction method, mean parent compound recovery from four apple leaf samples (each 100 g) treated only with standard imidacloprid solution (0.046 mg kg $^{-1}$), then agitated and left to dry, was 73% (level of detection 0.009 mg kg $^{-1}$). The results have not been corrected for imidacloprid recovery.

Statistical analysis

Imidacloprid residue data from crown quadrant leaf samples were analyzed using mixed model and executed using GLIMMIX procedure in SAS 9.3 (SAS Institute, 2011). Spatial and temporal distribution residue data were log transformed prior to statistical analysis. Temporal distribution of imidacloprid was analyzed with repeated measures best adjusted using heterogeneous autoregressive covariance structure of 1st order. When the main factor effects or their interactions were found to be statistically significant (p<0.05), slicing i.e. examination of interactions within main effects was performed, tested with F-tests, and pairwise or specific time or treatment comparisons were conducted using t-tests (α=0.05).

Imidacloprid residue concentration patterns

Spatial distribution of imidacloprid in apple leaf canopy was shown on the level of crown quadrant in figure 2 (A-C), while distribution on the level of cardinal direction of the crown was shown in figure 3 (A-D). Lower-case mean separation letters represent statistical differences between the cardinal directions of the crown within one of the four different injections and within one time point. Upper-case mean separation letters represent statistical differences of the same cardinal direction of the crown between the four different injections and within one time point (Figure 3).

Results

The number of trunk injection ports, time as days after injection (DAI), and crown orientation, all affected imidacloprid concentration in leaves of the injected apple trees (Figure 2, 3; F=3.35, $p\leq$0.0201; F=68.07, $p\leq$0.0001; and F=8.57, $p\leq$0.0001). Crown height (i.e., upper vs. lower crown position) did not affect leaf imidacloprid concentration (F=3.62, $p\geq$0.0588) and interactions of crown height and other effects were not significant ($p\geq$0.4318). Therefore crown positions within one cardinal direction of the crown were combined and averaged to simplify data analysis and presentation (Figure 3). Strong interactions between all effects of number of injection ports, time after injection and crown orientation (F=1.88, $p\geq$0.0169; F=2.85, $p\geq$0.0101; F=3.55, $p\leq$0.0004), were examined by comparisons within each of the effects. Simple effect comparisons are shown in figure 3.

Spatial distribution of imidacloprid

Consistently during the experiment 1 and 2-port injections showed spatially non-uniform imidacloprid distribution in the crown while 4 and 8-injections showed spatially uniform compound distribution (Figure 2A-C, 3A-D, lower-case letters). The level of uniformity in spatial distribution of the compound rose with the increase of the number of trunk injection ports. However, there was no improvement in uniformity of spatial distribution in 8-port injection versus the 4-port injection showing that there is a point of diminishing returns on the number of injection ports (Figure 3A-D). Non-uniform spatial distribution in 1 and 2-port injections reached its strongest expression at 28 DAI, since there was a higher incidence of significant differences

between cardinal crown directions than at 14 DAI (Figure 3A, B, 2B). These findings are further confirmed when cardinal directions of the crown are compared between all four injections within one time point (Figure 3A-D, upper-case letters).

Temporal distribution of imidacloprid

Significant differences in comparison of the same cardinal direction of the crown between 14, 28, and 42 DAI, within one injection treatment, showed that temporal distribution of imidacloprid in apple leaf canopy was overall non-uniform (Figure 3A-D, asterisk and dagger symbols). In all four injections at 28 DAI, imidacloprid concentration increased significantly in at least one cardinal direction of the crown when compared to 14 DAI (Figure 3A-D, 2B) and was the highest recorded during the study. At 42 DAI, imidacloprid concentration decreased significantly in at least one of the cardinal directions in all the injection treatments (Figure 3A-D, 2C). With the exception of one crown direction, concentration patterns at 42 DAI overall showed that in 1 and 2-port injections compound levels dropped to statistically similar levels detected at 14 DAI (Figure 3A, B). A similar trend was detected in 4 and 8-port injections but with more crown directions as exceptions (Figure 3C, D).

The significant changes in concentration showed varying levels of non-uniformity in temporal distribution of imidacloprid. In 4 and 8-port injections temporal distribution was the most non-uniform since concentrations significantly increased in all cardinal directions of the crown at 28 DAI (Figure3C, D). In 1-port injection non-uniform temporal distribution was less pronounced since at 28 DAI concentration significantly increased only in two cardinal directions of the crown (Figure3A). The lowest level of non-uniform temporal distribution of imidacloprid

was in 2-port injection since concentration significantly increased in only one cardinal direction of the crown (Figure3B).

Temporal distribution was heavily coupled with dose splitting and the number of injection ports. With more injection ports used per tree and with the lower dose delivered per injection port, imidacloprid distributed to more cardinal directions of the crown where concentration significantly changed in time. With fewer injection ports and with the higher dose delivered per injection port imidacloprid distributed to fewer cardinal directions of the crown where concentration significantly changed in time.

Imidacloprid residue concentration patterns

The patterns of spatial distribution of imidacloprid after 1 and 2-port injections (Figure 2A, B) showed concentration gradients in the crown which indicated that apple trees have a helical transport of this compound in the wood xylem. Namely, the lower crown quadrants of 1-port injection at 14 DAI showed a partial concentration gradient rising from west, to south, to east (Figure2A). The upper crown quadrants showed a partial concentration gradient declining from east, to north, to west (Figure2A). These gradients, with no imidacloprid detected in lower north quadrant (Figure2A) and its detection in the upper north quadrant, indicated that the helix of vertical imidacloprid transport in apple trees has the display of counterclockwise spiral. This transport led to high concentration accumulation in all four lower and upper south and north crown quadrants in the 2-port injection at 14 DAI (Figure 2A). With 4 and 8-port injections this shows that as more injection ports are added and helical transport pathways occur from them,

there is overlapping of these pathways and their concentrations in the upper crown quadrants, thus resulting in a wider breath of spatial distribution in the canopy.

Implications of imidacloprid distribution in apple leaf canopy

In the case of spatially non-uniform imidacloprid distribution provided by 1 and 2-port injections, low or no insect control is to be expected in directions of apple crown weakly or not supplied by imidacloprid. Further, in south and north crown directions, oversupplied with imidacloprid in these injections (28 and 42 DAI), the excess represents unneeded amount of injected compound to provide required control mortality. Quite opposite, at 28 DAI, highly efficient insect control throughout the crown is to be expected in 4-port and 8-port injections with spatially uniform compound distribution in the crown.

For sucking tree pests such as aphids (*Myzus*, *Aphis*), LC_{95} for imidacloprid is around 0.15 ppm (Elbert et al. 1991). In this case due to much higher imidacloprid concentrations than LC_{95}, efficient aphid control in trunk-injected apple trees should be achieved in all the injections except in the north crown direction of 1-port injection at 14 and 28 DAI (Figure2A, B). However, for different pest species lethal doses are different and the injected product should not be delivered variably throughout the crown, at too low or too excessive doses, thus not providing control or allowing compound waste on the same tree, respectively. Efficient pest control depends on delivered compound dose in space and temporal persistence in its activity.

Table 2. The gradient mobile phase flow used for imidacloprid HPLC/MS residue analysis.

Time (min.)	Flow rate (µ/min.)	Solvent A(%)	Solvent B(%)
	0.30	80	20
1.00	0.30	80	20
4.00	0.30	40	60
4.10	0.30	80	20
8.00	0.30	80	20

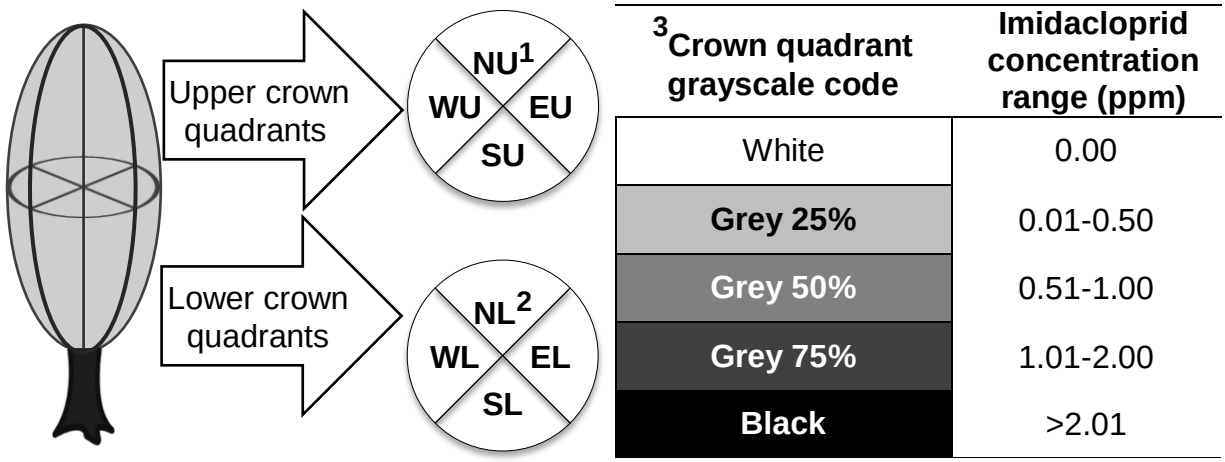


Figure 1. Horizontal to vertical representation of eight apple tree crown quadrants repeatedly sampled through time in spatial distribution treatments with trunk-injected imidacloprid (left). [1]NU: north upper, WU: west upper, SU: south upper, EU: east upper; [2]NL: north lower, WL: west lower, SL: south lower, EL: east lower. [3]Grayscale code used for depicting different imidacloprid concentration ranges in the crown quadrants [ppm] (right).

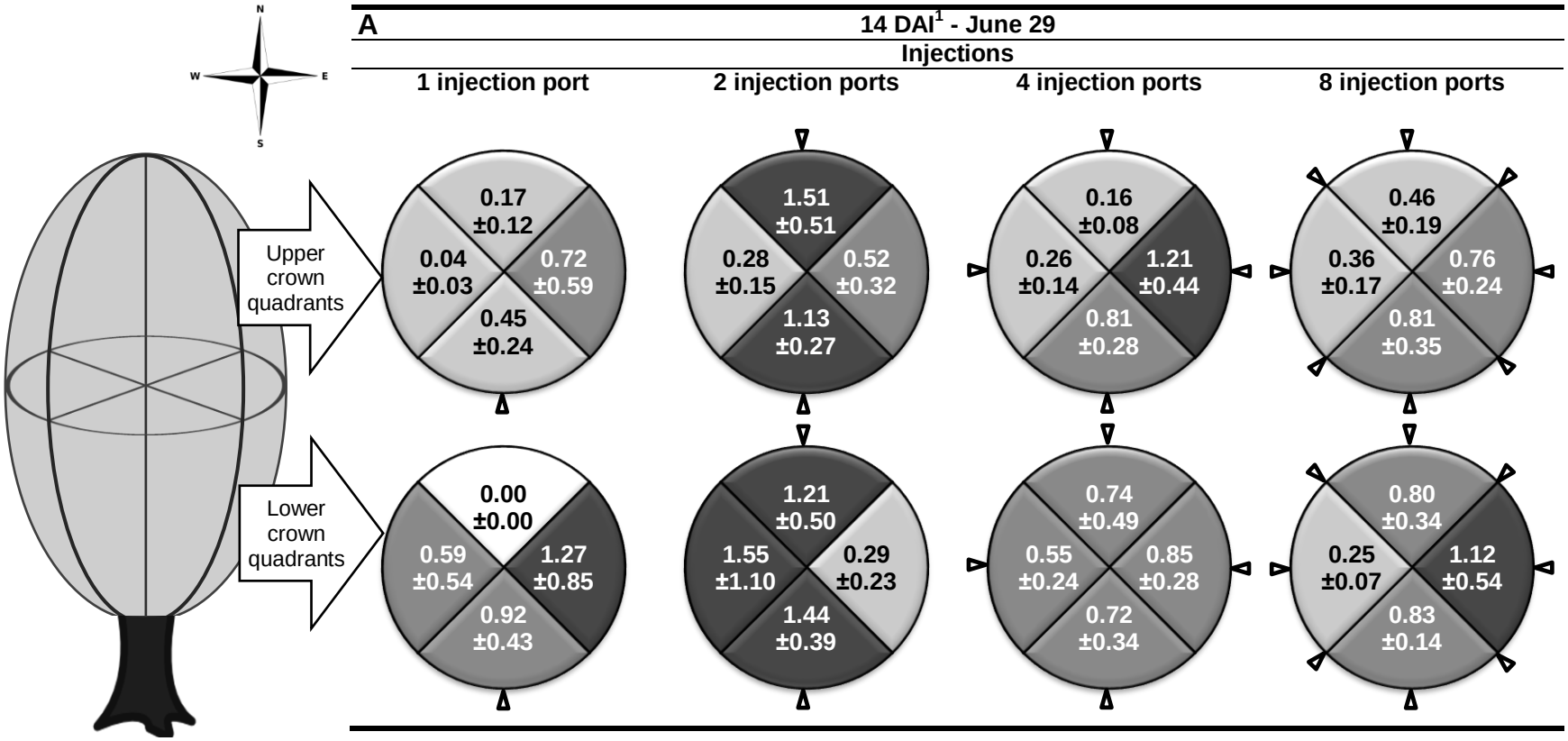
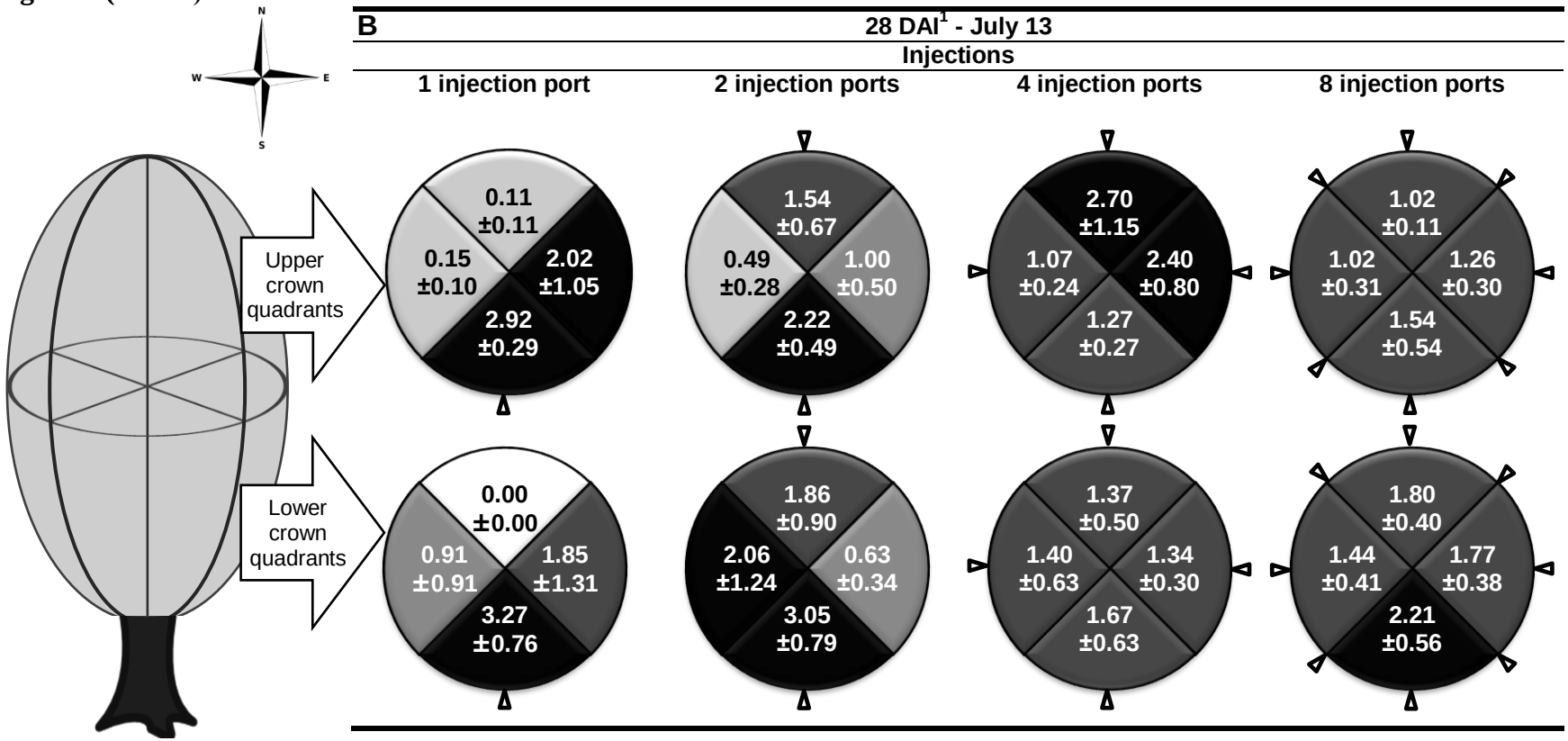


Figure 2. Spatial distribution of trunk-injected imidacloprid (1 g a. i. per tree) at 14 (A), 28 (B) and 42 (C) days after injection ([1]DAI), in apple leaf canopy divided vertically into upper and lower crown hemispheres and then into crown quadrants according to cardinal directions. Distribution resulted from delivery through 1, 2, 4 and 8 injection ports. Triangles on periphery of the crown quadrants depict the cardinal and intermediate positions of injection ports on the trunk below. Darker quadrants depict higher imidacloprid concentration [mg kg $^{-1}$]. Mean concentrations of quadrants are followed by standard error of the mean (±SEM). Main effect of vertical crown position, with lower and upper crown hemispheres, was not significant ($p \geq 0.0588$).

Figure 2 (cont'd)



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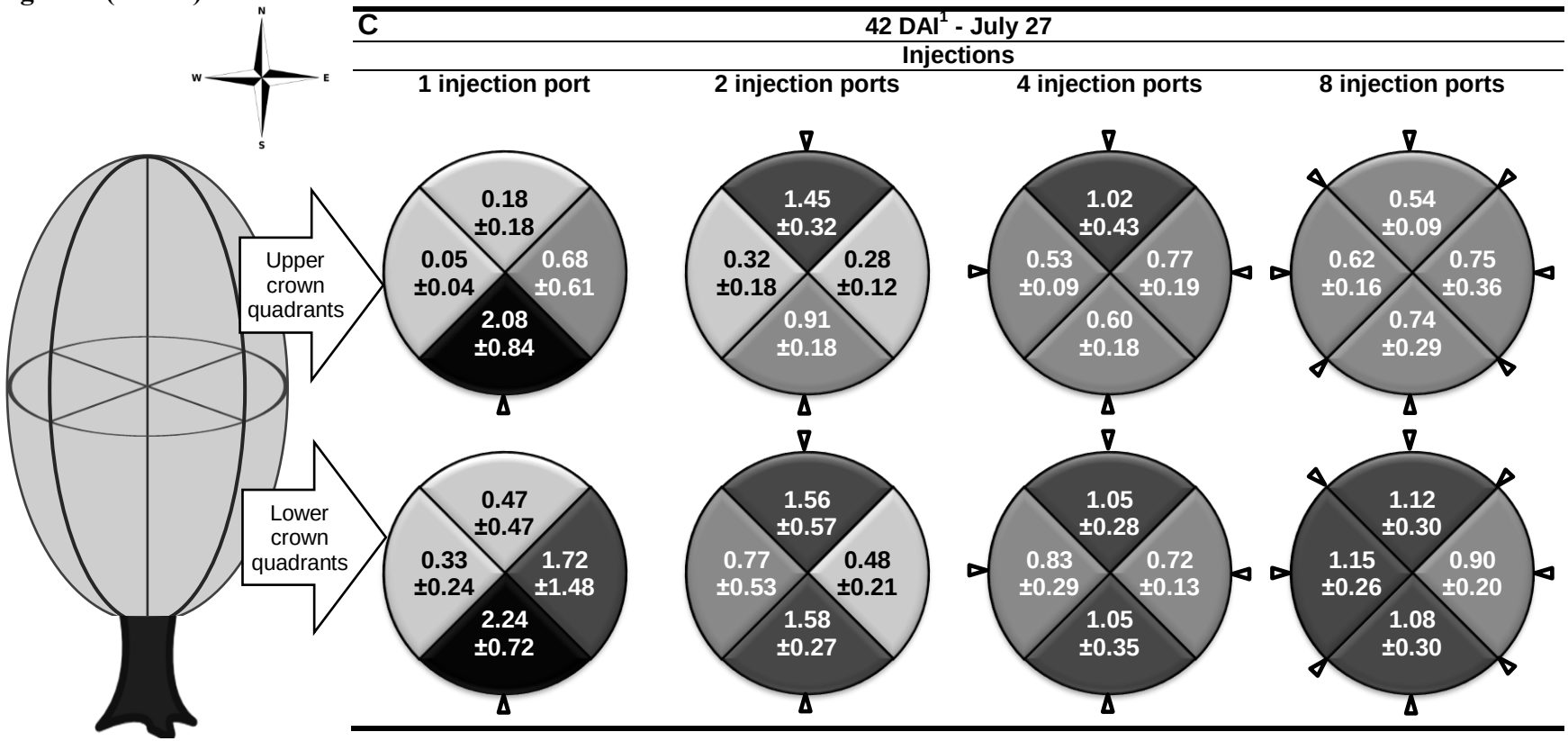


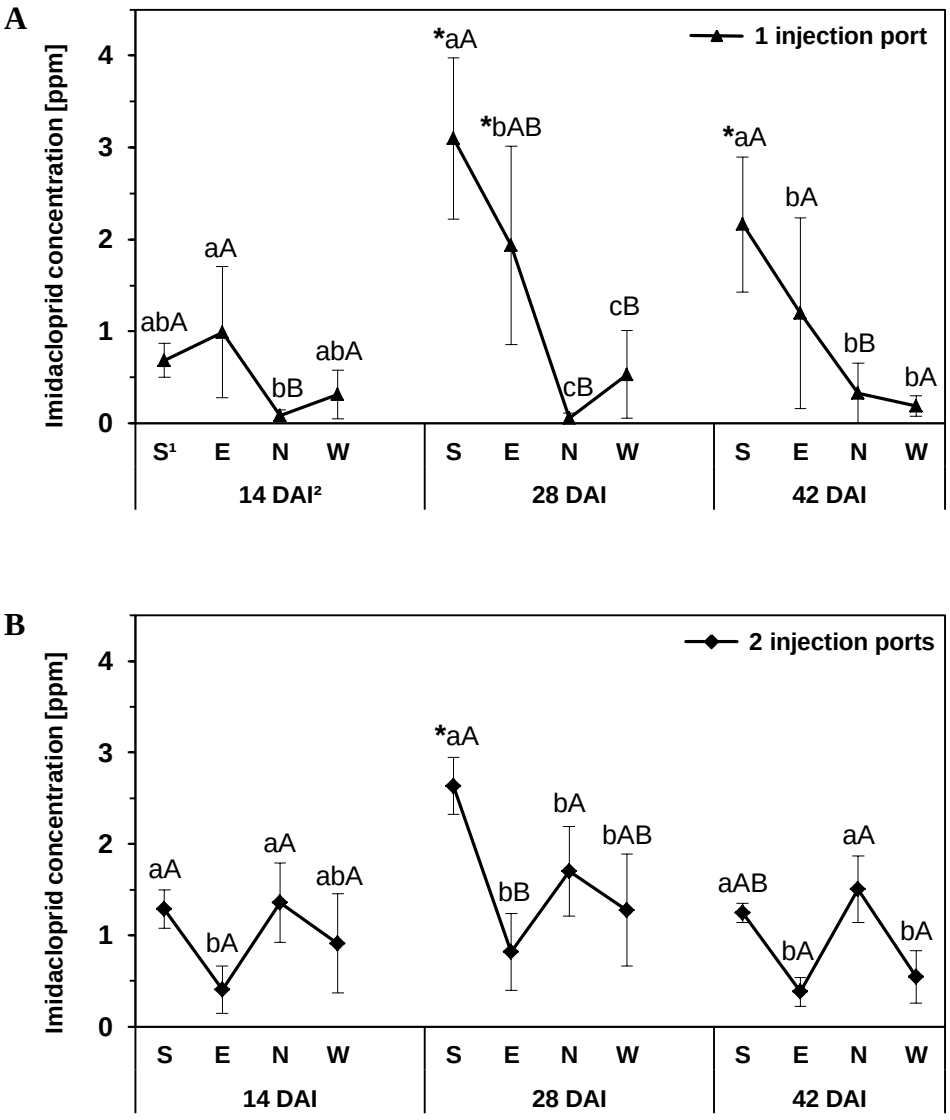
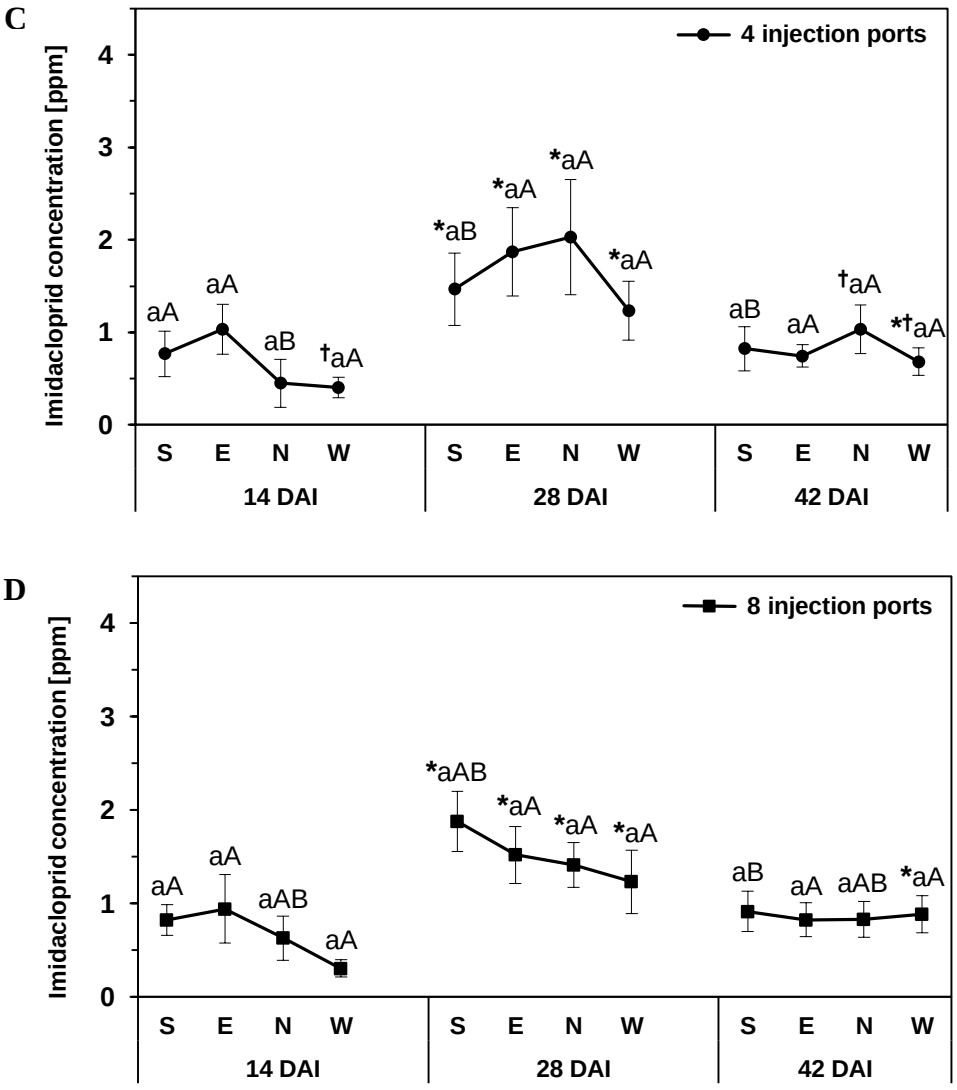


Figure 3. Temporal and spatial distribution of imidacloprid (1 g a. i. per tree) in apple leaf canopy divided in cardinal directions after delivery with 1 (A), 2 (B), 4 (C) and 8 (D) injection ports. [1]Cardinal directions: S, E, N, W - south, east, north, west. Mean concentration of one cardinal direction within one injection treatment followed by an asterisk or a dagger is significantly different between the time points ([2]DAI - days after injection). Graph line depicts spatial distribution of imidacloprid. [3]Means followed by different lower-case letters within one time point and one injection treatment are significantly different. [4]Means of one cardinal direction followed by different upper-case letters, between injection treatments and within one time point are significantly different (*t*-tests, $p<0.05$). Quadrant concentrations in each cardinal direction (Figure 2A-C) are averaged. Comparisons are based on log transformed data (means shown untransformed). Error bars represent standard error of the mean (SEM).

Figure 3 (cont'd)



Discussion

The results of this study provide important insights into the spatial and temporal transport and distribution of trunk injected compounds in apple trees. Patterns of imidacloprid residue concentration in the crowns of apple trees following trunk injection are influenced by several key factors, including the number and position of injection ports, time after injection, xylem anatomy and chemical properties of the compound.

The influence of number of injection ports on imidacloprid distribution in apple canopy

Our findings prove that increasing the number of trunk injection ports provides more uniform spatial distribution of imidacloprid in apple tree canopy. Four injection ports were sufficient to provide uniform distribution and more ports did not improve spatial uniformity. Unexpectedly, spatial distribution showed similar accumulation of imidacloprid in the upper and lower crown positions. Further, treatment specific uniform and non-uniform distributions established early and lasted during the experiment. This disproved the hypothesis that spatial uniformity achieved through more injection ports would require longer time to express in the canopy than non-uniform distribution achieved through fewer injection ports.

Non-uniform distribution of trunk-injected imidacloprid throughout the crown has been previously hypothesized as the main cause of low efficiency in pest and disease control (Percival & Boyle 2005; J.J. Doccola et al. 2007). Multiple injection delivery ports are required for uniform systemic distribution of trunk-injected dyes in the canopy and especially for tree species with highly sectored hydraulic pathways in the xylem (Larson et al. 1994). In arboriculture,

number of trunk injection delivery ports is directly related to the product volume or recommended dose per tree and frequency of seasonal application. However, correlation between the number of delivery ports and distribution of the injected compound in the canopy is indirectly determined through insect control efficiency after trunk injection of insecticides (J.J. Doccola et al. 2007). Our study is the first to show how distribution of a compound varies in apple canopy depending on number of injection ports.

The influence of time on distribution of imidacloprid in apple tree canopy

Our data show that injected imidacloprid has non-uniform temporal distribution in apple canopy. The temporal patterns of increase in concentration suggest that some imidacloprid undergoes more immediate translocation in the xylem, while the remainder is diffused radially in surrounding woody tissue and slowly released over time for transport in xylem. The slow increase in concentration can be attributed to the reservoir effect in the trunk governed by the physical and chemical interactions of imidacloprid with the cell walls of tracheids and vessels or with the xylem parenchyma of living symplast (Doccola et al. 2012; Tanis et al. 2012).

Following peak residue levels in the canopy, a decrease in imidacloprid concentration over time is likely a result of several contributing factors. The first and previously reported main cause of imidacloprid decline in leaves over time is degradation through plant metabolic processes (Mota-Sanchez et al. 2009; Tanis et al. 2012) and photodegradation (Lindquist 1965; Scholz & Reinhard 1999; Applegate & Esters 2002; Sur & Stork 2003). The parent compound breakdown leads to formation of several active metabolites of imidacloprid in ash and hemlock trees (Mota-Sanchez et al. 2009; Tanis et al. 2012; Coots 2012). Photocatalytic degradation of

imidacloprid by UV radiation is an efficient process proven in agricultural field conditions after topical application and under natural sunlight (Agüera et al. 1998; Scholz & Reinhard 1999; Wamhoff & Schneider 1999; Malato et al. 2001; Černigoj et al. 2007).

The second contributor is depletion of imidacloprid reserves temporarily stored in the trunk xylem after injection (Tanis et al. 2012). This could be due to the influence of continued compound dilution with new water uptake and translocation through transpiration driven sap flow in the tree. It is also possible that certain amount of imidacloprid is lost into the atmosphere through the process of volatilization driven by evapotranspiration from the canopy (Applegate & Esters 2002).

Another possible contributor is that transport of imidacloprid in apple tree xylem weakens in intensity (Harrell 2006). If true, this weakening is fine-tuned by the level of spatial dose splitting through the number of injection ports. In mature apple trees of cv. 'Empire', water use expressed through transpiration rate and estimated by sap flow measurements, gradually declines after mid-July in humid climate (Dragoni et al. 2005). Seasonal decline in water use could have caused the weakening of intensity in imidacloprid transport and allowed the decrease in concentration at 42 DAI. However, since the decline in water use is gradual over a long period of time and the accuracy of transpiration measurements is under question, it is less likely that this was significant contributor to decrease in imidacloprid concentration.

Time plays an important role in accumulation of efficient doses of trunk-injected compounds within the crown but also leads to their decline. Temporal distribution of injected compound is an important parameter indicating duration of plant protective activity (Schutte et al. 1988; Tattar et al. 1998). However, more research is needed to develop injection systems and

timing schedules that will take full advantage of the fundamental mechanisms at work within apples trees and allow temporally stable, efficient and long-lasting dose delivery.

The influence of xylem anatomy and mechanisms contributing to imidacloprid transport and distribution in apple tree canopy

Overall, based on both 1 and 2-port injections at 14 DAI it can be concluded that there are two main mechanisms in transport of trunk-injected imidacloprid to the apple tree crown: 1) helical or 360º round upward transport from each injection port, spirally up the xylem vascular system, which corresponds well to the counterclockwise spiral orientation of xylem grain in the wood of mature apple trees (Burger 1941); and 2) radial diffusion from the injection ports into the surrounding xylem tissue, which corresponds well to the diffuse-porous xylem anatomy present in wood of *Malus* spp. (Beck 2010; Orians et al. 2004; Orians 2005; Zanne et al. 2006) and other hardwoods (Northcott 1957; Noskowiak 1963; Kozlowski & Winget 1963; Kozlowski et al. 1967; Waisel et al. 1972; Chaney 1986; Orians et al. 2004; Orians 2005; Zanne et al. 2006; Pallardy & Kozlowski 2008; Beck 2010).

Spiral orientation of vessels and fibers in the trunk is a common physical feature in growth of woody plants reported for almost 200 broadleaf and conifer tree species (Bannan 1966; Braun 1854). Only a few describe the helical nature of orientation and functioning of wood vessels and fibers (Burger 1941; Noskowiak 1963; Kozlowski & Winget 1963; Tae & Howlett 1965; Tuttle & Gotlieb 1985; Kubler 1991). One of the main functions of spiral grain in trees is uniform distribution of nutrients in the sap from each root to all branches and from each branch to many roots (Kubler 1991; Orians et al. 2004).

The properties of diffuse porous xylem type in other tree species (*Betula*, *Acer*, *Liriodendron*), such as very low level of compound flow sectoriality (more integrated compound flow), high density of vessels, increased vessel-to-vessel contact, higher lateral pitting of vessel element walls, larger pit size and high density of intervessel pits, all seem to contribute greatly to better radial and integrated vertical distribution of compounds in the crown (Orians et al. 2004; Orians 2005; Zanne et al. 2006), We argue that diffuse porous xylem of apple wood contributed substantially to imidacloprid radial distribution in the tree trunk and subsequently into the adjacent apple crown quadrants.

From the results of our study it can be concluded that non-uniform spatial distribution in the crown following the 1-port injection at 14 DAI is a direct consequence of simultaneous spiral ascent and radial diffusion of imidacloprid in the trunk, followed by sequential delivery into the respective crown quadrants. Further, the results confirm that imidacloprid, as a systemic and water soluble compound (CCME 2007), follows the pathway of spiral sap ascent in xylem after trunk injection in apple trees.

The influence of port position and dose splitting on distribution of imidacloprid in apple tree canopy

Concentration patterns at 28 DAI supported the two proposed mechanisms of imidacloprid transport. However, the expression of these transport mechanisms significantly changed compared to 14 DAI (Figure 3A, 2A, B). While at 14 DAI primary expression of upward transport of imidacloprid was helical ascent, at 28 DAI the helical transport pathway broadened as a result of further radial diffusion, thus substantially supplying crown quadrants

directly above the injection ports. In 1 and 2-port injections, a contributing factor to this change was the significant effect of port position on imidacloprid distribution in the apple crown, depicted mostly as faster sap flow on the south exposed tree side. Faster sap flow occurs naturally due to higher transpiration in the south crown direction which is associated with the larger foliar area, greater exposure to sun light, and denser xylem network on south trunk direction, all of which are reported on almond, apricot, pecan, and other fruit trees (Nortes et al. 2008; Nicolas et al. 2005; Fernández et al. 2006; Steinberg et al. 1990). Coupled with the effects of low number of injection ports and full and half split dose deliveries, injection port position on south direction of the trunk severely contributed to the non-uniform spatial distribution of imidacloprid in the crown (Figure 3A, B, 2B). In contrast, in 4 and 8-port injections the effect of port position on south was severely weakened by more injection ports and quarter and eighth split dose deliveries per port. Spatial dose splitting reduced the effect of faster sap flow in south direction of the trunk and provided spatially uniform imidacloprid distribution in the crown (Figure 3A-C).

Besides faster sap flow, the change in expression of helical imidacloprid transport is further supported by the high dose of 1 g a. i. in 1-port injection and more time allowed for radial diffusion. Extensive radial diffusion, typical for tree species with diffuse porous xylem, substantially increased the breadth of the helical transport front and the dose load transported upward in the apple tree (Orians et al. 2004; Beck 2010) (Figure 2B). In 2-port injection at 28 DAI, spatial distribution indicated that the broadening of helical pathway was significantly less expressed in north direction compared to the south direction of the crown (Figure 3B). This is probably due to an absence of faster sap flow on the north exposed tree direction and due to the lower dose of 0.5 g a. i. delivered in the north injection port. Notably, dose splitting in 4 and 8-

port injections allowed independent formation of 4 and 8 helical transport pathways in the trunk xylem at 14 DAI (Figure 2A). The effects of radial diffusion and the overlapping of concentration patterns in the upper crown quadrants greatly contributed to the spatially most uniform distribution of imidacloprid in the crown (Figure 3C, D, 2B).

The influence of compound properties and xylem transport capacity on distribution of imidacloprid in apple tree canopy

At 14 DAI, the results indicate that injected imidacloprid is stored in the trunk and released in time according to the chemical properties of this compound. It was implied that among others, critical properties which determine the movement of injected imidacloprid in the tree are water solubility and organic carbon-water partitioning coefficient (Koc) (Doccola & Wild 2012; J.J. Doccola et al. 2007; Doccola et al. 2012; Byrne et al. 2012).

When different doses of imidacloprid (1, 0.5, 0.25 and 0.125 g) were delivered to south trunk direction in all four injection treatments, imidacloprid concentrations in the south crown directions at 14 DAI were similar. This suggests that regardless of the dose load imidacloprid is held or stored equally in the trunk, thus limiting its upward transport to similar amounts between the treatments. Chemical properties of imidacloprid such as low water solubility of 510 mg/L (Mulye 1995) (at 20ºC) and medium-low Koc of ~350 (Cox et al. 1997), could explain these results by implying moderate compound adhesion in the xylem as discussed before on injected eastern hemlock (*Tsuga*) (Doccola et al. 2012). It is proposed that slow upward movement of imidacloprid in xylem is related to adsorption to cellulose of non-living apoplast or storage (absorption) within axial and radial parenchyma of living wood symplast (Doccola et al. 2012).

Imidacloprid concentration patterns in south crown direction at 14 DAI (Figure2A, 3A-D) serve as evidence for the temporal reservoir effect in the trunk that was first described in trunk-injected apple trees (Pinkas et al. 1973; Shabi et al. 1974), implicated in pine trees (*Pinus*) (Grosman et al. 2002; Takai et al. 2004; Grosman & Upton 2006) and experimentally proven in ash trees (*Fraxinus*) (Mota-Sanchez et al. 2009; Tanis et al. 2012). Besides binding to wood xylem tissues, the level of lateral pitting of vessel element walls and intervessel pits, depicted in number and size of pits (Orians et al. 2004; Zanne et al. 2006), could also influence the level of imidacloprid transport.

Conclusion

In summary, use of 4 injection ports allows uniform spatial distribution of imidacloprid within apple tree crowns of this size. Early achievement of both spatially uniform and non-uniform imidacloprid distributions in apple crown is long-lasting. Temporal distribution patterns using 1-8 injections of imidacloprid are not uniform. Crown concentration patterns indicate that imidacloprid transport in xylem occurs through radial diffusion and vertical 360º helical pathway with counterclockwise turn of the spiral.

In the light of our findings, however, many other knowledge gaps need to be addressed before trunk injection could be used in tree-based agriculture. Future research should evaluate the impact of injection port wounding on xylem functionality after multiple years of injection. Season-long measurements of sap flow rate in the xylem tissue around trunk injection ports, rather than of the extent of discolored wood, need to be conducted to quantify the impairment of tissue function. Nevertheless, recently developed needle-based injection systems are less

injurious to trees than the classic ones (Montecchio 2011; Montecchio 2013; Düker et al. 2006), thus allowing injection to be used more safely for several years. Research also needs to elucidate the impacts of trunk injection and delivered compounds on endophytic microorganisms in trees as well as evaluate its benefits by severely minimizing pesticide losses into the environment. Once this knowledge and new technologies for automated trunk-injection are available, the transition from topical pesticide application to trunk injection in commercial orchards could be assessed.

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CHAPTER 3. CONTROL OF FIRE BLIGHT (*ERWINIA AMYLOVORA*) ON APPLE USING TRUNK INJECTION DELIVERY OF PLANT RESISTANCE INDUCERS AND ANTIBIOTICS

Abstract

Management of *Erwinia amylovora* is difficult due to resistance development, the scrutiny on use of antibiotics in agriculture and limited efficacy of alternative control agents. Even though successful in control, preventive antibiotic sprays allow considerable compound losses into the environment, aiding the development of resistance in non-target bacteria which can be transferred to *E. amylovora*. Trunk injection as a target precise pesticide delivery system, which utilizes tree xylem to deliver the compound, could decrease antibiotic usage in the environment and increase the effect of plant resistance inducers in fire blight control. After 1-2 injections of apple trees, blossom and shoot blight were controlled with the injected antibiotics, potassium phosphites (PJ) and acibenzolar-S-methyl (ASM). Oxytetracycline gave excellent control of shoot blight severity indicating that trunk injection is a superior delivery for this antibiotic. Besides ASM, injected PJ significantly expressed PR-1, 2 and 8 protein genes showing resistance induction (SAR) in apple leaves under field conditions. The time separated SAR and fire blight control indicated that various defensive compounds synthesized and accumulated in the canopy suppress the disease after gene expression ceases. The accumulation of injected compounds most likely was higher in shoots than in flowers but the amounts were still sufficient to express their effects on the plant or the pathogen. Provided fire blight control was lower than expected in commercial orchards. With the development of injectable

formulations, proper dosing, and more time for translocation and accumulation in the canopy, the injection of protective compounds could serve as an effective option for fire blight control.

Introduction

Erwinia amylovora (Burrill) Winslow et al. is a devastating bacterial pathogen of rosaceous plant species which causes fire blight disease. In Michigan, a fire blight epidemic resulted in economic losses of $42 million in 2000 due to removal of 350,000 to 450,000 apple trees, while a previous outbreak in 1991 led to economic losses of $3.8 million (Longstroth 2001; Douglas 2006). In Washington and northern Oregon, economic losses on pome fruits were over $68 million (Stockwell et al. 2002).

Traditionally, management of fire blight depends on use of cultural practices and preventive copper and antibiotic sprays (Norelli et al. 2003). Currently, there are no synthetic compounds with systemic properties available to improve fire blight protection programs (Adaskaveg et al. 2011; Balaž et al. 2013). Another management difficulty is the occurrence and spread of *E. amylovora* resistance to antibiotics which limits their efficacy as plant disease control agents (Chiou & Jones 1991; 1995). Further, possible transfer of genes for antibiotic resistance from plant to human pathogenic bacteria via conjugative plasimds with transposable elements could have repercussions on human health (Sundin et al. 1995; Sundin & Bender 1996). These risks drive the efforts for rational antibiotic use and preserving the efficiency of antibiotics in human medicine, while imposing scrutiny on use of antibiotics for plant protection (McManus et al. 2002). To alleviate this conundrum scientific research in the past two decades focused on investigating plant resistance inducers and biological control agents in fire blight management

(Johnson et al. 2000; Maxson-Stein et al. 2002; Norelli et al. 2003; Sundin et al. 2009). However, both showed limited efficiency in fire blight control and need for frequent reapplication. As a result, plant resistance inducers are viewed solely as a supplement to antibiotic programs, to aid in delaying the occurrence of fire blight pathogen resistance.

E. amylovora management relies upon preventive sprays of plant protection products. This provides direct contact of the material with the pathogen, before or immediately after the pathogen reaches the apple flowers or shoots. Therefore, successful fire blight control is dependent on controlling the epiphytic pathogen populations before they enter the host xylem and spread in the endophytic phase of pathogenicity (Koczan et al. 2009). However, airblast ground sprayers are inefficient in topical compound delivery with pesticide solution losses into the environment of up to 44-71% (Steiner 1969). Research shows that when non-target bacterial populations are exposed to broadcast application of pesticides in the agro-ecosystem, they can acquire resistance genes and then transfer these genes to target organisms, such as *E. amylovora*, thus hastening the development of resistance (Chiou & Jones 1993; Sundin et al. 1995). Further, even under the best spray coverage, the activity of topically applied protective products is negatively affected by variable weather conditions (rainfall, sunlight, temperature), specific properties of the phyllosphere, and a limited rate of absorption and subsequent movement in the plant (Windels et al. 1985; Scholz & Reinhard 1999; Percival 2001; Gozzo 2003; Cabrefiga et al. 2011). These difficulties bring into question the means by which we deliver materials for fire blight control and support investigation into alternative solutions (Brundtland 1987; McManus et al. 2002; Düker et al. 2006).

Trunk injection is an alternative approach for the delivery of plant protective compounds in tree fruit crops. It is based on harnessing the vascular transport capacity of a tree, which

allows active ingredient (a. i.) translocation and subsequent distribution into the canopy, where the protection is needed. The majority of tree injection technologies are based on compound delivery into the xylem. Originally developed for the purposes of plant protection and nutrition in landscape tree care, trunk injection offers numerous advantages that could enhance disease management on fruit trees. The most important advantage is that trunk injection is a target precise pesticide delivery system, facilitating compound deployment in a contained manner, without direct pesticide losses into the environment. These properties could be particularly effective for control of *E. amylovora* which spreads through the xylem and cortical parenchyma (Bogs et al. 1998; Perino et al. 1999).

Research on trunk injection of bactericides and plant resistance inducers in apples is limited. Recent investigation showed that trunk injection of mono- and di-potassium salts of phosphorous acid (Arborfos) on 'Paula Red' apple trees provided significant reduction of shoot blight for 67%, while the injected prohexadione-calcium (Apogee) was ineffective (Spitko 2008). Another study on 'Gala Must' and 'White Transparent' showed that trunk-injected prohexadione-carboxylic acid provided significant control of *E. amylovora* infections on flowers, comparable to the sprayed SS (Düker & Kubiak 2011a). In landscape tree care, trunk injection of bactericides and plant resistance inducers such as oxytetracycline and phosphites is used for fire blight control on sensitive varieties of crabapples (*Malus* spp.) (Swartz, P., 2012; Doccola, J., 2013, personal communication). In this study we aimed to investigate the effect of injected bactericides and plant resistance inducers on fire blight in apple orchards.

The leading hypothesis of our study was that significant control of fire blight and expression of PR protein genes should be achieved by 1-2 trunk injections of maximum seasonally allowed or lower doses of antibiotics and systemic acquired resistance (SAR)

inducers. Taking in consideration above described difficulties in fire blight control and weak effect of topically applied plant resistance inducers for this purpose, our first objective was to demonstrate performance of trunk-injected antibiotics and SAR inducers in control of *E. amylovora* on apple blossoms and shoots. The second objective was to demonstrate whether trunk-injected SAR inducers are capable in significantly upregulating PR protein genes in apple leaves and flowers, as markers of SAR response. PR-proteins are defined as plant host proteins which are produced only in response to attack by pathogens or by a related event (Loon et al. 1994). SAR is defined as a form of induced resistance in plants with a specific defense signaling pathway and which occurs after localized exposure to a pathogen or after spraying by a synthetic or a natural compound, commonly known as inducer (Hammerschmidt 2007). Our goal was to answer the question whether trunk injection can enhance the activity of protective compounds in fire blight control, and especially of SAR inducers (Heaton & Dullahide 1990; Wicks & Hall 1990; Guest et al. 1995).

Materials and methods

Control of blossom and shoot blight incidence

Chemical materials

Orchard experiments were conducted in 2012 at the Michigan State University's (MSU) Plant Pathology Research Center in Lansing, MI (GPS: N42° 41' 34.71", W84° 29' 32.43") in 2012, and in 2013 at the MSU Trevor Nichols Research Center in Fennville, MI (GPS: N42° 35'

58.03", W86° 9' 16.67") in 2013. In 2012, 14-yr-old and in 2013, 21-yr-old 'Gala' apple trees, *Malus domestica* Borkh., were trunk-injected with compounds dosed according to Table 3. With the exception of few treatments, injections in 2012 were conducted on 26 March, at the tight cluster stage in apples or 21 days instead of 14 planned days before 80% bloom, and on 23 April at petal fall. With the exception of few treatments (Table 3), in 2013 injections were conducted on 1 May, at transition of half inch green to tight cluster or 13 days instead of 14 planned days before 80% bloom, and on 22 May at petal fall.

Each compound dose was amended with 520 ml of water per tree, except of phosphites (PHOSPHO-jet®, Arborjet Inc., Woburn, MA) and imidacloprid (IMA-jet™, Arborjet Inc., Woburn, MA) which were formulated for direct trunk injection. Arborbiotic (MFG Scientific Inc., Royal Palm Beach, FL) dose was amended with 2.52 ml of water per 25.4 mm of tree DFH (trunk Diameter at one Foot Height or 30.48 cm) i.e. as 11.1% dilution.

Recommended spray dose of Actigard of 56.7g/378.5 L of water per 0.405 ha, amounting to 0.34 g/tree, was used based on our previous injection experiments (see Appendix 1). Phosphojet dose of 22.5 ml/tree was based on previous injection research on apple cv. 'Paula Red' with generic formulation ArborFos™ (Mauget Inc., Arcadia, CA) (Spitko 2008). In 2012, injected 'Gala' trees ranged from 6.86-14.73 cm (average 11.43 cm) in diameter at one foot or 30.48 cm of trunk height (DFH). In 2013, 'Gala' trees ranged from 16-25.4 cm (average 19.99 cm) in DFH. By taking the upper limit of range of 'Gala' trees in 2012 and a lower limit of range in 2013, a fixed value of 15.24 cm of DFH (6 inches) was chosen to calculate the used dose of 45 ml of Arborfos per one 'Paula Red' apple tree (Spitko 2008). After multiplying this dose per tree with 250 trees commonly planted in Michigan per 0.405 ha, a dose of 11,250 ml (380.45 fl. oz./acre) of Arborfos was calculated. Due to potential of high Phosphojet doses for causing

phytotoxicity after injection (VanWoerkom 2012), we used slightly lower dose of 11,236.6 ml/0.405 ha (380 fl. oz./acre) or 44.95 ml per one 'Gala' apple tree, which was then split-delivered in time as two injections of 22.5 ml per tree (Table 3).

Imajet dose providing 1 g imidacloprid per tree aligns with the maximal allowed dose per season of 0.91 g/0.405 ha for foliar application on apple (Provado® 1.6; Admire® Pro™, Bayer Crop Science, Research Triangle Park, NC).

Arborbiotic dose was chosen based on previous results in 2012 shoot blight control experiment on 'Kit Jonathan' apples, to determine whether a slightly higher dose than in this previous experiment will be able to provide yearlong control of blossom blight. 'Gala' apple trees as experimental units injected with Arborbiotic ranged from 16.0-18.03 cm (average 16.66 cm) in DFH. Agrimycin dose per tree was derived from the higher recommended rate of 453.6 g/378.5 L of water per 0.405 ha for one spray treatment (200 ppm), divided by 250 apple trees per 0.405 ha. Kasumin® 2L (Arysta LifeScience North America, LLC., Cary, NC) dose of 7.6 ml per tree, has been calculated by dividing one spray treatment dose of 1,892.48 ml per 0.405 ha by 250 apple trees per 0.405 ha.

The two Copper Chelate treatment doses (copper chelate 5% - Baicor® Chelates, Baicor® L.C. Expert Plant Nutrition, Logan, UT) were chosen after recommendation from Arborjet Inc. (Woburn, MA) to reduce risks of causing phytotoxicity on apple trees (Table 3).

In 2012, four replicate trees per treatment were arranged in a randomized complete block design (RCBD). Blocking controlled variable crown sizes in trees (large, medium, medium-small, and small) since different transpiring leaf areas modulate the speed of compound

translocation and accumulation in the canopy after injection. In 2013, four replicate trees per treatment were arranged in a completely randomized design (CRD).

Trunk injection

Four cardinally oriented injection ports per tree, positioned approximately 10-15 cm above the ground level, were created by drilling 25.4 mm deep into the xylem tissue and 9.53 mm in diameter, with a cordless 1500 rpm drill (DeWalt Industrial Tool Co., Baltimore, MD) (Aćimović et al. 2014). Ports were sealed with Arborplug no. 4, using screwdriver-like plug tapper and a hammer, with plug positioned just below the bark level to to allow port closure with cambium (Arborjet Inc., Woburn, MA).

Due to cold weather conditions, undeveloped leaf canopy and hence weak transpiration pull of sap in the wood xylem, injections on 26 March 2012 were conducted with Viper air/hydraulic micro-injection system® (Arborjet Inc., Woburn, MA). Trunk injections on 23 April were conducted using Tree IV® air/hydraulic micro-injection system (Arborjet Inc., Woburn, MA), since the leaf canopy was more developed. Injection needles of both devices were inserted into the Arborplugs through the septum thus allowing delivery of protective solution into the four freshly drilled ports. Total injected volume per tree was divided equally among the four ports. Due to warm weather conditions, partially developed leaf canopy, substantial root pressure (Chaney 1979; 1986) and good transpiration pull of sap in the xylem, both trunk injections on 1 and 22 May 2013 were conducted using Tree IV injection system.

Inoculation

Late in the afternoon on 16 April 2012 (80% bloom), flowers were spray-inoculated with distilled water suspension of *E. amylovora* strain Ea110 (5.4×10^6 CFU/ml) using hand-sprayer (Solo® 457 handheld sprayer with 11.36 L tank, Solo Inc., Newport News, VA). During late evening and night on 14 May 2013 (80% bloom), spray inoculations were done with distilled water suspension of the same *E. amylovora* strain at 0.7×10^6 CFU/ml, using hand-sprayer (Roundup® Multi-Use Cart Sprayer with 11.36 L tank, The Fountainhead Group Inc., New York Mills, NY). Serial dilutions of bacterial suspension were conducted in 0.5X PBS buffer (Appendix 3) and plate counted on LB agar medium.

Disease evaluations

In 2012, blossom blight incidence was evaluated 3 times on 22 and 29 May and 5 June, and in 2013, on 11, 18 and 25 June. We randomly chose blossom clusters on spurs and counted the number of diseased and healthy blossom clusters in a 100-cluster sample. Blossom blight incidence was calculated as blossom blight percent in per tree basis. Since fire blight spread from infected flowers onto the intensively growing shoots, blossom blight driven shoot blight was also evaluated but only 2 times in 2012 on 29 May and 5 June, and at the same time points in 2013 when the blossom blight was rated. After counting the numbers of randomly chosen blighted and healthy shoots in a 100-shoot sample per tree, shoot blight incidence was calculated as shoot

blight percent in per tree basis. For each treatment blossom and shoot blight incidence means were calculated from 4 replicate trees.

PR protein gene expression in leaves and flowers

Sample collection

From injected 'Gala' apple trees for blossom blight control in 2012 and 2013, 21 leaves and 21 flowers per tree were collected for pathogenesis related (PR) protein gene expression analysis. In 2012, leaves were collected on 5 and 16 April, 7 and 21 May, 4 and 18 June, and 2 July, and flowers on 16 April. In 2013, leaf samples were collected on 10, 14, 23 and 31 May, and flowers on 14 May. Samples were cooled and stored at -80ºC.

Out of 4 tree replicates per treatment in 2012, samples from only 3 were used for PR protein gene expression analysis through all sampling times. For 2012, only 2 replicates per treatment are shown in results section. Out of 4 replicates per treatment in 2013, samples from 3-4 were used for gene expression analysis through all sampling times. For 23 and 31 May 2013, only 2 and all 4 replicates per treatment, respectively, are shown in the results section.

RNA extraction and gene expression

In both years, gene expression analyses were conducted for the controls and Actigard, Imajet and Phosphojet treatments (Table 3). For flower samples, generative parts were separated from vegetative parts (Gasic et al. 2004). Sampled leaves and vegetative flower parts were hand

ground and homogenized within closed plastic bags and 100 mg of tissue per sample was finely ground in liquid nitrogen. RNA was extracted from tissue using E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek Inc., Norcross, GA; Plant RNA Protocol II for difficult samples). RNA purification was conducted using TURBO DNA-free™ Kit (Ambion®, Life Technologies Corp., Carlsbad, CA). All RNA samples were diluted to 81.9 ng/µL in 2012, and to 235.8 ng/µL in 2013. cDNA was synthesized with TaqMan® Reverse Transcription Reagents (Invitrogen™, Life Technologies Corp., Carlsbad, CA) in PTC-100™ Programmable Thermal Controller (MJ Research Inc., Waltham, MA). RNA and DNA concentrations were determined with NanoDrop 1000 Spectrophotometer for RNA-40 and DNA-50 (Thermo Fisher Scientific Inc., Wilmington, DE). Using cDNA, SYBR® PCR Green Master Mix (Applied Biosystems Inc., Foster City, CA) and Step OnePlus™ Real-Time PCR machine (Applied Biosystems Inc., Foster City, CA) expression levels of PR-1 (unknown function), PR-2 (β-1,3-glucanase) and PR-8 (chitinase type III) genes was quantified and normalized to apple actin gene (primers by Invitrogen™, Life Technologies Corp., Carlsbad, CA). Normalization by including actin as an invariant control i.e. reference gene was done to improve the reliability of qPCR since this corrects for sample to sample variations in qPCR efficiency and for errors in sample quantification (Pfaffl 2001; Bustin 2002). For each biological replicate i.e. apple tree, qPCR was ran in three technical replicates of 20 µL reaction for each set of primers. Pair Wise Fixed Reallocation Randomisation Test© was used to compare the expression levels among treatments (Multiple Condition Solver REST-MCS©, version 2) (Pfaffl 2001).

Control of shoot blight severity

Chemical materials

Orchard experiment was conducted in 2012 and 2013, at Michigan State University's Plant Pathology Research Center in Lansing, MI (GPS: N42° 41' 34.71", W84° 29' 32.43"). On 23 April 2012 (petal fall), mature 12-yr-old 'Kit Jonathan' apple trees, *Malus domestica* Borkhausen, were trunk injected with oxytetracycline hydrochloride (ArborBiotic™, MFG Scientific Inc., Royal Palm Beach, FL). We injected 0.28 g of Arborbiotic per each 25.4 mm of trunk DFH, amended with 2.52 ml of water per each 25.4 mm of trunk DFH (10% dilution). Total dose per tree depended on each tree's unique DFH. In 2013, the same apple trees injected in 2012 were re-injected on 22 May 2013 (petal fall) using the same dosing method, common for landscape tree care. Control treatment was injection of 2.52 ml of water per each 25.4 mm of trunk DFH.

Trunk injection

Trunk injections were conducted using Quik-jet® micro-injection system (Arborjet Inc., Woburn, MA) after creating injection ports using the same method described above for blossom blight control experiments. DFH-unique dose for each tree was equally divided and delivered through four trunk injection ports. In 2012, injected 'Kit Jonathan' trees ranged from 7.11-10.16 cm (average 8.02±0.36 cm) in DFH. In 2013, the same trees ranged from 7.75-11.43 cm (average

8.84±0.45 cm) in DFH. Four replicate trees were arranged in a completely randomized design (CRD).

Inoculation

Shoot inoculations on 7 May 2012 (14 DAI) and on 30 May 2013 (8 DAI) were conducted with scissors dipped in PBS suspension of *E. amylovora* (2012: 4.7×10^7 CFU/ml; 2013: 5×10^8 CFU/ml). Total of 10 randomly chosen shoots per each 'Kit Jonathan' tree were inoculated, while additional 10 shoots on the same tree replicate were inoculated with distilled water as a negative control. Using scissors the upper third of the leaf blade of second or the third youngest leaf on the shoot tip were cut off (Koczan et al. 2009). (Koczan et al. 2009)

Disease evaluations

For each inoculated shoot, p(Koczan et al. 2009)ercent of shoot blight severity was calculated from the ratio of necrotized shoot length and total shoot length [cm]. Total shoot length [cm] was measured on negative control shoots. Measurements of total shoot length were first taken on 6 May 2012 and 30 May 2013, before the inoculation. Shoot and necrosis lengths were then measured in 7 day intervals on 14, 21 and 28 May and on 4, 11 and 18 June 2012, and on 10, 17 and 24 June and on 1, 8 and 15 July 2013. Measurements ceased at terminal bud set on shoots. For each treatment, shoot blight severity mean per tree (%) was calculated from 10 shoot replicates and grand average shoot blight severity was calculated from 4 replicate trees.

Statistical analysis

Data were analyzed using MIXED procedure in SAS 9.3 (SAS Institute, 2012). Blossom blight data in 2012 were transformed using ARCSINE (ARSIN) transformation to normalize the residuals. The main effects of treatment and time on blossom blight incidence in 2012 were analyzed using repeated measures best adjusted to spatial power variance covariance structure (α=0.05). The main effects on blossom blight driven shoot blight incidence in 2012 were analyzed using Type 3 Tests of Fixed Effects (F-test, α=0.05) for each time point independently. The main effects on blossom blight incidence in 2013 were analyzed with time as a fixed factor since no variance covariance structures reduced AIC and BIC criterions. The main effects on blossom blight driven shoot blight incidence in 2013 were analyzed using repeated measures best adjusted to compound symmetry variance covariance structure with heterogeneous variances (α=0.05).

Shoot blight severity data on 'Kit Jonathan' trees in 2012 were transformed using SQUARE ROOT (SQRT) transformation to normalize the residuals. Main effects of Arborbiotic and time on shoot blight severity in 2012 were analyzed using CRD with repeated measures best adjusted to heterogeneous autoregressive variance covariance structure of first order (α=0.05). In 2013, the main effects of Arborbiotic and time on shoot blight severity were analyzed using CRD with repeated measures best adjusted using spatial power variance covariance structure (α=0.05).

Variance-covariance structures in blossom blight, blossom blight driven shoot blight and shoot blight experiments, for all monitored parameters, were decided by lowering the AIC and BIC fit statistics values after model fitting with different suitable structures. Tree was used as subject of repeated measurements through time. When the main effects or their interactions were

found to be statistically significant (p<0.05), main effect examination and interactions slicing examination by main effects was performed, tested with F-tests (α=0.1 or 0.05), and pairwise or specific time or treatment comparisons were conducted using *t*-tests (α=0.1 or 0.05) or Tukey's HSD test (α=0.05).

Results

Control of blossom and shoot blight incidence

The injected compounds in 2012 affected blossom blight incidence (α=0.1) (Table 4). At a medium infection pressure in 2012, injected Agrimycin and Phosphojet significantly reduced blossom blight and provided some level of disease control (*t*-tests, α=0.1) (Figure 4). Actigard 1 and 2 provided statistically similar effect to these compounds. Imajet did not affect blossom blight.

Fire blight has spread from flowers onto the shoots. The injected compounds affected blossom blight driven shoot blight incidence only on 29 May 2012 (α=0.1) (Table 4). Phosphojet and Agrimycin provided good control of shoot blight incidence on this date, while Imajet and Actigard 1 and 2 had no effect (*t*-tests, α=0.1) (Figure 5). During 2012 experiment, Phosphojet caused limited phytotoxicity expressed as scorching and burn on flowers and shoots of 1-3 branches, on 2 out of 4 replicate trees.

The injected compounds in 2013 affected blossom blight incidence (α=0.05) (Table 4). At a high infection pressure in 2013, all injected compounds provided significant reduction of blossom blight incidence (Figure 6). The best blossom blight control was achieved by injected Arborbiotic which significantly outperformed all other compounds. The next best control was

achieved by Kasumin and Agrimycin which outperformed both doses of Actigard and Copper (Figure 6). Phosphojet effect was statistically similar to these two antibiotics but also to Actigard 1 and 2, but it provided better control from Copper 1 and 2. Copper 1 and 2 provided significant reduction of blossom blight incidence, similar to Actigard 1 and 2, but yielded poor disease control.

Fire blight has spread from flowers onto the shoots (Figure 7). The injected compounds affected blossom blight driven shoot blight incidence in 2013 (α=0.05) (Table 4). Since treatment and time effects on shoot blight incidence interacted, the interaction slicing examination by both main effects was performed using *t*-tests (α=0.05) (Table 5).

Within each time point all treatments showed significant shoot blight reduction (Table 5). Arborbiotic again was significantly the best providing good shoot blight control. In comparison to Arborbiotic, all the other compounds provided weaker shoot blight control and the effects were largely similar between each other. Kasumin and Agrimycin reduced disease incidence the most after Arborbiotic. During 2013 experiment, Copper caused limited level of phytotoxicity expressed as scorching and burn on flowers and shoots of 3-6 branches, on 3 out of 4 replicate trees. However, Phosphojet in 2013 did not cause any adverse effects on flowers and leaves.

Overall, the majority of injected compounds did not provide satisfactory fire blight control levels achieved with topical compound application in commercial orchards. Arborbiotic, as the only exception, provided relatively satisfactory control of fire blight with just a single injection per season.

PR protein gene expression

Gene expression in apple leaves

Injected Actigard provided significant upregulation of PR-1, 2 and 8 protein genes in two consecutive years (Table 6, Figure 8 and 9). Injected Phosphojet provided significant upregulation of PR-1, 2 and 8 protein genes only in 2013 (Figure 10). In 2012, Phosphojet significantly upregulated only PR-8 protein gene (Figure 8). Trunk injection of Imajet significantly upregulated only PR-2 and PR-8 protein genes (Table 6, Figure 8).

SAR induction effect by Actigard occurred 10 days after the first injection (DAFI) in 2012 (Figure 8), and 22 DAFI and 1 day after the second injection (DASI) in 2013 (Figure 9). SAR induction by Phosphojet in 2013 occurred 30 DAFI and 9 DASI (Figure 10).

In apple leaves collected on 5 April 2012, Actigard showed from 3 to almost 5-fold of the expression in water injected control for all three PR protein genes, while Phosphojet showed almost 22-fold of the expression in the same control for PR-8 gene (Figure 8). Imajet provided 3.5 to 4.3-fold of the expression in water injected untreated control for PR-2 and PR-8 protein genes, respectively (Figure 8). In apple leaves collected on 23 May 2013, Actigard 2 provided from 2 to almost 3-fold of the expression in water injected control for all three PR protein genes (Figure 9).

In apple leaves collected on 31 May 2013, Actigard 1 provided up to almost 5-fold of the expression, while Actigard 2 provided from 13 to almost 24-fold of the expression in non-injected non-inoculated control for PR-2 and PR-8 protein genes (Figure 10). Surprisingly,

Phosphojet provided from 3 to almost 25-fold of the expression in non-injected non-inoculated control for all three PR protein genes (Figure 10).

At all other time points in 2012 and 2013, significant PR protein gene expression was rare (2 July 2012) and mostly inconsistent among tree replicates.

Gene expression in apple flowers

Gene expression on 16 April 2012, or 51 DAFI and 23 DASI, showed that only Phosphojet and Actigard 2 significantly upregulated only PR-8 protein gene (Table 6, Figure 11). All other compounds did not upregulate PR protein genes. In non-injected non-inoculated control significant downregulation of PR-2 protein gene was detected ($p \leq 0.001$) (Figure 11). Phosphojet provided 2-fold and Actigard 5-fold of the expression in water injected control for PR-8 protein gene. Non-injected non-inoculated control showed 0.5-fold of the expression in water injected untreated control for PR-2 protein gene (Figure 11).

Gene expression on 14 May 2013, 14 DAFI, showed no significant upregulation of the three PR protein genes.

Control of shoot blight severity

The main effect of injected Arborbiotic on shoot blight severity was significant for two years consecutively ($\alpha=0.05$) (Table 7, Figure 12). This antibiotic formulated for injection stopped the infection and provided significant, season-long control of shoot blight after each injection (Figure 12A: interaction slicing examination by Tukey's HSD test, and 12B: interaction

slicing examination by *t*-test, α=0.05). Fire blight necrosis significantly increased through time only in both water injected controls (Figure 12).

Table 3. Plant protective products and doses used for trunk injection on 'Gala' apple trees for control of blossom blight and blossom blight driven shoot blight in 2012 and 2013.

Product/ treatment	Active ingredient	Dose	Date(s) of injection(s) in 2012	2013
Actigard 1	acibenzolar-S-methyl 50%	1 x 0.34 g/tree	26 March	1 May
Actigard 2		2 x 0.34 g/tree	26 March 23 April	1 and 22 May
Phosphojet	mono- and di-potassium salts of phosphorous acid 45.8%	2 x 22.5 ml/tree	26 March 23 April	1 and 22 May
Imajet	Imidacloprid 5%	2 x 10 ml/tree	26 March 23 April	-
Arborbiotic	oxytetracycline hydrochloride 39.6%	0.31 g/25.4 mm DFH*	-	1 and 22 May
Agrimycin	streptomycin sulfate 22.4% (equivalent to 17% streptomycin)	2 x 1.82 g/tree	26 March 23 April	1 and 22 May
Kasumin	kasugamycin hydrochloride 2.3% (equivalent to 2.0% kasugamycin)	2 x 7.6 ml/tree	-	1 and 22 May
Copper chelate 1	water soluble copper (Cu) 5%	2 x 5 ml/tree	-	1 and 22 May
Copper chelate 2		2 x 15 ml/tree	-	1 and 22 May
Water injected untreated control	-	2 x 520 ml/tree	26 March 23 April	1 and 22 May
Non-injected non-inoculated control	-	-	-	-

Table 4. The main effects and their interactions after trunk-injection of 'Gala' apple trees with compounds for control of fire blight in 2012 and 2013 (α=0.05).

Fire blight stage / Year		Main effects	*F*	DF	*P*
Blossom blight incidence	**2012**	Treatment	3.13	15	**≤0.0393**
		Time	5.76	23.5	**≤0.0092**
		Treatment*Time	0.49	26.7	≥0.8817
	2013	Treatment	68.49	30	**≤0.0001**
		Time	39.33	60	**≤0.0001**
		Treatment*Time	1.17	60	≥0.3131
Shoot blight incidence	**29 May 2012**	Treatment	6.74	10.1	**≤0.0052**
	2013	Treatment	17.87	30.1	**≤0.0001**
		Time	174.73	44.4	**≤0.0001**
		Treatment*Time	2.08	48.8	**≤0.0219**

Table 5. Blossom blight driven shoot blight control in 2013 with injected protective products (*t*-tests, α=0.05). [1]WIC - water injected untreated control, Ninja - non-inoculated and non-injected control. [2]Treatment means within one date followed by different upper-case letters are significantly different. [3]Means within one treatment across the three dates followed by different lower-case letters are significantly different.

Treatment	Date / mean shoot blight incidence [%]		
	6/11/2013	6/18/2013	6/25/2013
WIC[1]	60 A[2] a[3]	66.8 Ab	78.8 Ac
Phosphojet	41.8 Ba	54.3 Bb	61.5 Bc
Copper 5	41.8 Ba	53.3 Bb	60 BCc
Actigard 1	41 Ba	51.5 Bb	57.5 BCDb
Copper 15	35.5 BCa	45.3 BCb	55 BCDc
Actigard 2	36.8 BCa	51.5 Bb	53.8 BCDb
Agrimycin	35.5 BCa	48.5 BCb	46.5 DEb
Kasumin	32 Ca	38 Cb	49 CDEc
Arborbiotic	22.3 Da	24.3 Da	34.3 Fb
Ninja	13 Ea	22.5 Db	39.8 EFc

Table 6. Significance in the expression of Pathogenesis Related (PR) protein genes in apple leaves and flowers after trunk-injection of 'Gala' trees with plant resistance inducers for control of fire blight. Significance was relative to water injected or non-injected non-inoculated control and normalized to actin gene (Pair Wise Fixed Reallocation Randomization test, α=0.05).

Plant organ	Date	Compound	Protein gene	*p* value
Leaves	5 April 2012	Actigard	PR-1	**≤0.042**
			PR-2	**≤0.042**
			PR-8	**≤0.042**
		Phosphojet	PR-1	≥0.946
			PR-2	≥0.946
			PR-8	**≤0.001**
		Imajet	PR-1	≥0.944
			PR-2	**≤0.001**
			PR-8	**≤0.001**
	23 May 2013	Actigard	PR-1	**≤0.046**
			PR-2	**≤0.046**
			PR-8	**≤0.046**
	31 May 2013	Actigard	PR-1	≥0.492
			PR-2	**≤0.042**
			PR-8	**≤0.042**
		Phosphojet	PR-1	**≤0.045**
			PR-2	**≤0.045**
			PR-8	**≤0.045**
Flowers	16 April 2012	Actigard	PR-1	≥0.431
			PR-2	≥0.894
			PR-8	**≤0.042**
		Phosphojet	PR-1	≥0.603
			PR-2	≥0.946
			PR-8	**≤0.001**

Table 7. The main effects and their interactions after trunk-injection of 'Kit Jonathan' apple trees with Arborbiotic (oxytetracycline hydrochloride) in 2012 and 2013 (α=0.05).

Shoot blight severity	**2012**	Treatment	10.68	6.1	**≤0.0167**
		Time	4.47	4.56	≥0.0744
		Treatment*Time	1.68	4.56	≥0.2981
	2013	Treatment	49.14	6.8	**≤0.0002**
		Time	12.5	29.8	**≤0.0001**
		Treatment*Time	9.09	29.8	**≤0.0001**

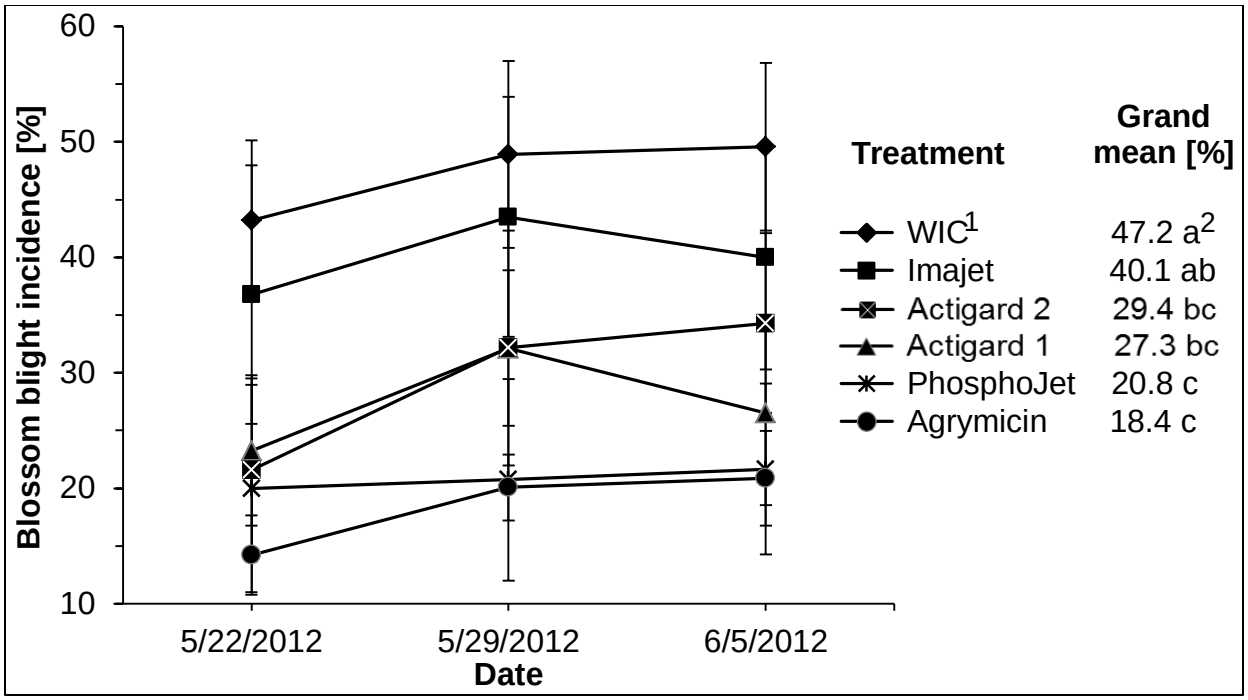


Figure 4. Blossom blight control in 2012 after 1-2 trunk injections of 'Gala' apple trees on 26 March and 23 April with protective products. [1]WIC - water injected untreated control. [2]Blossom blight incidence means across the three time points within one treatment followed by different letters are significantly different (*t*-tests, $p<0.1$). Error bars represent standard error of the mean (SEM).

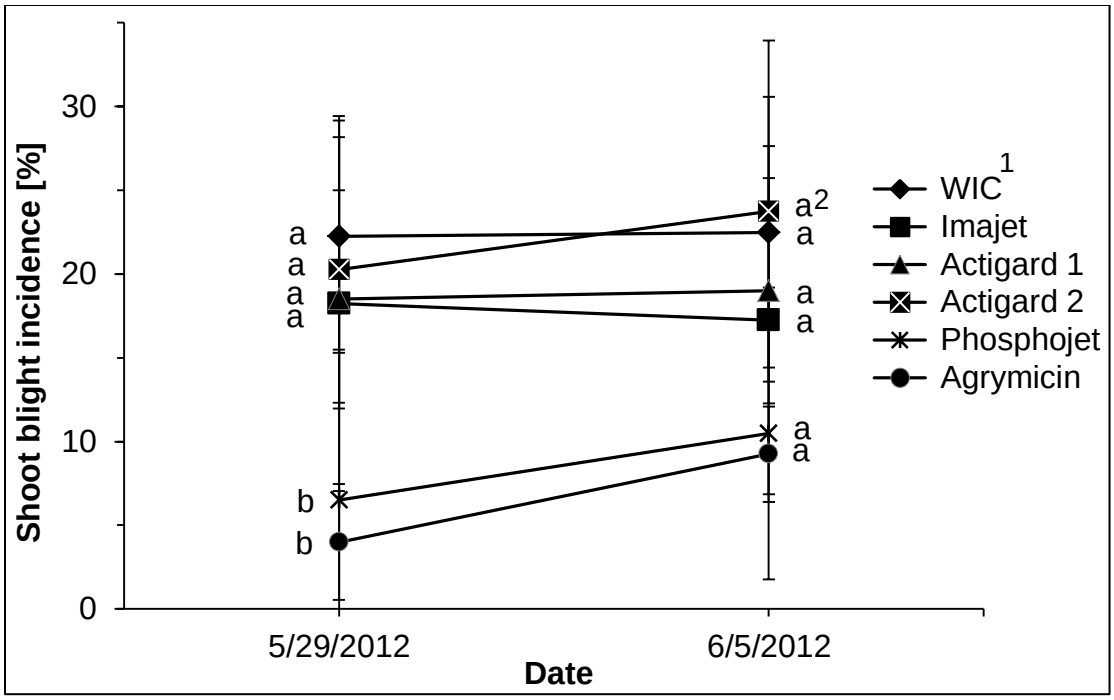


Figure 5. Shoot blight control in 2012 after 1-2 trunk injections of 'Gala' apple trees on 26 March and 23 April with protective products. [1]WIC - water injected untreated control. [2]Shoot blight incidence means between treatments within one time point followed by different letters are significantly different (*t*-tests, $p<0.1$). Error bars represent standard error of the mean (SEM).

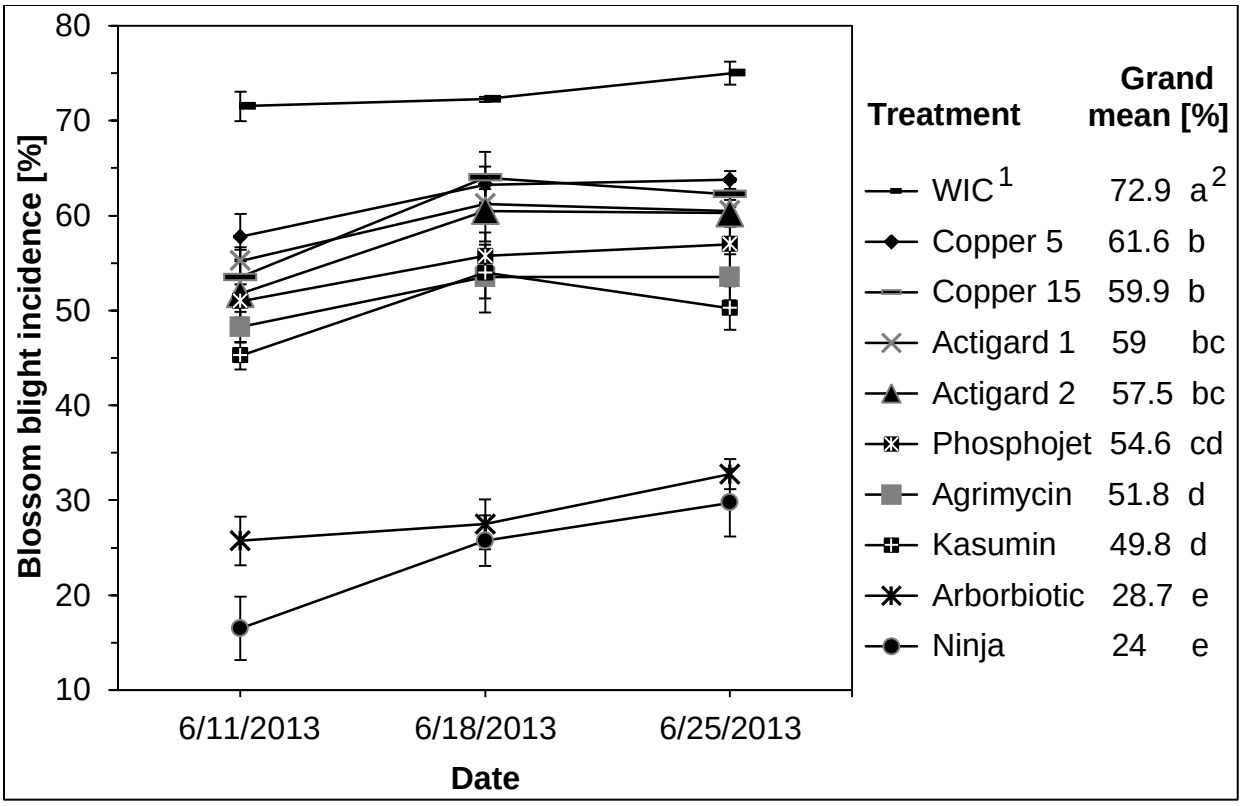


Figure 6. Blossom blight control in 2013 after 1-2 trunk injections of 'Gala' apple trees on 1 and 22 May with protective products. [1]WIC - water injected untreated control, Ninja - Non-inoculated and non-injected control. [2]Blossom blight incidence means across the three time points within one treatment followed by different letters are significantly different (*t*-tests, $p<0.05$). Error bars represent standard error of the mean (SEM).

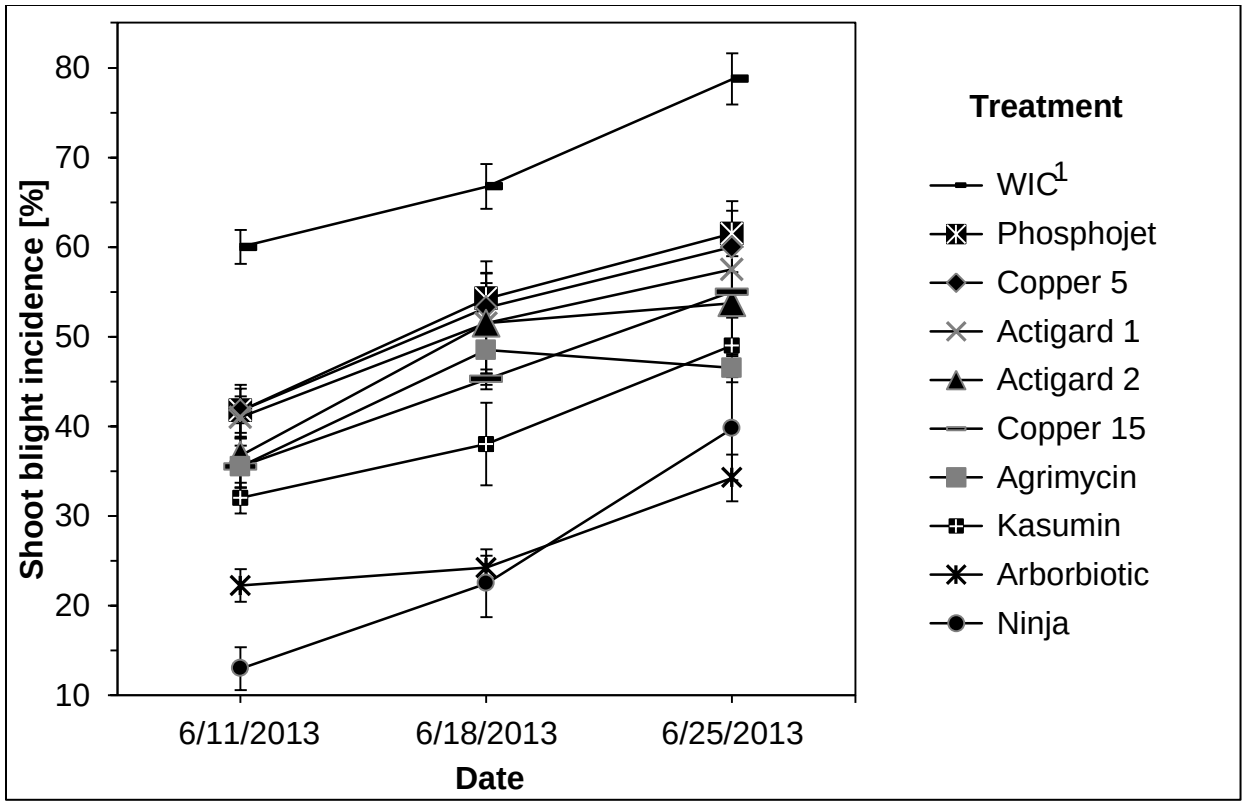


Figure 7. Shoot blight control in 2013 after 1-2 trunk injections of 'Gala' apple trees on 1 and 22 May with protective products. [1]WIC - water injected untreated control, Ninja - Non-inoculated and non-injected control. Error bars represent standard error of the mean (SEM).

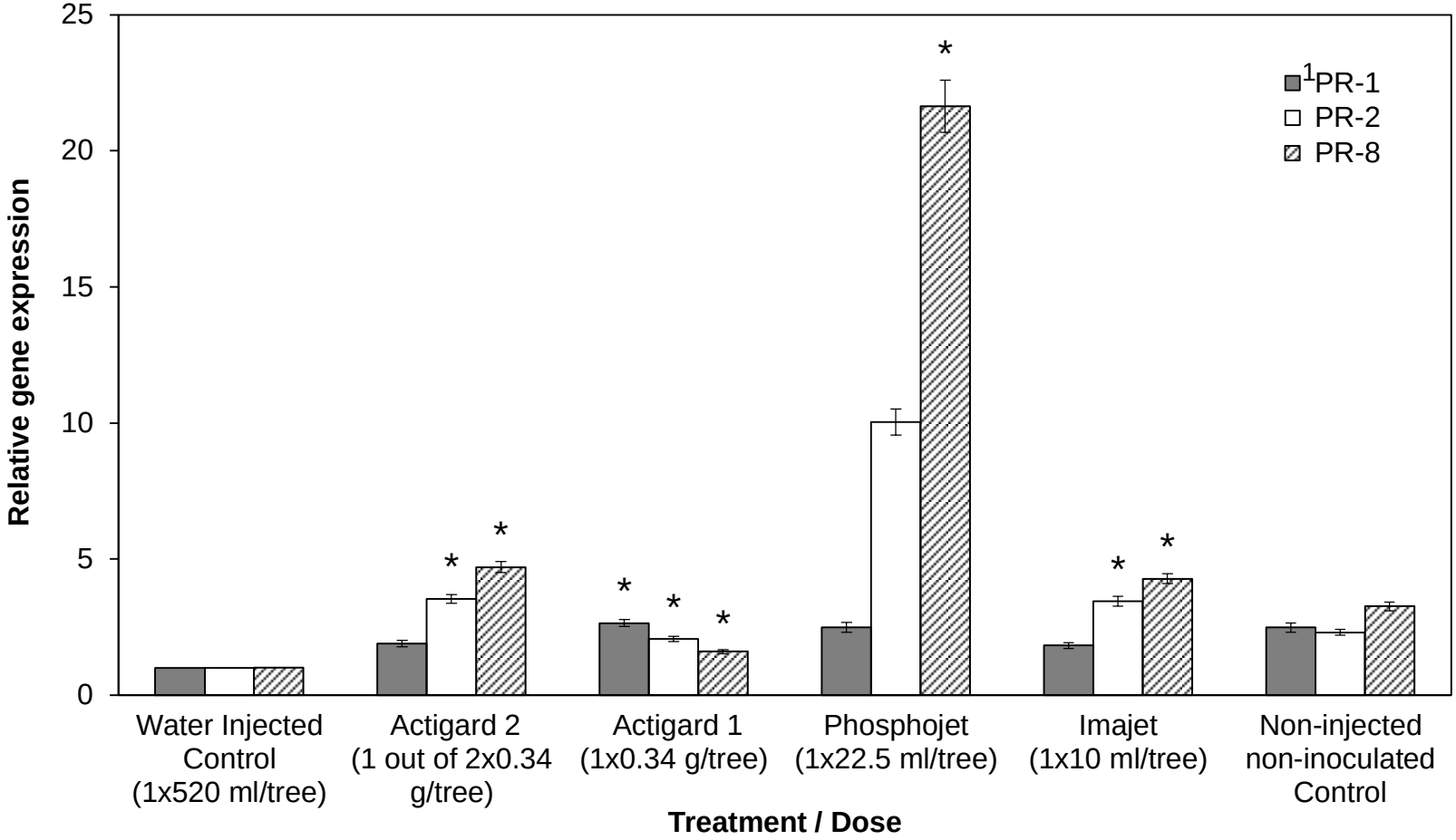


Figure 8. Gene expression in leaves on 5 April 2012 after first trunk injection of 'Gala' apple trees with acibenzolar-S-methyl (Actigard), potassium salts of phosphorous acid (Phosphojet), imidacloprid (Imajet) and water on 26 March 2012 (tight cluster). [1]PR - pathogenesis related proteins. *Mean of gene expression followed by an asterisk is significantly upregulated relative to water injected untreated control and normalized to actin housekeeping gene (Pair Wise Fixed Reallocation Randomization test, α=0.05). Error bars represent standard error of the mean (SEM).

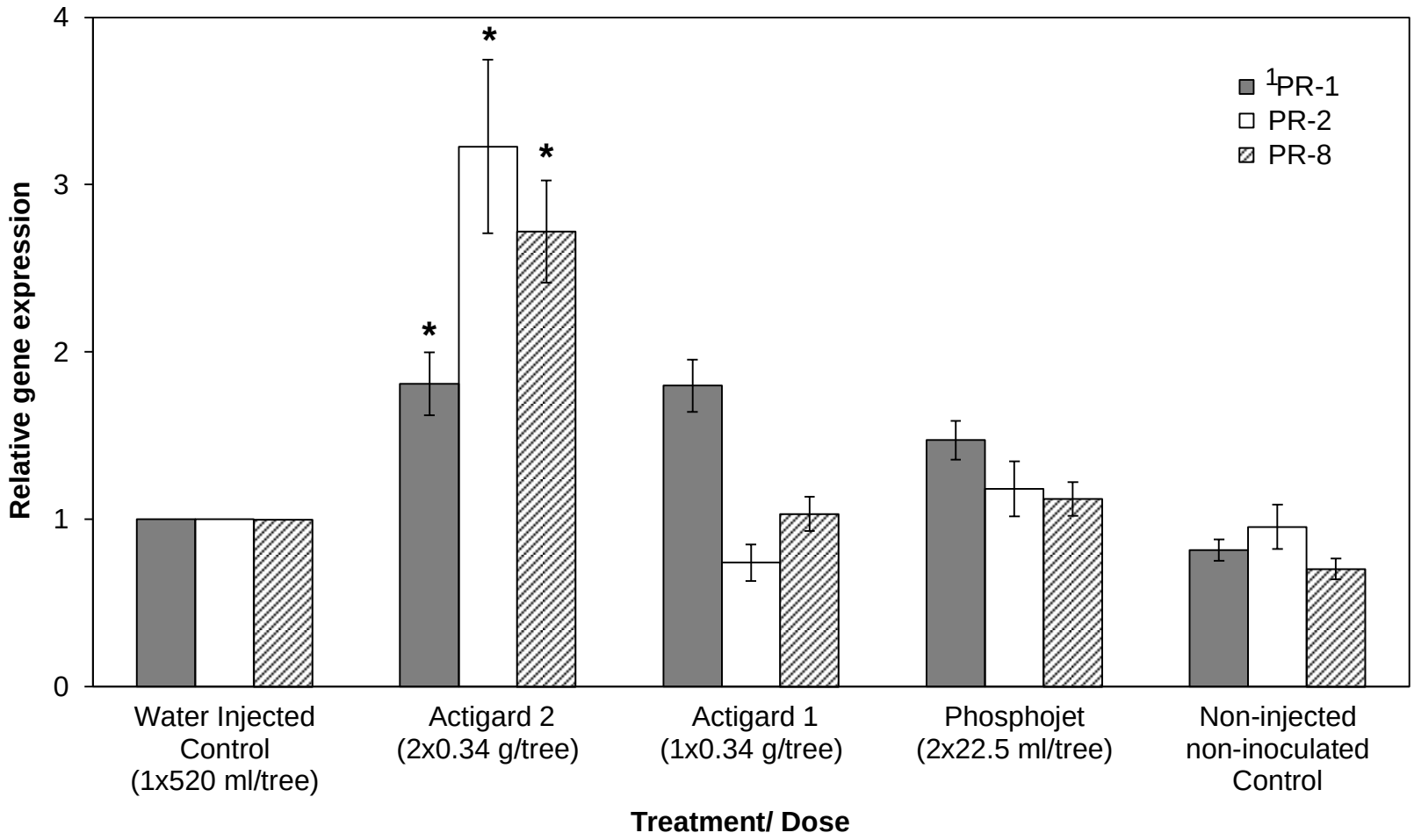


Figure 9. Gene expression in leaves collected on 23 May 2013 after two trunk injections of 'Gala' apple trees with acibenzolar-S-methyl (Actigard), potassium salts of phosphorous acid (Phosphojet) and water on 1 May (half inch green/tight cluster) and 22 May 2013 (petal fall). [1]PR - pathogenesis related proteins. *Mean of gene expression followed by an asterisk is significantly upregulated relative to water injected untreated control and normalized to actin housekeeping gene (Pair Wise Fixed Reallocation Randomization test, α=0.05). Error bars represent standard error of the mean (SEM).

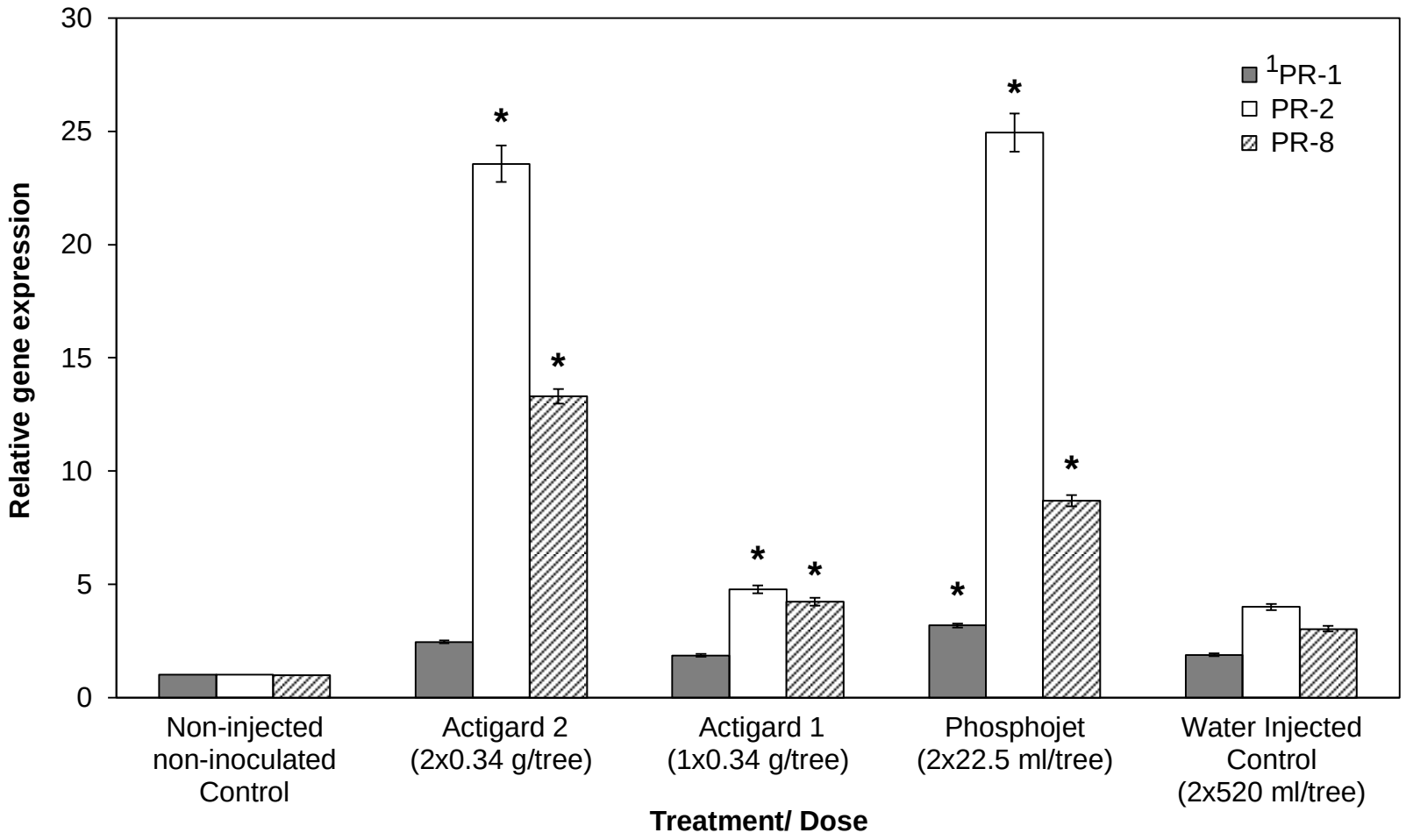


Figure 10. Gene expression in leaves on 31 May 2013 after two trunk injections of 'Gala' apple trees with acibenzolar-S-methyl (Actigard), potassium salts of phosphorous acid (Phosphojet) and water on 1 May (half inch green/tight cluster) and 22 May 2013 (petal fall). [1]PR - pathogenesis related proteins. *Mean of gene expression followed by an asterisk is significantly upregulated relative to non-injected non-inoculated control and normalized to actin housekeeping gene (Pair Wise Fixed Reallocation Randomization test, α=0.05). Error bars represent standard error of the mean (SEM).

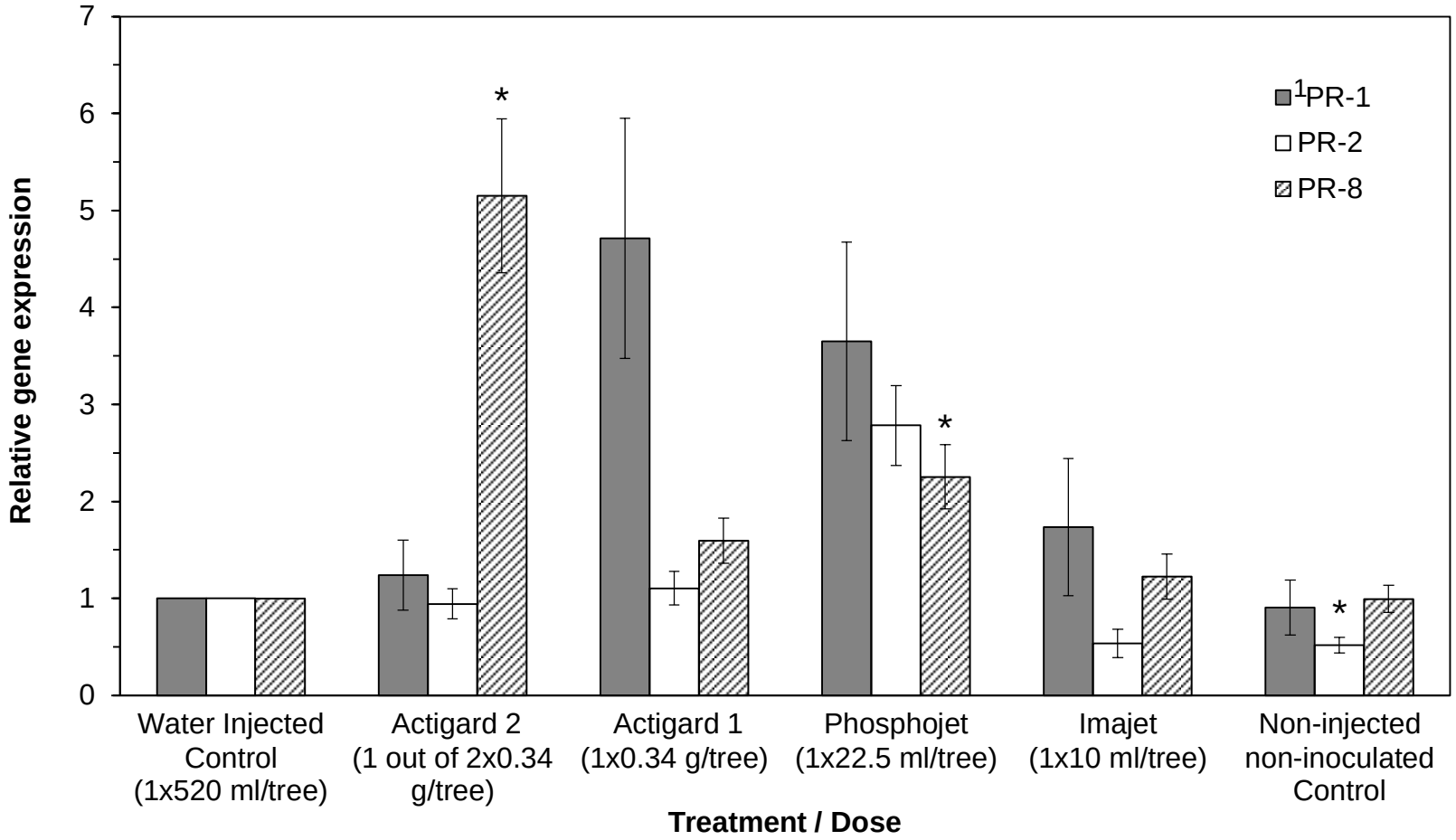
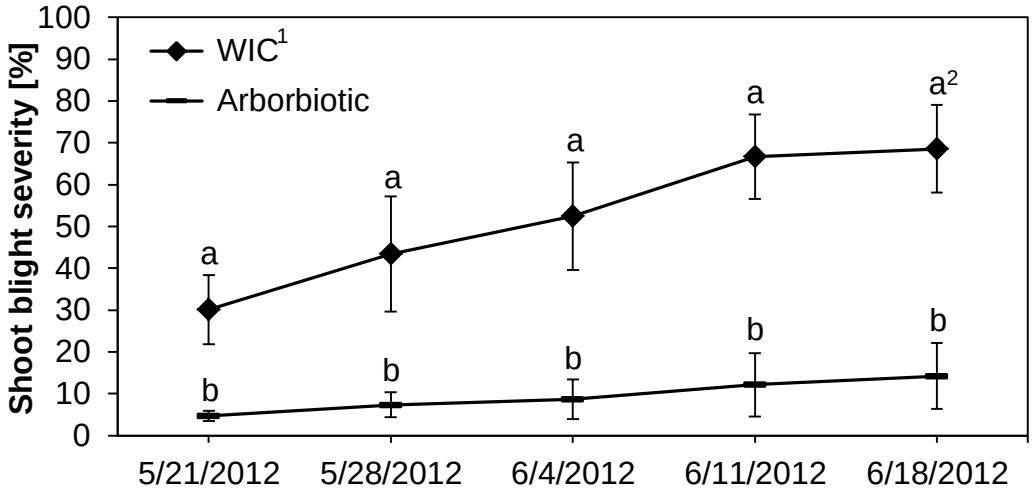


Figure 11. Gene expression in green flower tissues on 16 April 2012 after first trunk injection of 'Gala' apple trees with acibenzolar-S-methyl (Actigard), potassium salts of phosphorous acid (Phosphojet), imidacloprid (Imajet) and water on 26 March 2012 (tight cluster). [1]PR - pathogenesis related proteins. *Mean gene expression followed by an asterisk is significantly upregulated relative to water injected untreated control and normalized to actin housekeeping gene (Pair Wise Fixed Reallocation Randomization test, α=0.05). Error bars represent standard error of the mean (SEM).

(A)



(B)

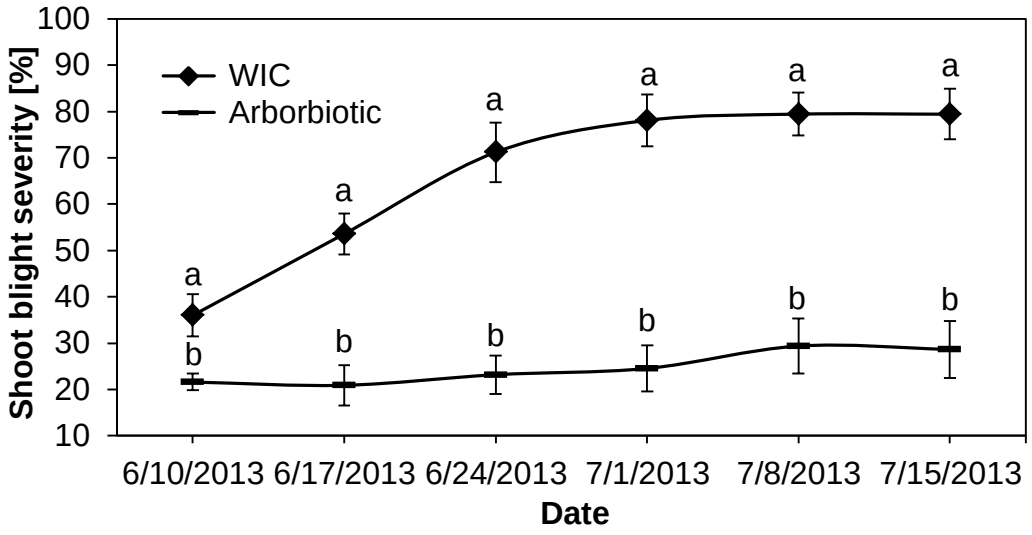


Figure 12. Shoot blight control in 2012 (A) and 2013 (B) after single injection of 'Kit Jonathan' apple trees per season with Arborbiotic (oxytetracycline hydrochloride). [1]WIC - water injected untreated control. [2]Shoot blight severity means between treatments within one time point followed by different letters are significantly different (2012: Tukey's HSD test, 2013: *t*-test, $p<0.05$). Error bars represent standard error of the mean (SEM).

Discussion

This study contributes new knowledge on fire blight management using tree injection as an alternative delivery approach for plant protective compounds. Trunk-injected bactericides and plant resistance inducers can control fire blight on apple flowers and shoots. This is the first study demonstrating significant fire blight suppression through PR-protein gene expression by injected acibenzolar-S-methyl (ASM) and potassium salts of phosphorous acid (PJ) on mature apple trees, under field conditions.

Blossom blight incidence control under medium and high disease pressures was consistently best with injected antibiotics and Phosphojet, and then with Actigard. However, injected Agrimycin and Kasumin were not as good as their spray applications which control blossom blight to incidences of 0.2-3.5% and 1.5-5.6%, respectively (Sundin et al. 2009; McGhee & Sundin 2011). This suggests that injected antibiotics either do not reduce bacterial populations on flowers as after topical application because they do not reach the surface of stigmas, favorable for *E. amylovora* growth, or they do reach these surfaces but too late for better effect. However, once *E. amylovora* invades the inner flower tissues, the injected bactericides accumulated in the tissues most likely acted therapeutically, but only to stop further pathogen spread on other flowers and into the spurs and twigs. Single injection of Arborbiotic versus 1-2 injections of all other compounds gave best fire blight control. Depending on the study evaluating spray application of oxytetracycline, injection of this antibiotic showed to be either slightly or much better than the sprayed oxytetracycline calcium complex (17% oxytetracycline, Mycoshield, Nufarm Limited, Melbourne, Australia) which allows 29-37% or 57-67% of infected flowers (McManus & Jones 1994; Stockwell et al. 2007). This shows either a better

effect of 0.8 g/tree of injected oxytetracycline versus 0.3 g/tree received after spraying, or that the Arborbiotic formulation of oxytetracycline can reach the surface of flower stigmas and significantly reduce bacterial populations. The disease control in this study indicates that injection is a superior form of delivery for oxytetracycline, allowing its prolonged activity in comparison to spraying, where oxytetracycline levels decline fast on plant surfaces due to short half-life and bacterial populations reestablish (Johnson & Stockwell 1998; McManus et al. 2002).

Blossom blight control with injected Phosphojet and Actigard occurred probably due to PR-8 protein accumulation in vegetative flower parts (2012) or due to full-PR-gene systemic acquired resistance (SAR) which was expressed much earlier or later than at full bloom, when we expected it and when it was not detected (2013). This can be explained by the fact that in *Malus* spp. vegetative flowers parts and later fruits have 10- to 100-fold lower frequency of stomata on epidermis in comparison to abaxial epidermis in leaves (Blanke & Lenz 1989). Therefore, the transpiration rate per surface unit is much weaker, leading to slower accumulation of injected compounds in flowers than in leaves and hence delayed SAR expression which reduced blossom blight. Previous research on 'Golden Delicious' shows that 2-4 Actigard sprays (100 and 200 mg/L; 75, 150 and 200 mg of a. i./L) provide 3-52% of blossom blight control, while on 'Rome Beauty' 74-91% of control (Brisset et al. 2000; Thomson, Gouk, et al. 1998). Sprayed Actigard (0.024 and 0.012%) on 'James Grieve' gave 56-68% of blossom blight control (Zeller & Zeller 1998). Our 1-2 times injected Actigard gave control of around 14-20%, indicating that injection does not improve the effect of this compound on flowers. The only investigation of blossom blight control with trunk-injection of plant resistance inducer, evaluated prohexadione-carboxylic acid (PCA), the free acid of prohexadione-calcium (Apogee, BASF

Corp., Research Triangle Park, NC) (Düker & Kubiak 2011a). Injected PCA (10-40 mg/tree) provided 13.6-17.5% of blossom blight control on 'White Transparent' and 'Gala Must' apple trees (Düker & Kubiak 2011a). PCA also caused expected shoot stunting. All the above implies that the injected compounds for blossom blight control must translocate and accumulate more rapidly in flowers to be effective, or injected much earlier to allow enough time for ample translocation.

Shoot blight incidence was best controlled with the injected antibiotics and then with Actigard and Phosphojet which induced resistance in the trees. Consistently, at the first date of disease rating, Agrimycin and Phosphojet in 2012, and all the injected compounds in 2013, showed better fire blight suppression on shoots than on flowers. The driver of this effect was probably the high transpiration rate of shoots which hold the largest leaf area in the apple canopy. This implies that shoots rapidly accumulate high amount of injected compounds, controlling the disease early after injection. Weakening of control effects at later ratings can be explained by the compound dilution effect facilitated by tissue mass increase through shoot growth (Long et al. 1989) or metabolic processes leading to a. i. decline (Lindquist 1965). Arborbiotic was best, since as injectable formulation it moved rapidly through the xylem and accumulated abundantly in the shoots. Other antibiotics followed behind most likely due to formulations designed for topical application, hampering their translocation and accumulation. Injected Arborfos (Mauget Inc., Arcadia, CA), a Phosphojet generic, showed shoot blight control of 67% on inoculated 'Paulared' apple trees (Spitko 2008). In our study, with the same dose per tree delivered in 2 split injections of Phosphojet, we achieved disease reduction of only 14-16%. This implies that temporal dose splitting allowed weakening of shoot blight control by Phosphojet and most likely by Actigard. On inoculated shoots, 3-6 Actigard sprays (0.15g/L)

provided shoot blight control of 2.8 and 50.7%, respectively, while Agrimycin gave 56% control (Maxson & Jones 1999). On naturally infected 'Jonathan' apple trees, 6 Actigard sprays (75 mg/L) provided 50% of shoot blight control which was similar to Agrimycin with 57% of control (Maxson-Stein et al. 2002). We show that 1-2 injections of Actigard provided only 18.5-21.2% of shoot blight control. Therefore, it seems that two-time injection does not significantly improve shoot blight control by Actigard.

Even thought formulated for trunk injection, Imajet's failure to reduce blossom and shoot blight incidence in 2012, could be either because imidacloprid did not accumulate in flowers and shoots, as rapidly as the other compounds, or it did but it triggered different pathways in plant SAR induction that are not active against *E. amylovora* (Ford et al. 2010). Imidacloprid induced SAR is effective against bacterial citrus canker caused by *Xanthomonas citri* subsp. *citri* (Dowson) and a fungal pathogen *Golovinomyces orontii* (Castagne) V.P. Heluta, causing powdery mildew on *Arabidopsis* (Francis et al. 2009; Ford et al. 2010; Graham & Myers 2011). In 2013, copper at a lower and higher doses provided significant reduction of blossom blight incidence for 11.3% and 13%, respectively. It also reduced shoot blight incidence for 13.5-18.8% at a lower dose, and for 21.5-24.5% at a higher dose. Besides the fact that these effects were similar to the injected Actigard, this indicates that copper in a chelate form can readily move in the appe tree xylem and accumulate at effective levels in the apple canopy after trunk injection.

Excellent control of shoot blight severity by injected Arborbiotic implies that oxytetracycline in this formulation most likely exerts therapeutic effect on *E. amylovora* in apple trees. This is supported by the fact that in 2013, unlike in 2012, time between injection and inoculation was insufficient for Arborbiotic to distribute spatially uniform and reach all the inoculated shoots and impact the pathogen. Therefore, infections have progressed to about 20%

on 10 June. However, after that, the infections were stopped by Arborbiotic and this effect lasted until the end of the experiment. Even though its mode of action is bacteriostatic, we show that oxytetracycline injected once per season has the ability to express its effectiveness longer and better than after spraying (Johnson & Stockwell 1998; McManus et al. 2002). Hence, trunk injection enhances the activity of trunk-injected oxytetracycline in shoot blight control.

Finally, we show that the injected phosphites as Phosphojet can induce SAR in apple leaves and confirm this effect for Actigard, but when these compounds are injected on mature apple trees. SAR allowing or aiding the disease control was implicated after evaluation of phosphites on different plant species (Schutte et al. 1988; Smillie et al. 1989; Heaton & Dullahide 1990; Grant et al. 1990; 1995; Guest & Bompeix 1990; Guest & Grant 1991; T. Jackson et al. 2000; Pilbeam 2003; Reuveni et al. 2003; Shearer et al. 2006; Gentile et al. 2009). Phosphojet did not show SAR in 2012, probably because the leaf sampling times were too far apart or too close to the injection dates, thus not allowing the detection of this effect. Hence, at sampling times in 2012, accumulated Phosphojet doses in leaves were most likely either too low to cause significant PR-protein gene expression or the gene expression has already ceased. Further, it is also possible that *E. amylovora* potentially suppressed the expression of certain PR-protein genes by its dspA/E type III effectors and hrp regulator proteins (Pester et al. 2012).

PR-protein gene expression indicating induction of SAR occurred before the detected fire blight control effects which were more persistent in flowers than in shoots, and showed that trunk injections were properly timed for disease control. Time separated SAR induction and fire blight control effects indicate that probably the myriad of accumulated defensive compounds long-lastingly suppressed the disease. Injected Actigard consistently expressed PR-1, PR-2 and PR-8 protein genes in both years of the study. Based on research on tobacco including PR-1, then

on apple including PR-2, and on cucumber including PR-8, these genes code for proteins with an anti-oomycete-activity, β-1,3-glucanase hydrolase activity (on cell walls of fungi and oomycetes), and class III chitinase i.e. lysozyme with hydrolysis activity (on cell walls of bacteria), respectively (Alexander et al. 1993; Neuhaus 1999; Brisset et al. 2000; Métraux et al. 1988). The same three PR-protein genes were significantly expressed on 'Jonathan' apple seedlings after Actigard spray treatment (250 mg a. i./L) (Maxson-Stein et al. 2002). Similar results were also achieved with Actigard (100 and 200 mg a. i./L) on 'Golden Delicious' apple seedlings (Brisset et al. 2000). However, in 1-year-old 'Gala' apple trees treated with Actigard (250 mg a. i./L), no significant induction of PR-1a, PR-2 and PR-8 genes was detected in shoots (Bonasera et al. 2006). In this study, PR-2 and PR-8 were only induced in shoots after *E. amylovora* inoculation, and the difference from results of induction in seedlings were attributed to the different development stage and responses of the treated tissues. In the present study, we show that very soon i.e. 1-10 days after injection, Actigard induces PR-protein gene expression in leaves on mature 'Gala' apple trees. In 2012, this occurred before and in 2013 after the inoculation of flowers. In 2012, Actigard induced SAR and fire blight control effects were more distant in time than in 2013 indicating that control occurred due to secreted and accumulated PR-1, 2, and 8 proteins in the apoplast after the gene expression ceased (Bonasera et al. 2006; Gau et al. 2004). In 2013, Actigard induced SAR was closer in time to the recorded effects on fire blight while the Phosphojet induced SAR occurred later than the Actigard's and even closer to the control effect on fire blight. Nevertheless, in flowers it seems that accumulated PR-proteins persisted better in disease suppression than on the infected shoots where the SAR effect faded gradually over time most likely due to diluting effect of newly forming leaf mass.

In summary, fire blight control indicates that injected compounds accumulate at sufficient amounts in the apple tree canopy and express their putative effects on the plant or the pathogen. The results imply that accumulation of injected compound in the crown is a time dependent process, potentially not exposing epiphytic bacterial populations to the compound. Injected antibiotics provided best fire blight control but, excluding Arborbiotic, were probably hampered in translocation, accumulation and thus activity by formulations for topical application. Injected Actigard provided weaker fire blight control but consistent SAR effect in mature apple trees, while Phosphojet, besides good control, showed that potassium phosphites can induce SAR. Overall, the results indicate that injected bactericides could cure the infected orchards and that tree injection could decrease antibiotic usage in the open environment, thus reducing the potential for side effects to the environment (Zhang et al. 2011).

The next research step in trunk injection of these compounds is the analysis of their temporal residue profiles in the canopy and the salicylic acid (SA) accumulation in leaves after PJ injection. Only with these quantifications one can be fully certain in claiming that the effects on fire blight and their fluctuation depending on treatment were due to differential accumulation of the injected compound in the crown. Before trunk injection can be used in tree-based agriculture, the timing of application, compound doses and formulations (Kondo 1978), and trunk injection technology, all need to be adjusted or redesigned and improved to meet the unique requirements of fruit tree production. The most important requirements in fruit production are the high compound efficiency in disease control and fair time of its lasting, pesticide residues levels in fruit below the EPA determined MRL's, and a short time of application with acceptable costs.

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CHAPTER 4. TRUNK-INJECTED POTASSIUM PHOSPHITES AND OTHER FUNGICIDES PROVIDE TWO SEASONS OF CONTROL OF APPLE SCAB *VENTURIA INAEQUALIS* ON 'RED DELICIOUS' APPLE TREES

Abstract

Trunk injection of pesticides is a target-precise pest control approach which could eliminate the negative drift-driven effects of topical ground sprayer applications on the environment. It is not known whether acceptable control of apple scab fungus *Venturia inaequalis* can be achieved with trunk injection of fungicides. This study evaluated whether four injections of phosphite biopesticides and other fungicides can provide acceptable and season-long foliar apple scab control. The study also aimed to determine whether trunk injection can enhance the efficiency of phosphites in apple scab control so that they can provide similar effect to the sprays of standard fungicides. Injected potassium phosphites controlled apple scab on leaves for two consecutive seasons and at some times provided comparable control to two seasons of standard topical spray applications. Control of apple scab on shoots, in the year of injection, was better than on the spurs indicating that higher intensity of transpiration from shoot leaves led to the higher accumulation of injected compounds than in spurs. Good control of apple scab with potassium phosphites indicated on good translocation and accumulation in leaves which extends over two seasons. Trunk injection provides superior delivery of phosphite biopesticides by enhancing their activity to be relatively similar in effect to two years-worth standard topical sprays. The results suggest that with optimization of injection timing and compound dosing, trunk injection could provide acceptable control of apple scab for 1-2 seasons.

Introduction

Topical pesticide applications from ground sprayer in fruit production creates drift-driven pesticide losses into the environment (Pimentel 2005; Perry 1998; Ecobichon 1999). Commonly used air blast sprayers can waste 44-71% of the pesticide solution into the agro-ecosystem (Steiner 1969; Pimentel & Levitan 1986; Pimentel 1995; Zhu et al. 2006). Scientists estimate that with spraying less than 0.1% of the applied pesticide reaches the target pest, thus allowing 99.9% of the pesticide to be dispersed and washed away into the off-target end points (Pimentel & Levitan 1986; Pimentel 1995; Düker et al. 2006). This contaminates water, soil and air, leading to undue exposure of non-target organisms and humans to pesticides (Helling et al. 2000; Hamilton et al. 2004; Yen et al. 2009).

In humid continental climate, the majority of applied pesticides in apple production are fungicides for control of apple scab fungus *Venturia inaequalis* (Cooke) Wint. To preserve high yield and blemish-free fruit, frequent fungicide sprays are required during the season and can lead to excessive accumulation of pesticide residues in fruit (FAO & WHO 2004; Hamilton et al. 2004; Rawn et al. 2007). This poses a serious risk for the health of consumers and applicators (Pimentel et al. 1992; Pimentel & Lehman 1993; Mills 1998). Public concern over pesticide residues in food and environment has put apple as the most sprayed culture on the forefront of attention from the 1960's till today (Sutton 1996).

Due to negative effects of pesticide application, conventional apple production has the lowest level of environmental and economic sustainability (Reganold et al. 2001). Besides many novel approaches such as integrated pest management (IPM) and organic production, and less harmful pesticide chemistries, which aim to minimize pesticide use and their negative impact,

conventional fruit production is still a major source of fruits for the world (Garcia 2002; Willer & Yussefi 2012).

To significantly reduce off-target pesticide losses, new technologies for topical application based on sensor-guided spraying, high-volume directed air-jet systems, and recycling sprayers have been investigated for rational and target-precise compound delivery to fruit trees (Holownicki et al. 2000; Theriault et al. 2001; Llorens et al. 2010). However, trunk injection technology which provides target-precise delivery of compounds for efficient tree protection in landscapes is weakly investigated for agricultural purposes. Trunk injection utilizes tree xylem to distribute the pesticide into the trunk, branches and the canopy where the protection is needed. This approach could decrease pesticide usage in the open environment and improve the performance of some compounds currently used for pest control (Zhang et al. 2011).

Previous research shows that single trunk injection of fungicides like propiconazole, penconazole and thiabendazole can significantly reduce *V. inaequalis* incidence in the canopy, either for one or two seasons (Pinkas et al. 1973; VanWoerkom 2012; Percival & Boyle 2005). Also, trunk injected fosetyl-aluminium and potassium phosphites significantly reduced *V. inaequalis* on apple (Percival & Boyle 2005; VanWoerkom 2012). These results indicated that the efficiency of injected fungicides depended on time and number of injections, chemical properties of the injected compound, accumulated concentration in the crown and the uniformity of compound distribution in the tree (Shabi et al. 1974; VanWoerkom 2012; Aćimović et al. 2014). Nevertheless, it is undetermined how commercially acceptable apple scab control can be achieved in one season with trunk injection of fungicides or biopesticides.

Using apple scab as a model, we aimed to determine whether multiple trunk injections of fungicides or biopesticides per season, can provide commercially acceptable and season-long

apple scab control. A single injection per season of these compounds gave weak but significant apple scab suppression on 'MacIntosh (RedMax)' apple trees (VanWoerkom 2012). Our second objective was to the determine whether trunk injection of phosphite biopesticides can enhance their efficiency in apple scab control so that their effect can be similar to sprays of standard fungicides (Heaton & Dullahide 1990; Wicks & Hall 1990). We chose phosphites since their activity in foliar control of *V. inaequalis* has not been satisfactory for commercial use (Percival et al. 2009; Mitre et al. 2010; Percival 2010; Jamar 2011). However, when injected they activate systemic acquired resistance (SAR) in apple leaves aiding the plant to control disease (Aćimović et al. *unpublished*) and have direct toxic effects on some fungal species (Deliopoulos et al. 2010).

Our leading hypothesis was that 4 injections of potassium phosphites or other fungicides will provide comparable control to the standard spray program. We hypothesized that trunk injection will significantly enhance the activity of phosphites and provide two seasons of *V. inaequalis* control comparable to the effects of two years of seasonal sprays of standard fungicides. Overall, the goal of this study was to investigate whether trunk injection can be an efficient alternative to the conventional spray management programs.

Materials and Methods

An orchard experiment was conducted in 2012 at Michigan State University's Trevor Nichols Research Center (TNRC) in Fennville, MI (GPS: N42° 36' 27.34", W86° 9' 27.19"). From 21 March onwards, between half inch green to tight cluster growth stage, we trunk-injected 14-yr-old 'Red Delicious' apple trees 4 times with the compounds and doses shown in Table 8.

Except for Alamo and Phosphojet (Arborjet, Inc., Woburn, MA), all compound doses were delivered with 1000 ml of water to ease translocation and increase accumulation in the canopy. Alamo was amended with 30 ml of water per 25.4 mm of DFH (29.14% solution). No water was amended with Phosphojet. Doses of Prophyt (Helena Chemical Company, Collierville, TN) and Nutrol (LidoChem, Inc., Hazlet, NJ) were adjusted to match the a. i. amount in Phosphojet 1 treatment (Table 8). The dose of potassium bicarbonate (MilStop®, BioWorks Inc., Victor, NY) was based on one spray label rate of 907.2 g per 0.405 ha (1 acre) for spray application divided by 250 apple trees planted per acre as a standard in Michigan. The dose of hydrogen peroxide (OxiDate®, BioSafe Systems LLC., East Hartford, CT) was similarly derived from the spray label rate of 3,785 ml per 0.405 ha. Spray standard was delivered with 189.25 L of water/ 0.405 ha.

Injected trees ranged from 13.97-19.05 cm with the average of 16.71±0.25 cm in DFH. We compared the effect of injected compounds on apple scab to the spray standard of Penncozeb™ 75DF (Cerexagri Inc., King of Prussia, PA) and Rubigan® E.C. (Dow AgroSciences LLC, Indianapolis, IN) applied in accordance with the alerts from apple scab forecast system through Enviro-weather station at TNRC in Fennville, MI (http://enviroweather.msu.edu). We used completely randomized design (CRD) with 4 replicate trees per treatment.

For each injection date, a separate set of four cardinally oriented injection ports per tree were created by drilling 25.4 mm deep into the xylem tissue and 9.53 mm in diameter (1500 rpm cordless drill, DeWalt Industrial Tool Co., Baltimore, MD) (Aćimović et al. 2014). The first set of ports was positioned approximately 20-25 cm above the ground level, while the following ones were positioned between and above the former ones. All ports were sealed with Arborplug®

no. 4 (Arborjet Inc., Woburn, MA). Phosphojet was injected with Quik-jet micro-injection system® (Arborjet Inc., Woburn, MA). Due to weakly developed leaf canopy and weak transpiration, injections on 21 March were conducted with Viper air/hydraulic micro-injection system® (Arborjet Inc., Woburn, MA). Later injections were conducted with Tree IV® air/hydraulic micro-injection system (Arborjet Inc., Woburn, MA). Injection needles of these devices were inserted through the Arborplugs and the total injected volume per tree, for one time of injection, was divided and delivered equally among the four ports.

We monitored the efficiency of compounds injected in 2012 by rating apple scab control on leaves during both 2012 and 2013. The standard fungicide sprays were conducted in both 2012 and 2013 on the same trees. We examined 20 spurs and 20 terminal shoots per tree replicate and rated the leaf scab incidence (Ehret et al. 2010). Due to frosts during bloom in 2012 and 2013, apple fruits were lost and fruit scab was not rated. In 2013, we discontinued evaluating apple scab control by potassium dihydrogen phosphate, hydrogen peroxide and potassium bicarbonate since preliminary rating indicated no significant difference in comparison to the water injected untreated control.

The apple scab control in 2012 and 2013 was analyzed with a mixed model using the MIXED procedure in SAS 9.3 (SAS Institute, Cary, NC, 2011). Before the statistical analysis, the data on spur and shoot leaf scab incidences in 2012 were square root and log transformed, respectively. The data on shoot leaf scab incidences in 2013 were log transformed. We analyzed the spur and shoot leaf scab control in 2012 with repeated measures best adjusted using unstructured and spatial power covariance structures, respectively. In 2013, the continued spur and shoot leaf scab control was analyzed using repeated measures best adjusted with unstructured and heterogeneous compound symmetry covariance structures, respectively. Tree

was the subject of repeated measurements. When the effects of injected compound and time or their interaction were found to be statistically significant ($P < 0.05$), slicing, i.e. examination of interaction within main effects, was performed, *F*-tests were carried out and pairwise or specific comparisons of injection devices were conducted using *t*-tests ($\alpha = 0.05$).

Results

The injected compounds and time significantly affected apple scab on leaves in both 2012 and 2013 (Table 9).

The injections of phosphites, potassium dihydrogen phosphate and hydrogen peroxide provided largely similar and significant reduction of apple scab on both spur and shoot leaves (Table 10). On shoots, the scab control was better with these compounds since at some time points the effects were similar or even better than in the spray standard. Shortly after injection, propiconazole caused phytotoxicity as scorching of young leaves which prevented scab rating until 14 June when trees re-foliated (Table 10). However, this fungicide provided similar scab control as the other injected compounds.

In 2013, continued disease rating showed prolonged significant control of apple leaf scab with phosphites with no additional injections in contrast to the regularly applied spray standard in this season (Table 11). Propiconazole failed to provide continuous prolonged control of leaf scab during 2013. Similarly, at most of the time points in 2013 apple scab control with phosphites was similar to the spray standard (Table 11).

Overall, spray standard provided the best control of spur leaf scab in 2012 and 2013. Injected phosphites were very similar to or have outperformed the spray standard in shoot leaf scab control.

Table 8. Biopesticides and fungicides for control of apple scab used for trunk injection and spraying of 'Red Delicious' apple trees in 2012. [1]DFH - trunk diameter at one foot height (30.48 cm). *Dates followed by an asterisk indicate application of fenarimol.

Treatment	Active ingredient	Dose	Dates of injection or spraying			
Phosphojet 1	mono- and di-potassium salts of phosphorous acid 45.8%	2.59 ml/ 25.4 mm DFH[1]	21 March	20 April	25 May	22 June
Phosphojet 2		5.17 ml/ 25.4 mm DFH				
Prophyt	potassium phosphite 54.5%	2.18 ml/ 25.4 mm DFH				
Nutrol	potassium dihydrogen phosphate 50%	3.56 ml/ 25.4 mm DFH				
Milstop	potassium bicarbonate 85%	3.63 g/tree				
Oxidate	hydrogen peroxide 27%	15 ml/tree				
Alamo	propiconazole 14.3%	12.5 ml/ 25.4 mm DFH				
Water injected untreated control	-	30 ml/ 25.4 mm DFH				
Spray standard: Penncozeb + Rubigan	mancozeb 75% + fenarimol 12%*	2.72 kg/ 0.405 ha + 354.88 ml/ 0.405 ha	***22 March, 3, 13, 18* April, and 1 May 2012 22 April, 1, 8, 16*, 21 May and 4 June 2013***			

Table 9. The effects and interactions of trunk-injected compounds and time on apple scab control on 'Red Delicious' trees.

Experiment		Main effects	*F*	DF	*P*-value
Control of apple scab on	spur leaves in 2012	Treatment	21.24	24	**≤0.0001**
		Time	7.94	24	**≤0.0095**
		Treatment*Time	2.04	24	≥0.0915
	shoot leaves in 2012	Treatment	8.13	27.5	**≤0.0001**
		Time	69.18	56.5	**≤0.0001**
		Treatment*Time	4.78	60.3	**≤0.0001**
	spur leaves in 2013	Treatment	28.39	5.21	**≤0.0009**
		Time	122.3	8.4	**≤0.0001**
		Treatment*Time	40.89	4.6	**≤0.0008**
	shoot leaves in 2013	Treatment	16.61	18.2	**≤0.0001**
		Time	96.36	34.8	**≤0.0001**
		Treatment*Time	4.43	41.4	**≤0.0001**

Table 10. Control of apple scab on leaves with trunk-injected biopesticides and fungicides on 'Red Delicious' apple trees in 2012 (*t*-tests, α=0.05). [1]WIC: water injected untreated control, SpraySTD: spray standard. [2]Treatment means within one date followed by different upper-case letters are significantly different. [3]Means within one treatment across the four dates followed by different lower-case letters are significantly different. [4]Missing ratings due to phytotoxicity caused by injected compound.

Treatment	Mean apple leaf scab incidence [%]				
	Spurs	Shoots			
	Mean for 4 and 18 May	4 May	18 May	14 June	17 August
WIC[1]	81.93 A	35.89 A[2] a[3]	68.91 Ab	74.56 Ab	24.23 ABc
Milstop	72.36 AB	26.69 ABa	39.74 Bb	62.11 ABc	17.5 BCd
Oxidate	61.1 BC	11.41 DEa	38.57 Bb	45.26 Bb	21.01 ABc
Phosphojet 1	60.82 BC	21.65 BCa	32.37 Bb	24.18 Cab	13.04 CDc
Nutrol	60.42 BC	16.79 BCDa	32.82 Bb	45.91 Bc	16.81 BCa
Prophyt	53.49 C	12.72 CDEa	26.12 Bb	22.59 Cb	12.35 CDa
Phosphojet 2	47.67 C	16.33 CDEa	28.11 Bb	20.38 Ca	9.23 Dc
Alamo	-[4]	-	-	29.95 Ca	15.11 BCb
SpraySTD	16.68 D	9.5 E	14.06 Cb	28.19 Ccd	30.78 Ad

Table 11. Prolonged control of leaf scab in 2013 with biopesticides and fungicides injected in 2012 on 'Red Delicious' apple trees (*t*-tests, α=0.05). [1]WIC: water injected untreated control, SpraySTD: spray standard. [2]Means within one date followed by different upper-case letters are significantly different. [3]Means within one treatment across the four dates followed by different lower-case letters are significantly different.

Treatment	Mean apple leaf scab incidence [%]					
	Spurs		Shoots			
	13 June	3 July	13 June	3 July	30 July	26 August
WIC[1]	35.85 A[2]b[3]	75.63 Aa	33.81 Ab	65.57 Aa	71.94 Aa	79.99 Aa
Alamo	34.07 Ab	58.61 Ba	25.21 ABc	52.97 ABb	47.05 Bb	77.93 Ba
Phosphojet 1	12 Ba	27.4 CDa	12.9 BCc	22.94 CDb	28.43 CDb	46.84 Ca
Prophyt	11.56 Bb	31.65 Ca	14.34 BCb	40.96 BCa	36.02 BCa	44.52 CDa
Phosphojet 2	8.28 BCb	21.75 Ca	9.39 Cc	23.97 Db	20.03 Db	31.82 Da
SpraySTD	3.59 Ca	9.63 Da	2.75 Dd	13.06 Dc	21.32 Db	37.92 CDa

Table 12. Chemical properties of injected compounds for apple scab control. [1]Koc: organic carbon-water partitioning coefficient (ml/g or µg/g).

Active ingredient	Water solubility	Koc[1]
mono- and di-potassium salts of phosphorous acid	500 g/L (phosphorous acid: 3100 g/L)	228 - 587
potassium phosphite		
potassium dihydrogen phosphate	100 - 250 g/L	375 - 440
propiconazole	100 - 150 mg/L	382 - 1817
hydrogen peroxide	1,000 g/L	0.2 - 13.2
potassium bicarbonate	263,000 - 337,000 mg/L	1

Discussion

This study provides new insight on the effect and lasting of trunk-injected phosphite biopesticides and other fungicides on apple scab fungus *V. inaequalis*. After four injections in one season, control of leaf apple scab with potassium phosphites lasted for two seasons and at some time points provided comparable control to two seasons of standard spray applications. With few exceptions in 2013, control of apple scab on shoot leaves was better than on the spur leaves. The data imply that, if improvements are made in the timing of injections, the compound dosing and their formulations, trunk injection could provide acceptable control of apple scab for 1-2 seasons.

Good control of apple scab with the injected potassium phosphites indicates on their substantial accumulation in leaves and their translocation from trunk to the canopy extending over two seasons. This shows that trunk injection is a superior way for delivering phosphite biopesticides because it enhances their activity to be partially similar in effect to two-year worth standard sprays against apple scab. If the injection timeline and doses are improved to provide more efficient scab control, this could lead to a significant reduction of fuel and pesticide costs within apple disease management. Similar effect, of injected fungicides on apple scab on leaves and fruit extending to even 3 years of activity was reported on 'Crown Gold' apples (Percival & Boyle 2005). However, in this study, used scale for scab severity rating was primarily depicting aesthetic impact on the tree. This is a less precise indication of the true economic impact of apple scab on the tree canopy in agriculture, where apple scab incidence rating is used for this purpose (Ehret et al. 2010). Therefore, future research should determine whether the injected phosphites also provide acceptable fruit scab control.

With the weaker effects provided by potassium dihydrogen phosphate, propiconazole, and hydrogen peroxide, good control with phosphites implies that specific chemical properties of the injected compounds govern variable speed of their translocation and accumulation and hence disease control in the apple canopy. The two most important properties for trunk injection are water solubility and organic carbon-water partitioning coefficient (Koc) which influence the amount of mobile compound in the sap and the level of compound adhered to the carbon rich compounds in the xylem, respectively (Doccola et al. 2012; Doccola & Wild 2012). Once in xylem, the compound interacts with the chemical components of apoplast and living symplast and with high Koc and low water solubility the binding is stronger and release and translocation of the compound into the canopy is slower over time. This effect is known as the temporal reservoir effect in the trunk and was described in trunk-injected apple trees and proven in *Pinus* and *Fraxinus* species (Pinkas et al. 1973; Shabi et al. 1974; Takai et al. 2003; 2004; Mota-Sanchez et al. 2009; Tanis et al. 2012). The water solubility and Koc values of injected compounds shown in Table 12 align well with the achieved effects on apple scab in 2012 and 2013 (European Commission 2003; Herner & Acock 2003; BCP Council 2011; EFSA 2012; HSDB 2012; Norman et al. 2012; Sigma-Aldrich 2012). However, since the injected hydrogen peroxide and potassium bicarbonate did not perform in accordance with their chemical properties, this indicates on their metabolic change in the plant and too low accumulation in leaves or that these compounds' modes of action are not potent enough for *V. inaequalis*.

Since apple scab control on shoots was overall better than on spurs, this indicates on differential accumulation of the injected compounds between shoot and spur leaves. This can be assigned to the higher rate of water transpiration occurring from shoots versus spurs, because the leaf area on shoots is larger and increases in time with growth in comparison to spurs. Higher

transpiration through more stomata drives the injected compound to accumulate more in shoots than in spurs, thus reducing *V. inaequalis* infections. The number of stomata varies across organs in the canopy and can change during the season. For example, vegetative flowers parts in *Malus* spp. have 10- to 100-fold lower frequency of stomata on epidermis in comparison to abaxial epidermis in leaves (Blanke & Lenz 1989). These authors report that stomatal frequency declines with fruit expansion and formation of lenticelles, which leads to decrease in permeability of fruit epidermis. We speculate that the accumulation of trunk-injected compounds in fruits should be lower than in leaves and should decline over time due to fruit development. Another contributing factor to the weaker control of spur leaf scab is that spur leaves unfold first at the beginning of the season and reach their full size very early, when total tree transpiration is very low. Hence the translocation and accumulation of injected compounds in spur leaves was likely very low and allowed higher disease incidence (Chaney 1979; 1986). However, in 2013 this higher scab incidence on spurs was expressed less than in 2012 probably because with more time the accumulation of injected compound in spurs equalized with the amount accumulated in shoots and thus the effect on apple scab was more similar between them. The spur leaves emerging in 2013 received doses which controlled primary scab infections in spring. Thus, the movement of protective compounds after trunk injection is a time-consuming process which needs to be taken into account for maximizing the effect in relation to the pathogen life cycle.

Significant decline in apple leaf scab incidence from June to August 2012, in all treatments except the spray standard, indicated on the leaf drop from basal shoot sections due to scab infections and on the development of new leaves on shoot termini. However, the magnitude i.e. the slope of this decline varied between treatments because of the variable protection effects provided by the injected compounds. Hence, it is likely that the levels or accumulated

compounds in basal shoot leaves varied upon their chemical properties and were lower due to early season development of basal leaves when transpiration driven accumulation was weak. In 2013, the leaf drop at the end of the experiment was absent probably because of prolonged accumulation of injected compounds in leaves which provided good and long-lasting control of all the leaf strata on the shoots. At last, formation of new leaves on shoot tips contributed to the reduction of the apple scab incidence in August. These leaves get much less or no scab infections because the ascospores of *V. inaequalis* in continental climate stop being discharged from pseudothecia between the end May and beginning of July (Smith et al. 1988). In our injection experiment, secondary infections with conidia of *Spilocaea pomi* Fr. & Fr. were most likely prevented or severely reduced by the injected compounds. Significant increase of apple scab incidences on shoot leaves in 2013 suggests that the accumulated amount of injected compounds was weakened in effect by new leaf growth which "diluted" their effect and led to fading of scab reduction.

Propiconazole caused phytotoxicity on leaves probably because the total dose per tree in 2012 was very high and its viscosity increased due to addition of 1 L of water with each injection. This substantially diluted the compound and increased its mobility in the xylem, thus leading to the higher accumulation in the apple crown.

In summary, trunk injection of potassium phosphites and maybe other fungicides could be a useful option for apple scab control during 2 seasons. Besides reducing the costs of application and used compound, trunk injection would enable decrease of pesticide usage in the open environment and increase the effect of injected compound (Zhang et al. 2011). However, injectable formulations of fungicides are few and only available for efficient disease and pest control in landscape tree care industry. If more injectable compound formulations are discovered,

and the timing and doses of injection are optimized for efficient control, *in planta* delivery of plant protective compounds could provide acceptable management of fruit tree diseases and pests in agriculture.

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CHAPTER 5. SEASONAL AND CROSS-SEASONAL TIMING OF FUNGICIDE TRUNK INJECTIONS ON APPLE TREES TO OPTIMIZE MANAGEMENT OF APPLE SCAB FUNGUS *VENTURIA INAEQUALIS*

Abstract

To optimize the number and timing of injections for acceptable seasonal control of *V. inaequalis* we evaluated 1-2 and 4 cross-seasonal and 1-2 seasonal injections of potassium phosphites and synthetic fungicides. Fungicide residues in leaves and fruit were quantified to determine the accumulation timing and alignment with the effects on apple scab. The fungicide, and number and timing of injections significantly affected apple scab control and residue profiles in the canopy. Injected phosphites aligned better with the apple scab effects and provided better control than propiconazole, cyprodinil and difenoconazole. Due to different transpiration footprints and chemical properties of the fungicides, the effects varied among canopy organs with best scab control on shoot leaves, fruit, and then spur leaves. Overall, only 2-3 injections in spring provided good disease control. Higher residue accumulation after fall injection followed by 1 or 3 spring injections did not improve apple scab control due to overall ineffective concentrations. Residues of synthetic fungicides in fruit were far below the MRLs and declined towards the end of the season. The fruit residues of potassium phosphites increased over time but are exempt from food tolerances. Due to their higher mobility in xylem, injected phosphites accumulated in the canopy at a much higher concentrations than all the synthetic fungicides. After spraying and injection, the phosphite residues were similar while the synthetic fungicides had much higher residues after spraying. Injected phosphites showed better or equal and more

persistent apple scab control with fewer injections than sprays per year, while the sprayed synthetic fungicides provided better control but weakened in effect towards the end of the season. Overall, trunk injection of fully systemic and injection formulated fungicides could be a viable option for cheaper and eco-friendly apple scab control.

Introduction

The efficiency of protective compounds in plant disease control is crucially dependent on the timeline of compound application. The treatments against plant pathogens are scheduled in accordance with their life cycles and the critical growth stages of the plant. For maximum effect, the protective compounds are applied primarily before the major infection periods of the pathogen. For the most important fungal pathogen of apple, *Venturia inaequalis* (Cooke) Wint., which causes apple scab, frequent fungicide sprays are required during the season to protect the leaf canopy and yield and to secure blemish-free fruit to be marketable at a high price. From the beginning of discharge of *V. inaequalis* ascospores in spring the sprays are usually repeated every 7-10 days or just before each of the many ascospores discharge cycles. Therefore, the used fungicides for apple scab management represent the largest cost in apple production. Each of the spray treatments is efficient for only a limited period of time due to development of new tissues that require protection. The goal of frequent sprays is to prevent the establishment of primary ascospore infections on fruit and leaves which could lead to formation of multiple generations of conidia and enable further devastating secondary infections. Once the ascospore discharge from pseudothecia ceases, which in continental climate usually occurs between the end of May and beginning of July, further sprays are rarely needed if the primary infections were successfully

controlled (Smith et al. 1988). However, if the protection from primary infections was weak due to improper timing of sprays, further treatments are needed to protect any non-infected fruits from secondary infections and reduce the inoculum potential on leaves for the following season. In this case, the overall efficiency of seasonal of apple scab management is reduced and overly expensive due to minimized return in high quality yield. To correctly align the spray treatments before the infection periods, ascospore maturity and release is monitored and computerized forecast models for apple scab based on pathogen ecology and weather conditions are used (MacHardy & Gadoury 1985; Sutton et al. 2000; Berrie & Xu 2003; Holb et al. 2003). However, these systems and forecast are often unavailable to many users and can be of variable accurateness in different climates due to variability of distant *V. inaequalis* populations and the influence of specific weather conditions. Overall, studies comparing accuracy of different models are lacking and model revisions are often needed to improve their predictive preciseness (MacHardy & Gadoury 1989; Beresford & Spink 1992; Denzer 1996; Magarey et al. 2007).

Besides these difficulties in disease control, frequent topical application of pesticides creates drift-driven pesticide losses which contaminate soil, air, wildlife and fruit (Pimentel 2005; Pimentel & Lehman 1993; Ecobichon 1999). Fungicide application can lead to excessive accumulation of their residues in fruit (FAO & WHO 2004; Hamilton et al. 2004; Rawn et al. 2007). This increases health risks for the applicators and consumers (Pimentel et al. 1992; Mills 1998; Helling et al. 2000; Pauker 2003; Hamilton et al. 2004; Yen et al. 2009). Public concern over the pesticide residues in fruit has put apple, one of the most sprayed trees in agriculture, at the center of attention (Sutton 1996). The majority of sprayed pesticides on apple are fungicides for control of *V. inaequalis*.

Being that the air-blast sprayers used for tree protection deliver only 29-56% of the pesticide solution to the canopy, it is of prime importance to investigate more target-precise delivery methods for increasing the level of sustainability in fruit-tree agriculture (Steiner 1969; Pimentel & Levitan 1986; Reganold et al. 2001). Even though the movement and accumulation of systemic compounds in trees is a time consuming process (Aćimović et al. 2014), trunk injection as an *in planta* delivery method allows pesticides to persist longer in their effect and potentially protect new growth during longer periods within one or even for 2-3 seasons (Percival & Boyle 2005; J. J. Doccola et al. 2007; Smitley et al. 2010; Byrne et al. 2014). Trunk injection is a target-precise compound delivery to trees which utilizes tree xylem to distribute compounds into the canopy and could significantly reduce the use of pesticides in the open environment (Zhang et al. 2011). This method of delivery is widely used for control of insect pests and pathogens in landscape tree care (Haugen & Stennes 1999; Doccola & Wild 2012).

Trunk injection could provide unprecedented benefit in apple production by replacing numerous spray treatments with one to several injections of pesticides (Percival & Boyle 2005; Spitko 2008; Düker & Kubiak 2011a). Previous research in apple scab management by trunk-injected fungicides showed that a single injection of potassium phosphites, trialzoles and other compounds can provide 1-3 seasons of significant disease reduction on leaves and fruit (Percival & Boyle 2005; VanWoerkom 2012). Our experiments showed that 4 seasonal injections of potassium phosphites and propiconazole, conducted in spring and month apart, provide two years of apple scab control on leaves, mostly comparable to two-year worth standard fungicides sprays (Aćimović et al., *unpublished*). However, it is unclear when and how many fungicide injections are needed to achieve economically acceptable apple scab control on fruit and leaves and whether the injection in fall of the previous season could increase the efficiency of apple scab

control in the following season. Further, the research demonstrating relation between the effects on *V. inaequalis* and the fungicide residues in leaves and fruit is limited (Pinkas et al. 1973; VanWoerkom 2012). Since the movement of pesticides in xylem after trunk injection is dependent on tree physiology and chemical properties of the injected compound (Doccola et al. 2012; Aćimović et al. 2014), to maximize efficiency it is important to optimize the number of injections and their seasonal timing in accordance with fungicide residues in the canopy and thus achieve good apple scab control during major infection periods. Similar work on trunk-injection control of avocado thrips, *Scirtothrips perseae* Nakahara, emphasizes the importance of injection timing and accumulated insecticide residues in leaves for optimal effects (Byrne et al. 2014).

Aiming to improve the efficiency of trunk injected fungicides in apple scab control, our first objective was to determine and compare the efficiency of 1-4 cross-seasonal or within-seasonal fungicide injections at different doses. The second objective was to determine relationship between apple scab control and the temporal residue profiles of injected fungicides in leaves and fruit. This would indicate how the injection timing schedule can be optimized for maximizing the effect on pathogen. Our leading hypothesis was that 4 versus 1-2 injections of potassium phosphite and triazole fungicides will provide significantly better apple scab control. Further, we hypothesized that due to more time allowed for compound translocation in xylem, fall injection will improve the control of apple scab in the spring. At last, we predicted that the effects on apple scab after injection will align well with the fungicide residues in leaves and fruit, and will be comparable to standard fungicide spray program or the sprays with the same compounds used for injection.

The main goal of our these experiments was to determine whether different timing of trunk injection will significantly enhance the activity of injected fungicides and provide

significant control of *V. inaequalis* (Heaton & Dullahide 1990; Wicks & Hall 1990; Guest et al. 1995).

Materials and Methods

Trunk injection and apple scab evaluation

Two-year orchard experiments were conducted at Michigan State University's Trevor Nichols Research Center (TNRC) in Fennville, MI (GPS: N42° 35' 56.64", W86° 09' 14.06"). Mature, 29-yr-old trees of 'Mac Spur' apple, *Malus domestica* Borkhausen, were trunk-injected either 4 times across two calendar years or 1-2 times across two of within one calendar year using the compounds and doses in Table 13. On 11 April 2012, injected trees were in 50% bloom growth stage and on 21 April 2013 they were in silver tip growth stage.

The injection doses of propiconazole and potassium phosphites (Arborjet Inc., Woburn, MA) were based on our previous research (Aćimović et al. *unpublished*; Vanwoerkom, 2012), while the dose of difenoconazole and cyprodinil combination (Inspire Super® EW, Syngenta, Basel, Switzerland) was derived from dividing the maximum seasonally allowed dose of this fungicide according to the EPA label in USA by 250 apple trees grown per one acre (0.405 ha). Injection doses in 2011 were not amended with water while in 2012 only propiconazole was amended with 20 ml of water per 25.4 mm of trunk diameter at one foot height i.e. 30.48 cm or DFH (29.33%; 45.36%). Each dose of difenoconazole-cyprodinil combination (DCC) was injected with 500 ml of water per tree (1.4%). No water was added to injected potassium

phospites. Injected trees in 2012 ranged from 17.78-32.51 cm with the average of 26.6±0.72 cm in DFH and in 2013 they ranged from 21.59-35.56 cm with the average of 28.04±0.53 cm.

For 2012, we compared the effect of injected compounds on apple scab to the spray standard of Penncozeb™ 75DF (Cerexagri Inc., King of Prussia, PA) and Rubigan® E.C. (Dow AgroSciences LLC, Indianapolis, IN) and in 2013 to the spray standards of Agrifos® (Agrichem Manufacturing Industries PTY., Ltd., Loganholme, Queensland, Australia) and Inspire Super® SC. Each spray treatment in 2012 was delivered with 189.25 L of water per acre, while in 2013 Agrifos was delivered with 56.78 L of water per acre and Inspire Super SC with 94.63 L of water per acre. Timely sprays were conducted based on alerts from apple scab forecast model through Enviro-weather station at TNRC in Fennville, MI (http://enviroweather.msu.edu). All experiments were arranged in a completely randomized design (CRD) with 6 replicate trees per treatment.

At each injection date, a separate set of four cardinally oriented injection ports per tree were created by drilling 25.4 mm deep into the xylem tissue and 9.53 mm in diameter (1500 rpm cordless drill, DeWalt Industrial Tool Co., Baltimore, MD) (Aćimović et al. 2014). The first set of ports was positioned approximately 20-25 cm above the ground level, while the following ones were positioned between and above the former ones. All ports were sealed with Arborplug® no. 4 (Arborjet Inc., Woburn, MA). All injections in fall 2011 were conducted with Quik-jet micro-injection system (Arborjet Inc., Woburn, MA). On 11 April 2012, due limited leaf canopy on trees and thus weaker transpiration pull in the wood xylem, propiconazole was injected with Viper air/hydraulic micro-injection system® (Arborjet Inc., Woburn, MA). Since the leaf canopy was more developed, later trunk injections of propiconazole were conducted with Tree IV air/hydraulic micro-injection system® (Arborjet Inc., Woburn, MA). In 2012 and 2013,

Phosphojet was always injected using Quik-jet, while in 2013 all injections of DCC were injected using Tree IV. Injection needles of these devices were inserted through the Arborplugs and the total injected volume per tree, for one time of injection, was divided and delivered equally among the four ports.

Within each treatment we rated apple scab on leaves in 2012 and on leaves and fruit in 2013. We chose 20 spurs and 20 terminal shoots per tree replicate and rated the leaf scab incidence as described before (Ehret et al. 2010). Due to fruit loss by frost during bloom, fruit scab was not rated in 2012. In 2013, apple scab incidence on fruits was rated on 100 fruits per tree or if less than 100, all the fruits on tree were rated for fruit scab incidence. Usually, 25 fruits per each cardinal crown direction were rated.

The apple scab control effects in both years were analyzed with a mixed model using the MIXED procedure in SAS 9.3 (SAS Institute, Cary, NC, 2011). Prior to statistical analysis, data on control of spur and shoot leaf scab in 2012 were cosine and square root transformed, respectively. We analyzed control of the spur and shoot leaf scab in 2012 with repeated measures best adjusted using a first-order heterogeneous autoregressive covariance structure and a spatial power covariance structure, respectively. Prior to statistical analysis, data on control of spur leaf scab in 2013 were arcsine transformed. The control of spur leaf scab in 2013 was analyzed with time as a fixed factor while the control of shoot leaf scab in 2013 was analyzed with repeated measures best adjusted with a spatial power covariance structure. The data on fruit scab control in 2013 were cosine transformed and then analyzed with repeated measures best adjusted using a spatial power covariance structure. In all analyses tree was used as subject of repeated measurements. When the main effects or their interactions were statistically significant ($P < 0.05$), examination i.e. slicing of interactions within main effects was performed, *F*-tests were

conducted and pairwise or specific time or treatment comparisons were conducted using *t*-tests ($\alpha = 0.05$).

Fungicide residue analysis

In 2012, we collected only leaf residue samples and in 2013 we collected bud, leaf and fruit residue samples. We collected one composite sample of 40 uniformly chosen leaves or buds per replicate tree crown (10 leaves per each cardinal direction). Leaf samples in 2012 were taken at 0, 14, 28, 42, 56, 70 and 84 days after first injection in 2012 (DAFI). Leaf and bud samples in 2013 were taken 14 DAFI in fall 2012 and then at 0, 10, 24, 38, 52, 66 and 80 days after the second injection in 2013 (DASI). In 2013, depending on the fruit size during the season, we collected one composite fruit sample per tree consisting of 20 (spring) to 4 (summer) uniformly picked fruits across the tree crown (5-1 fruits per each cardinal direction). The average fruit sample weights at 40, 58, 80 and 133 DASI were 16.73, 55.61, 69.8 and 77.12 g, respectively.

We stored the residue samples for all the fungicides except potassium phosphites in 20, 40, 60, 75 or 90 ml of HPLC-grade dichloromethane (DCM, EMD Chemicals Inc., Gibbstown, NJ), depending on sample size, and held at 5ºC. To determine fungicide residues in all samples we conducted the extraction using previously reported protocol (Aćimović et al. 2014).

The samples for quantification of potassium phosphite residues were stored at -20ºC until the extraction. To each sample we added 60 ml of 1% sulfuric acid (Jade Scientific Inc., Canton, MI) and left them overnight on room temperature. The next day sulfuric acid solution was decanted through filter paper (Whatman, 150 mm, GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, UK) and to each sample we added 60 ml of isopropyl alcohol (J.T. Baker

Chemical Co., Phillipsburg, NJ) and left overnight on room temperature. The next day alcohol was decanted through a filter paper and into the same flask with the decanted sulfuric acid solution. For bud samples we added only 20 ml of each sulfuric acid and isopropyl alcohol. Each sample extract was separated from the solvents and dried at 50ºC using an R-114 rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland). Dry extract residue was dissolved in 2 ml of 1% sulfuric acid. To remove any remaining particulates, dissolved sample residue was collected and passed through an HPLC certified 0.45 μm Acrodisc CR 25 mm syringe filter with PTFE membrane (Pall Corp., East Hills, NY). Sample was stored at 5ºC in 2 ml HPLC glass vials (Agilent Technologies Inc., Santa Clara, CA) until HPLC/MS analysis.

Samples were analyzed for propiconazole residue (parent compound) with Agilent Technologies 7890A GC system/MSD-5973 Mass Selective Detector Network with oven program of 70ºC for 1 min then 20ºC per min to 300 ºC for 1.5 min The column was Agilent HP-5MS 5% phenyl methyl silox at 325ºC: 30 m × 250 μm × 0.25 μm (Agilent Technologies Inc., Santa Clara, CA). MS source was at 230ºC, maximum 250 ºC, and MS quad was at 150ºC maximum 200ºC. MS collision energy was 69.922. Auxiliary temperature was 280ºC. Monitored ions for propiconazole were 259.1 and 173.0 *m*/*z* (Da). The level of sensitivity for propiconazole was 0.05 mg kg^{-1} of active ingredient (AI), and level of detection was 0.165 mg kg^{-1}.

Samples were analyzed for difenoconazole, cyprodinil and phosphorous acid (parent compounds) with a Waters 2695 Separator module HPLC equipped with a Waters MicroMass ZQ2000 mass spectrometer detector and a Waters X-Bridge C_{18} reversed phase column 50×3.0 mm bore, 2.5 μm particle size (Waters Corp., Milford, MA). The mobile phase, solvent A, was water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. For difenoconazole and cyprodinil these was initially held at 75% solvent A and 25% solvent B and

followed by a gradient shown in Table 14. For phosphorus acid, these were initially kept at 90% solvent A and 10% solvent B, followed by a gradient shown in Table 14. The column temperature was 40ºC. Monitored ions for difenoconazole were 250.87 and 405.96 *m/z* (Da) and for cyprodinil 93.14, 118.51 and 226.18 *m/z* (Da) (ion mode was ES plus). Monitored ions for phosphorous were 38.5, 38.96, 80.07 and 80.7 *m/z* (Da). For phosphorus acid the level of sensitivity was 0.005 mg kg^{-1} and the level of detection was 0.0165 mg kg^{-1}. The levels of sensitivity for difenoconazole and cyprodinil were 0.00510 and 0.0505 mg kg^{-1} and the levels of detection were 0.01683 and 0.16665 mg kg^{-1}, respectively. The HPLC or GC level of quantification was 3.3 mg kg^{-1} AI. The results have not been corrected for AI recovery.

The residue data for each trunk-injected compound were analyzed with mixed models using the MIXED procedure in SAS 9.3 (SAS Institute, Cary, NC, 2012). Before statistical analysis, data on propiconazole and phosphorous acid residues in leaves in 2012 were square root and arcsine transformed, respectively. We analyzed the residues of these two AI-s with repeated measures best adjusted using an unstructured and a first-order heterogeneous autoregressive covariance structures, respectively.

The data on difenoconazole and cyprodinil residues in leaves in 2013 were logarithm 10 and square root transformed, respectively. We analyzed the residues of cyprodinil with repeated measures best adjusted using a heterogeneous compound symmetry covariance structure considering time as a fixed factor, while the residues of difenoconazole were analyzed with repeated measures best adjusted using an unstructured covariance structure. The apple fruit residue data on cyprodinil and difenoconazole were both logarithm 10 transformed. We analyzed the fruit residues of cyprodinil with repeated measures best adjusted using an unstructured

covariance structure, while the fruit residues of difenoconazole were analyzed with time as a fixed factor considering equal correlation between the disease rating times.

The data on phosphorous acid residues in apple leaves and fruits in 2013 were logarithm 10 and sine transformed, respectively. We analyzed the leaf residues with repeated measures best adjusted using an unstructured and a first-order heterogeneous autoregressive covariance structures, respectively. Tree was used as subject of repeated measurements. When the main effects or their interactions were statistically significant ($P < 0.05$), examination i.e. slicing of interactions within main effects was performed, *F*-tests were conducted and pairwise or specific time or treatment comparisons were conducted using *t*-tests ($\alpha = 0.05$).

Results

Apple scab control

In 2012, all the injected fungicides significantly reduced leaf apple scab on spurs and shoots (Tables 15 and 16). Spray standard provided the best scab control on spur and shoot leaves only on 1 May. On shoot leaves scab control has improved with time since more injection treatments provided comparable or even better control than the spray standard (Table 16). Overall, the injected potassium phosphites provided better leaf scab control than propiconazole and were most similar to even better than spray standard.

In 2013, the injected fungicides again significantly affected leaf apple scab (Table 15) but provided weaker control than in 2012 (Table 17). On spur leaves potassium phosphites provided better control than the difencoconazole-cyprodinil combination (DCC). Two injections of

phosphites provided better control than all the single injections and the sprays of these compounds. On shoots, the control with DCC was slightly improved after spring injections only (Table 17). The injected phosphites again improved in effect through time. Both treatments with two injections were similar and even better than the sprays of phosphites and the DCC. On fruits, phosphites injected twice or once in the spring provided comparable or better control than with the sprays of phosphites or DCC, and a single fall injection of phosphites (Table 17).

Residue profiles

In 2012, fungicide injections led to significant accumulation of propiconazole and phosphorous acid in leaves (Table 15). Residue concentration of propiconazole significantly increased over time (Figure 13A) but the two doses did not differ significantly. Phosphorous acid accumulated in leaves at a much higher concentration than propiconazole but did not change significantly over time (Figure 13B). The two doses of potassium phosphites significantly differed only in April 2012.

In 2013, the applied fungicides significantly affected the residue levels in apple buds, leaves and fruit and significantly interacted with time (Table 15). For reasons of clarity, differences between residue means across time points within each treatment are not shown (Figures 14-17). The injected cyprodinil and difenoconazole always accumulated more in leaves than in apple fruits. In apple buds and leaves, residues of injected cyprodinil and difenoconazole were 10- to 100-fold lower than after sprays (Figure 14A-C). The residues significantly increased in time in all treatments but this was more rapid after sprays than after injections. The time decline of residues was more rapid after sprays and much more gradual after injections. Overall,

injected cyprodinil accumulated in buds and leaves at a 10-fold higher concentration than difenoconazole (Figure 14B, C). Accumulation of cyprodinil in buds and leaves was much faster than of difenoconazole. Both injected fungicides show that fall injections allow significantly higher accumulation of residues in comparison to all single or double injections in spring (Figure 14B, C, Table 15).

In apple fruits, residues of injected cyprodinil and difenoconazole were only 10- fold lower than after sprays (Figure 15A-C). Over time, the residues declined more rapidly in spray treatment and fall injections versus the spring injections. Injected cyprodinil accumulated in fruits at a 10-fold higher concentration than difenoconazole (Figure 15B, C). Fall injection secured similar pattern of fungicide residue accumulation as after spraying but largely did not secure higher fungicide concentrations when compared to spring injections (Figure 15A-C). Overall, bud, leaf and fruit residues in all spring injections were similar in time regardless of the specific dose load.

The residues of phosphorous acid were always higher in comparison to cyprodinil and difenoconazole (Figures 14-17). Potassium phosphites injections led to largely similar accumulation of phosphorous acid in buds, leaves and fruit and its accumulation was much slower in time and peaked later when compared to cyprodinil and difenoconazole. The leaf residue profiles of injected phosphites were similar or higher than after sprays and depending on time, fall injections allowed similar and higher residue profiles than the spring injections and sprays. Their accumulation in fruits was slower and peaked later than in leaves. Phosphorous acid accumulation in fruits was largely similar between the injections and sprays.

Table 13. Biopesticides and fungicides for apple scab control used for trunk injection of 'Mac Spur' apple trees from 2011-2013. [1]DFH - trunk diameter at one foot height (30.48 cm).

Treatment	Active ingredient	Dose	Dates of injections or sprayings		
			in 2011	in 2012	
Phosphojet 1	mono- and di-potassium salts of phosphorous acid 45.8%	2.59 ml/ 25.4 mm DFH[1]	15 October	11 April	11 May 8 June
Phosphojet 2		5.17 ml/ 25.4 mm DFH			
Alamo 1	propiconazole 14.3%	8.3 ml/ 25.4 mm DFH			
Alamo 2		16.6 ml/ 25.4 mm DFH			
Water injected control	-	8.2 ml/ 25.4 mm DFH			
Spray standard	mancozeb 75% + fenarimol 12%*	2.72 kg/ 0.405 ha 354.88 ml/ 0.405 ha		27 March, 3, 13, 18* April, and 1 May 2012	
Treatments	**Active ingredient**	**Dose**	**Dates of injections or sprayings**		
			in 2012	**in 2013**	
Phosphojet 1	mono- and di-potassium salts of phosphorous acid 45.8%	5.17 ml/ 25.4 mm DFH	11 October	-	-
Phosphojet 2		2 x 5.17 ml/ 25.4 mm DFH	11 October	21 April	-
Phosphojet 3		5.17 ml/ 25.4 mm DFH	-	21 April	-
Phosphojet 4		2 x 5.17 ml/ 25.4 mm DFH	-	21 April	22 May
Agrifos spray		9 x 1892.71ml/ 0.405 ha on 1, 8, 16, 21, 31 May and 5, 11, 19, 26 June 2013			
Inspire Super 1	difenoconazole 8.4% + cyprodinil 24.1%	7 ml/ tree	11 October	-	-
Inspire Super 2		7 ml/ tree	-	21 April	-
Inspire Super 3		2 x 7 ml/ tree	-	21 April	22 May
Inspire Super spray		5 x 354.84 ml/ 0.405 ha on 1, 8, 16, 21, 31 May 2013			
Water injected control	-	500 ml/ tree	11 October	21 April	22 May

Table 14. The gradient mobile phase flow used for HPLC residue analysis.

Active ingredient	Time (min)	Flow rate (µl min^{-1})	Solvent A (%)	Solvent B (%)
		0.30	90	10
Phosphorous	1.00	0.30	90	10
acid	9.00	0.30	10	90
	9.10	0.30	90	10
		0.30	75.0	25.0
Difenoconazole	5.00	0.30	10.0	90.0
and	7.00	0.30	10.0	90.0
cyprodinil	7.50	0.30	75.0	25.0
	12.00	0.30	75.0	25.0

Table 15. The effects and interactions of trunk-injected fungicides and time on apple scab control on 'Mac Spur' trees and on residue levels in leaves and fruit (α=0.05).

Variable			Main effects	*F*	DF	*P*-value
Control of apple scab on	spur leaves in 2012		Treatment	122.7	10.1	**≤0.0001**
			Time	5.19	17.1	**≤0.0359**
			Treatment*Time	2.24	8.78	≥0.1407
	shoot leaves in 2012		Treatment	62.29	46.8	**≤0.0001**
			Time	173.05	88.9	**≤0.0001**
			Treatment*Time	5.44	94.8	**≤0.0001**
	spur leaves in 2013		Treatment	28.63	50	**≤0.0001**
			Time	28.21	50	**≤0.0001**
			Treatment*Time	1.15	50	≥0.3478
	shoot leaves in 2013		Treatment	60.50	53.2	**≤0.0001**
			Time	86.77	79.9	**≤0.0001**
			Treatment*Time	15.52	80	**≤0.0001**
	fruits in 2013		Treatment	17.92	45.2	**≤0.0001**
			Time	21.48	77.3	**≤0.0001**
			Treatment*Time	2.67	73.8	**≤0.0005**
Residues of	propiconazole	in leaves in 2012	Treatment	0.47	9.78	≥0.5095
			Time	78.36	9.28	**≤0.0001**
			Treatment*Time	1.98	9.28	≥0.1683
	potassium phosphites		Treatment	1.06	22.8	≥0.3149
			Time	2.31	18.3	≥0.0780
			Treatment*Time	4.27	18.3	**≤0.0074**
	cyprodinil	in leaves in 2013	Treatment	108.44	21.7	**≤0.0001**
			Time	130.83	21.2	**≤0.0001**
			Treatment*Time	21.43	32.7	**≤0.0001**
		in fruits in 2013	Treatment	31.15	6.57	**≤0.0003**
			Time	200.42	8.94	**≤0.0001**
			Treatment*Time	16.15	5.11	**≤0.0031**
	difenoconazole	in leaves in 2013	Treatment	218.11	20	**≤0.0001**
			Time	186.7	16	**≤0.0001**
			Treatment*Time	20.31	25.5	**≤0.0001**
		in fruits in 2013	Treatment	18.82	19.8	**≤0.0001**
			Time	29.11	31.7	**≤0.0001**
			Treatment*Time	2.46	29.1	**≤0.0475**

Table 16. Control of apple scab on leaves of 'Mac Spur' apple trees in 2012 injected with fungicides (*t*-tests, α=0.05). [1]WIC: water injected untreated control, SpraySTD: spray standard. [2]Treatment means within one date followed by different upper-case letters are significantly different. [3]Means within one treatment across the four dates followed by different lower-case letters are significantly different.

Treatment	Mean apple leaf scab incidence (%)				
	Spurs	Shoots			
	Mean for 1 and 16 May	1 May	16 May	13 June	15 August
WIC[1]	72.2 A	20.3 A[2]a[3]	50.5 Ab	72.8 Ac	72.5 Ac
Alamo 1	59.8 B	9.7 Ba	26.0 Bb	47.1 Bc	38.6 Bc
Alamo 2	52.5 BC	5.4 CDa	21.2 Bb	36.9 BCc	41.2 Bc
Phosphojet 1	47.8 CD	9.5 BCa	12.4 Cac	29.0 CDb	15.9 Cc
Phosphojet 2	39.3 D	7.5 BCa	7.3 Ca	24.9 Db	17.4 Cc
SpraySTD	15.5 E	3.9 Da	10.6 Cb	31.6 CDc	34.0 Bc

Table 17. Control of apple scab on leaves and fruits of 'Mac Spur' apple trees in 2013 injected with fungicides (*t*-tests, α=0.05). [1]DFH: trunk diameter at one foot height (30.48 cm) [2]WIC: water injected untreated control, IS: Inspire Super (difenoconazole+cyprodinil), PJ: Phosphojet (potassium salts of phosphorous acid), F: fall injection 2012, S: spring injection 2013. [3]Treatment means within one date followed by different upper-case letters are significantly different. [4]Means within one treatment across the four dates followed by different lower-case letters are significantly different.

Treatment/ Season/ Dose in ml per tree (IS) or per 25.4 mm of trunk DFH[1] (PJ)	Mean apple leaf scab incidence (%)				
	Spurs	Shoots			
	Mean for 17 June and 9 July	17 June	9 July	30 July	30 August
WIC[2]	88.3 A	83.0 A[3]c[4]	97.1 Ab	98.1 ABb	99.5 Aa
IS Spring+Spring 7+7	88.4 A	75.3 ABb	80.7 Bb	88.1 BCa	92.6 ABa
IS Spring 7	87.0 A	69.0 Bb	75.6 Bb	83.0 Ca	84.4 BCa
IS Fall 7	83.5 A	83.0 Ac	95.5 Ab	98.7 Aa	98.9 Aa
PJ Fall 5.17	69.5 B	56.6 Cb	73.7 BCa	71.7 Da	79.9 Ca
PJ Spring 5.17	65.8 B	48.2 CDab	44.5 Eb	48.0 Eb	58.2 Da
Agrifos Spray	63.4 B	45.4 Ea	29.6 Fb	34.0 Fab	38.8 Ea
PJ Spring+Spring 5.17+5.17	52.1 C	36.0 EFa	32.3 Fa	23.2 Gb	34.9 Ea
PJ Fall+Spring 5.17+5.17	47.4 C	38.2 DFb	57.0 Da	38.7 EFb	55.4 Da
IS spray	36.3 D	27.5 Ed	66.4 CDc	91.7 ABCb	97.2 Aba
	Fruit				
	17 June	9 July	30 July	30 August	
IS Spring+Spring 7+7	90.6 Abc	100.0 Aa	96.7 Aac	100.0 Aab	
IS Fall 7	89.0 Aa	98.2 Aa	97.9 Aa	99.5 Aa	
IS Spring 7	87.3 Aa	87.5 Aa	83.3 ABa	100.0 Aa	
WIC	84.6 Ab	98.0 Aa	99.2 Aa	100.0 Aa	
Agrifos Spray	60.5 Ba	60.1 BCa	72.5 Ba	78.6 BCa	
PJ Fall 5.17	53.6 BCc	77.0 Bb	93.4 ABa	87.5 ABa	
IS spray	36.1 Cc	62.0 BCDb	82.5 Ba	89.2 ABa	
PJ Spring+Spring 5.17+5.17	28.9 BCa	22.9 DEa	30.6 Ca	33.3 Da	
PJ Spring 5.17	27.4 Cb	26.7 CEb	29.6 Cb	58.5 CDa	
PJ Fall+Spring 5.17+5.17	17.2 Cb	25.7 Eb	47.1 Ca	45.3 Dab	

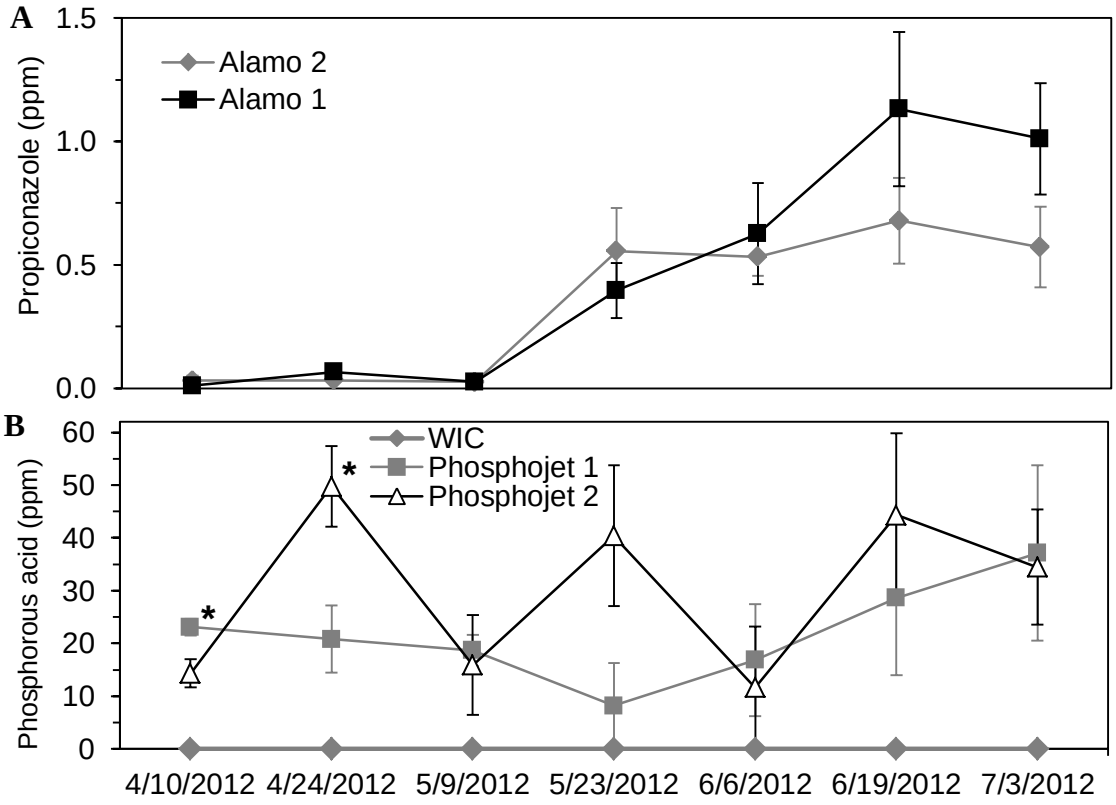

Figure 13. Leaf residue profiles of trunk-injected fungicides in 'Mac Spur' apples. (A) propiconazole (Alamo). (B) potassium phosphites expressed as phosphorous acid (Phosphojet). WIC: water injected control, 1: low dose, 2: high dose. *Means within one date followed by an asterisk are significantly different (*t*-tests, α=0.05). Error bars represent standard error of the mean (SEM).

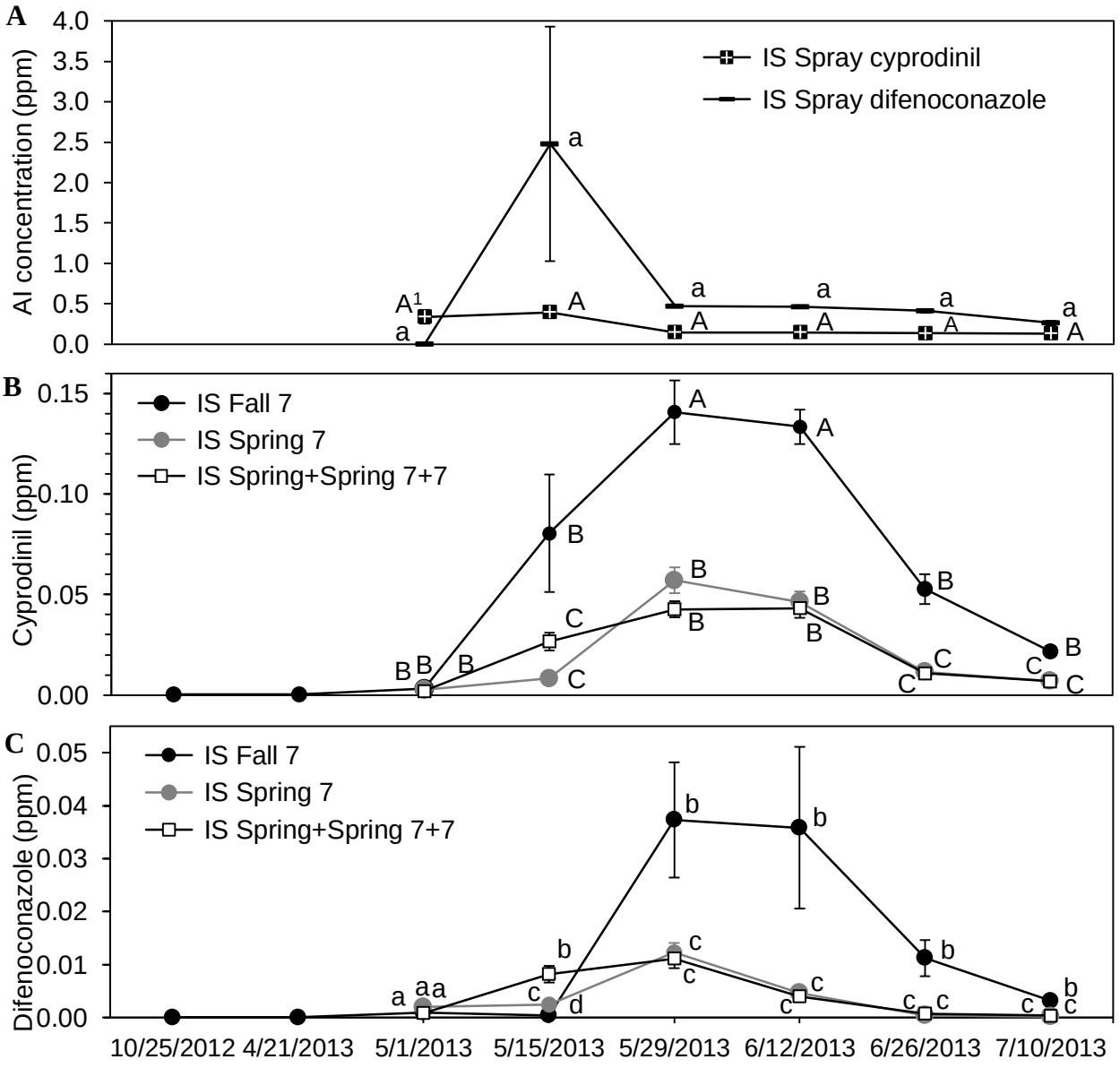

Figure 14. Leaf residue profiles of trunk-injected and sprayed fungicides in 'Mac Spur' apples. (A) sprayed cyprodinil and difenoconazole (IS: Inspire Super), (B) and (C) injected cyprodinil and difenoconazole, respectively. Fall/Spring: injection time, 7: dose per tree (ml). Residue means of one compound within one date followed by different upper- or lower-case letters are significantly different between the treatments and across the graphs (*t*-tests, α=0.05). Error bars represent standard error of the mean (SEM).

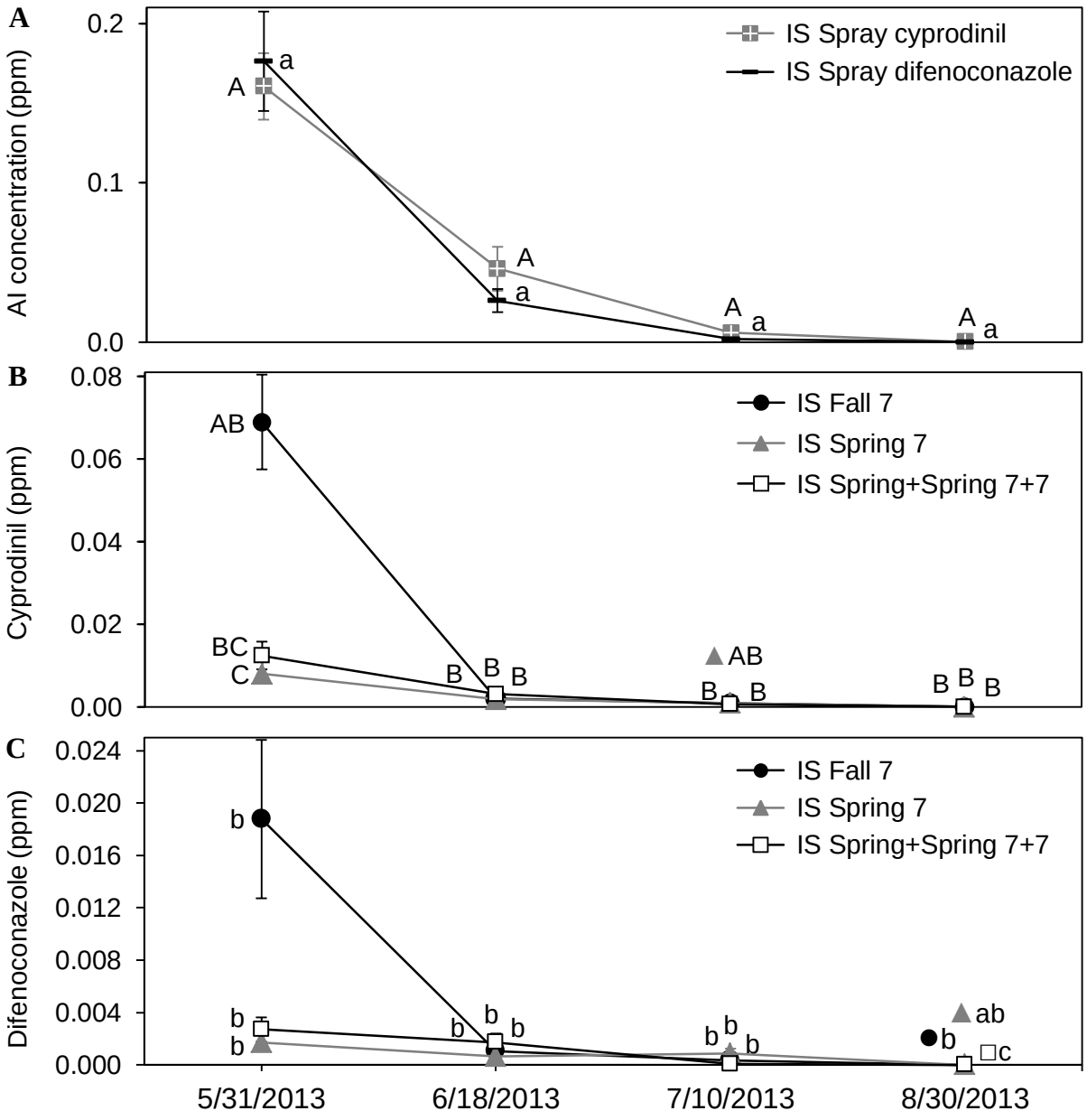

Figure 15. Fruit residue profiles of trunk-injected and sprayed fungicides in 'Mac Spur' apples. (A) sprayed cyprodinil and difenoconazole (IS: Inspire Super), (B) and (C) injected cyprodinil and difenoconazole, respectively. Fall/Spring: injection time, 7: dose per tree (ml). Residue means of one compound within one date followed by different upper- or lower-case letters are significantly different between the treatments and across the graphs (*t*-tests, α=0.05). Error bars represent standard error of the mean (SEM).

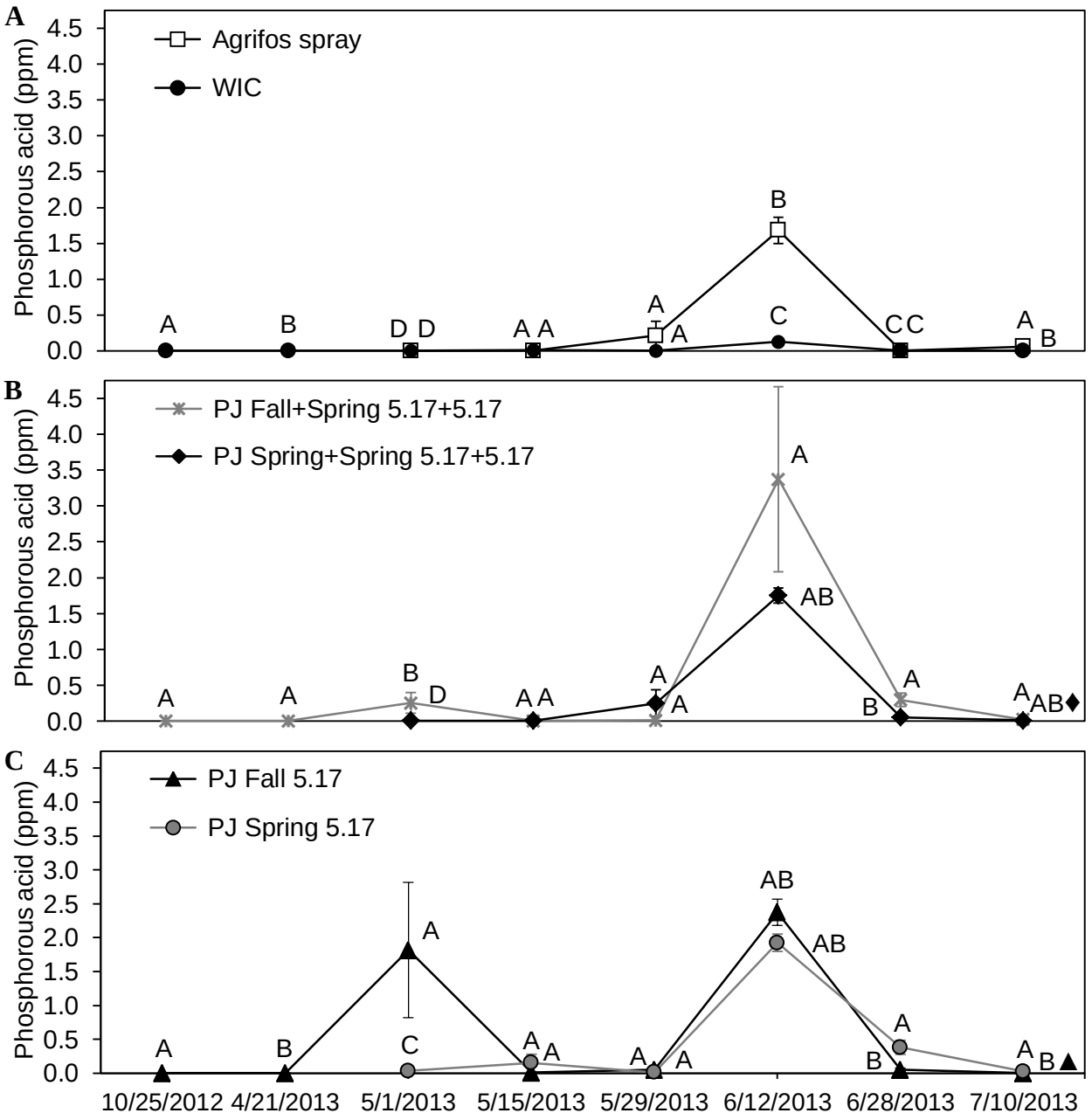

Figure 16. Leaf residue profiles of trunk-injected and sprayed potassium phosphites in 'Mac Spur' apples. (A) spray of potassium phosphites (Agrifos) with water injected control (WIC). (B) double injections and (C) single injections of potassium phosphites (PJ: Phosphojet). Fall/Spring: injection time. 5.17: dose per 25.4 mm of trunk diameter at 30.48 cm or 1 foot height (ml). Residue means within one date followed by different letters are significantly different between the treatments and across the graphs (*t*-tests, α=0.05). Error bars represent standard error of the mean (SEM).

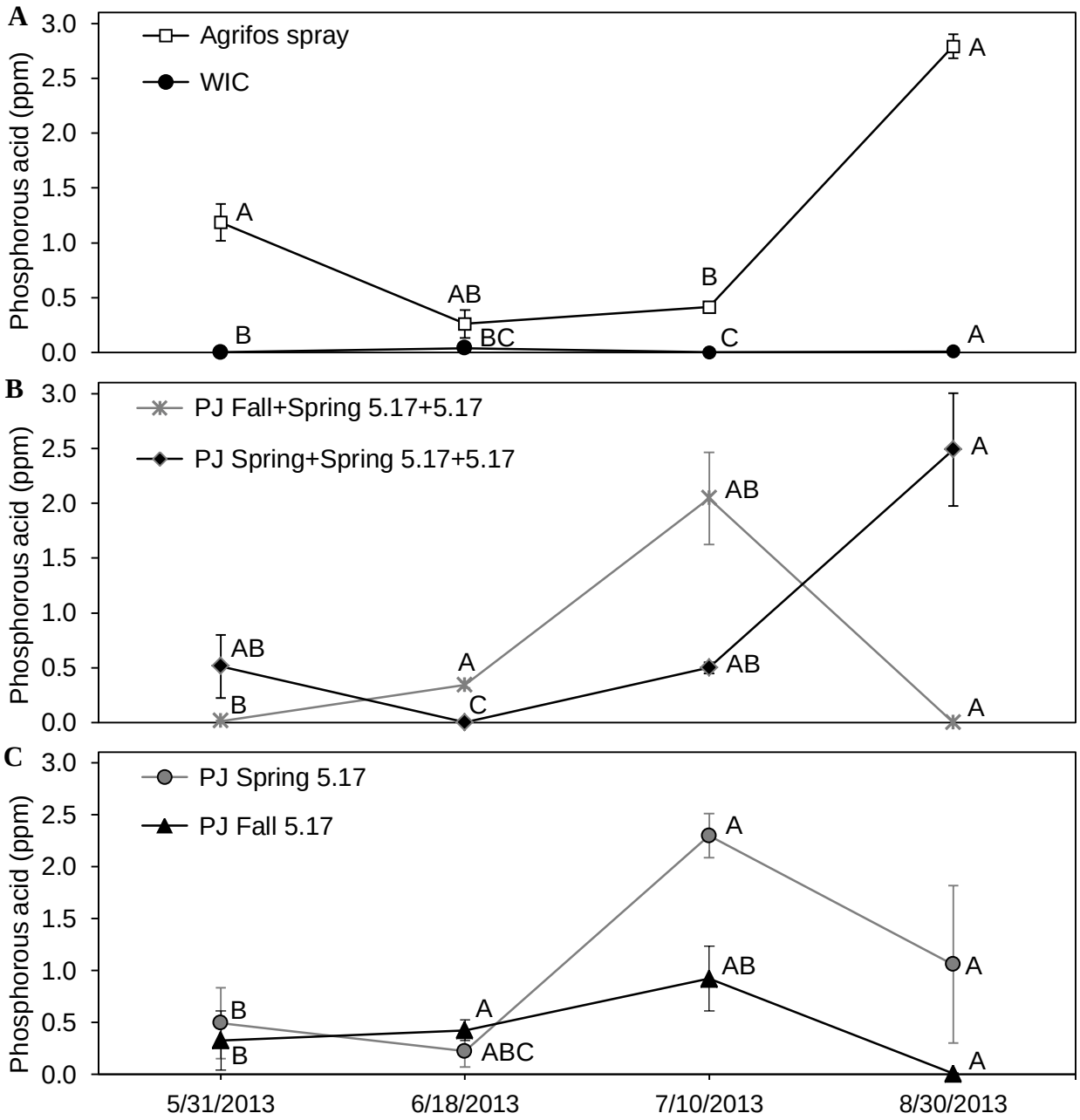

Figure 17. Fruit residue profiles of trunk-injected and sprayed potassium phosphites in 'Mac Spur' apples. (A) spray of potassium phosphites (Agrifos) with water injected control (WIC). (B) double injections and (C) single injections of potassium phosphites (PJ: Phosphojet). Fall/Spring: injection time. 5.17: dose per 25.4 mm of trunk diameter at 30.48 cm or 1 foot height (ml). Residue means within one date followed by different letters are significantly different between the treatments and across the graphs (*t*-tests, α=0.05). Error bars represent standard error of the mean (SEM).

Discussion

The present study provides new insight on apple scab control with differentially timed fungicide injections and their residues accumulated in the canopy. This serves as basis for improving eco-friendly and cheaper apple scab management via *in planta* compound delivery. Best apple scab control was achieved with 4 fungicide injections and varied depending on the injected fungicide and the organ in apple canopy. Fall injection of fungicides followed by 1 or 3 additional injections in the spring did not or only slightly improved leaf and fruit apple scab control in the coming season, respectively. The residues of potassium phosphites detected in leaves and fruit aligned better with the apple scab control effects than the other fungicide residues. Specific residue patterns in buds, leaves and fruit, along with the effect on apple scab, imply differential influence of tree and organ-specific physiology, fungicide properties, and the injection timing on accumulation of injected compound in the apple canopy. The results suggest that (1) depending on canopy organ, at least 1-2 early spring injections due to occurrence of major water transport in xylem can give good disease control, (2) fungicide accumulation and the effect on apple scab vary depending on the transpiration footprint of the organ, (3) accumulated amount and hence activity of the fungicide in apple canopy depends on its mobility in the environment rich in organic compounds, and (4) depending on the fungicide, better or equal apple scab control can be achieved with fewer injections than sprays per year.

Out of all the injected fungicides, potassium phosphites provided best apple scab control which indicates on their favorable properties for injection and accumulation in the canopy. Phosphites suppress *V. inaequalis* and can control other apple pathogens such as *Phytophthora cactorum* and *P. cambivora* (VanWoerkom 2012; Heaton & Dullahide 1990). They have more

than one mode of activity among which they directly affect the fungal pathogens and elicit plant resistance responses (Smillie et al. 1989; Walters & Bingham 2007; Deliopoulos et al. 2010). Apple scab incidence after injection of potassium phosphites and to a certain extent of other fungicides was the lowest on shoot leaves, somewhat higher on apple fruit, and the highest on spur leaves. This implies that injected compounds accumulate much more in shoot leaves than in spur leaves and that they accumulate more in fruits than in spur leaves. This is very likely the result of variable rate of water transpiration among these organs which govern the speed and rate of fungicide accumulation after injection. The total leaf area on spurs is smaller than on shoots and spur leaves develop first in the season, reaching their full development early in the spring. In contrary, shoots develop from petalfall onwards and have longer span of growth during which new leaves are constantly formed. Since more stomata are being formed on newly added leaves and transpiration rate increases in intensity, this drives accumulation of higher amounts of an injected compound and better suppression of *V. inaequalis*. It is known that apple fruits have 10- to 100-fold lower frequency of stomata on epidermis in comparison to epidermis on leaves (Blanke & Lenz 1989), thus explaining why apple scab incidence was higher on fruit than on shoots.

Contrary to expectations, fall injection of fungicides followed by additional injection(s) in spring did not crucially improve the control of leaf and fruit scab since a single or double spring injection provided comparable or better scab control. In 2012, when four cross-seasonal injections were used, better effect on leaf apple scab can be attributed primarily to the higher total dose of injected fungicides with 3 injections in the spring, than to the injection in fall in concert with them. This strongly implies that injections in spring are overall more effective in control of *V. inaequalis* than the fall injection alone or combined with spring injections, because

the most intensive water translocation in xylem occurs in spring and summer. Transpiration in apple trees gradually declines towards the end of the season (Dragoni et al. 2005). Thus, weak transpiration in fall does not lead to crucially abundant transport and higher accumulation of injected compounds in the canopy and contribution to a better scab control next year. Therefore, taking in consideration specific transpiration footprints of different canopy organs, optimal control of apple scab could be achieved with 2-3 early spring injections of fungicides.

Due to four injections, in 2012 more residues of potassium phosphites accumulated in leaves than in 2013. Phosphorous acid which has water solubility of 3100 g/L, accumulated more in leaves and fruit than the other fungicides because potassium phosphites have high water solubility of 500 g/L and low organic carbon-water partitioning coefficient (Koc) of 228-587 ml/g (EFSA 2012) which depicts the level of compound adsorption to carbon rich compounds. This suggests good vertical movement of phosphites in the tree xylem (systemic properties). Propiconazole, cyprodinil and difenoconazole have low to very low water solubilities of 100-150 mg/L, 15 mg/L and 13 mg/L, respectively (Herner & Acock 2003; Serafini 2003; Mensink 2008) and moderate to high Koc-s of 1086-1817 ml/g, 1550-2030 ml/g and 3870-11202 ml/g, respectively (Serafini 2003; BCP Council 2011; PPDB 2013). These chemical properties suggest medium low to very low mobility of these fungicides in the environment with high content of organic matter. Accordingly, difenoconazole is a local systemic and cyprodinil is systemic. They strongly bind to organic compounds of both symplast and apoplast in the tree xylem, which hampers their accumulation in the canopy. This is known as the reservoir effect and compound's Koc is an important parameter behind it (Tanis et al. 2009; 2012; Doccola et al. 2012). Further evidence for this effect was lack of difference between residue profiles of two different propiconazole doses and their effects on apple scab. At last, since pesticides need to be

formulated for tree injection (Kondo 1978; Doccola & Wild 2012; Montecchio 2013) it is possible that foliar formulation of cyprodinil and difenoconazole additionally hampered their abundant accumulation in the canopy and thus better effect on apple scab.

Good apple scab control with injected potassium phosphites, regardless of different residue accumulation in 2012 and 2013, could be assigned to the ability of these salts to induce resistance in apple leaves besides direct effect, thus aiding the control of *V. inaequalis* (Smillie et al. 1989; Guest & Grant 1991; Deliopoulos et al. 2010). Interestingly, the residue profiles in 2013 show that fall injection did provide significantly higher accumulation of injected fungicides in leaves and fruit at some time points. This could be due to substantial time for compound translocation after injection. It would be expected that due to this higher accumulation, control of scab should be better after fall injection. Based on spur leaves and fruit, it appears that the overall levels of accumulated fungicides injected in both fall and spring were out of the toxic range to *V. inaequalis* which could yield these expected control differences. Thus, fall injection improved fungicide accumulation only marginally and not enough to improve scab control. However, on shoot leaves as an exception, single spring versus single fall injection of difenoconazole-cyprodinil combination (DCC) shows better scab control opposite to the residue indicative patterns. Other unknown factors such as metabolic changes on a fungicide (oxidation, hydrolysis, conjugation) (Lindquist 1965) or the time of injection could have caused this unexpected reverse effect. Since leaf residue samples were not differentiating between the spur and shoot leaves any further explanation is difficult.

In 2012, the peaks of propiconazole residue profiles did not align with the best scab control on leaves. The residues indicate that the accumulation of propiconazole followed gradual spur leaf unfolding and shoot leaf development. Very low propiconazole concentrations detected

until 23 May provided significant scab control because the leaf mass at this period was limited in size and volume thus allowing significant impact on disease. With the increase in transpiring leaf area on spurs and shoots more of the residues accumulated in leaves and kept affecting the pathogen. However, this scab reduction effect was mostly weaker in comparison to phospates probably because the accumulated dose was insufficient for stronger pathogen suppression. Additional factors which might contributed to this weakening are *V. inaequalis* resistance to triazoles (Stanis & Jones 1985; Köller et al. 1997), the dose "dilution" with newly developed green mass, and plant metabolic processes which inactivate the fungicide (Lindquist 1965; Campana et al. 1979; Lamoureux & Frear 1979).

In 2013, the peaks of fungicide residue accumulation after injection occurred before the disease expressed and was rated on leaves and fruit. The effects on scab took place at the residue decline patterns and the peaks of accumulation driven by canopy transpiration were not fully aligned with the major scab infection periods and/or symptom expression. It can be concluded that the efficiency of injected compounds most likely was weakened by this un-aligning. In contrary, the sprays of DCC provided much better aligning of the fungicide residues on leaves with the scab onset. Due to direct deposition on the tree, spraying versus injection provided significantly higher and time-stable residues of DCC while potassium phosphite residues were very similar between these two methods of delivery. This indicates that translocation of injected fungicide to the canopy is a time consuming process strongly shaped by the tree physiology and resistance points in tissues, which is opposite to the immediate reaching of the canopy by fungicide sprays (Mäkelä 1986; McCulloh et al. 2003; Coomes 2006). However, even though the dose per tree in injection was 1.6-2 times higher than in sprays, the fact that phosphites injected only twice provided better control of fruit and spur leaf scab than their 9 sprays, indicates that

after injection, these salts have better persistency in activity. This effect, along with comparable control on shoots by injection and spraying, is impressive and proves that injection enhances the activity of potassium phosphites and can even extend their effect into the next vegetative season (Aćimović et al., *unpublished*). This offers an opportunity for cost reduction in apple scab control and lower risk for pathogen resistance development (Deliopoulos et al. 2010).

The fruit residues declined due to metabolic and environmental degradation and decrease in transpiration since fruit xylem gradually becomes dysfunctional towards the end of the season (Dražeta et al. 2004). The residue concentrations after injection were always below the apple fruit tolerances of 0.15 and 0.05 mg/kg for cyprodinil and 4.5 and 0.5 mg/kg for difenoconazole (US EPA, Codex Alimentarius) while potassium phosphites are exempt from food tolerances (US EPA 2006). Since vast majority of residues ends up in foliage versus fruit, trunk injection results in a discriminatory distribution of fungicides within an apple tree.

In summary, the results show that 2-3 trunk injections of fully systemic fungicides could be a valuable alternative to topical fungicide applications for control of *V. inaequalis*. Injected phosphites were a better model for optimizing apple scab control in the present work and emphasize the need for careful selection and formulation of future injectable pesticides.

Acknowledgements

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Injection Delivery". Special thanks to Dr. Joseph Doccola, head of Research & Development at Arborjet Inc. (Woburn, MA) for donating trunk injection equipment and the chemicals. I send a big thank you to my colleague and a friend Anthony VanWoerkom, who helped tremendously in conducting trunk injections on apple trees and in sample collection. Invaluable help in sample collection and disease rating was provided by Laura Lamb, research technician at TNRC, and an undergraduate student and a friend Christopher Meredith who also diligently and precisely worked on treatment trunk injections and sample processing for HPLC. Tremendous help in residue sample analysis and residue data processing was generously provided by Dr. Christine Vandervoort and research technician Thomas Garavaglia. A big thank you to Dr. Schilder Annemiek, research technician Jerri Gillett, Dr. Brad Day, Dr. Jim Miller and Dr. Randolph Beaudry for letting me use the resources and equipment of their laboratories at MSU. Finally, I thank all the staff of TNRC for generously helping me by maintaining and protecting the experimental plots used in this project.

CHAPTER 6. COMPARISON OF CURRENT AND DEVELOPED TRUNK INJECTION TECHNOLOGIES IN COMPOUND DISTRIBUTION AND SIMULATION OF SLOW TIME-RELEASE OF INJECTED COMPOUND FOR LONG-LASTING TREE PROTECTION

Abstract

The technology for topical pesticide delivery in tree-based agriculture creates drift-driven pesticide losses which contaminate the environment. Trunk injection of pesticides could greatly reduce these side effects. However, this technology is limited by the inability to provide temporally uniform release of injected compound over time and by limited research on performance comparisons of current trunk injection technologies. After simulation of slow time-release of injected imidacloprid by dose-splitting at 4 times, 14 days apart, a uniform temporal distribution of this compound was achieved in the apple canopy. After comparing the injection technology, the drill-based injection in combination with the liquid imidacloprid formulation provided higher concentrations in the apple canopy versus the needle-insertion injection and the slow-release formulation. Further comparison of seven trunk injection devices showed that all drill-based devices did not provide higher residue concentrations of the injected cyprodinil and difenoconazole in apple leaf canopy when compared to needle-insertion Bite, while Wedgle was similar to them. Chemical properties of the injected compound govern its temporal distribution. Imidacloprid was a better compound for comparing injection devices than the two fungicides. All injection devices facilitated largely similar apple scab control with both fungicides and their initially higher accumulation after Bite injections was not sufficient to provide better control.

The more additional tools and steps were involved in preparation for trunk injection with the device, the more time was required to conduct it. The shortest time for solution discharge during the injection was required by devices which used either more than 6 psi pressure to inject and which injected the lowest dose volume per tree. These findings offer basis for improving trunk injection as target-precise pesticide delivery for more sustainable tree-based agriculture.

Introduction

The technology for topical pesticide delivery in agriculture has changed little over the last 100 years. Although providing adequate pest control with frequent seasonal treatments, this technology carries excessive costs due to product losses into the environment from spray drift. Scientists estimate that less than 0.1% of the applied pesticide for plant protection contacts the target pest (Pimentel & Levitan 1986; Pimentel et al. 1992; Pimentel & Lehman 1993; Pimentel 1995; 2005; Shaaban 2009). Air-blast sprayers used in tree-based agriculture are inefficient means for delivering pesticides to their target, with only 29 to 56% of the applied spray solution being deposited on the tree canopy (Steiner 1969; Reichard et al. 1979; Perry 1998; Zhu et al. 2006). In order to achieve efficient and more target-precise delivery of active compounds to trees, new approaches and technologies such as trunk injection and drip irrigation have to be investigated for this purpose (Araujo et al. 1995; Ayars et al. 1999; Holownicki et al. 2000; Torii 2000; Takai et al. 2003; Düker et al. 2006; Llorens et al. 2010; Shang, Liao, et al. 2011; 2011).

As an *in planta* method for delivery of pesticides, trunk injection could be adapted from landscape tree care industry and used in tree-based agriculture for protection. This approach allows precise and confined pesticide delivery to trees and hence is an environmentally safer

alternative for pesticide application. Trunk injection utilizes tree's vascular system to translocate and distribute active compounds into the canopy (Wilson 1979; Barney et al. 1984; Barney et al. 1985; Navarro et al. 1992; Fernández-Escobar et al. 1993; Guillot & Bory 1997; Sánchez-Zamora & Fernández-Escobar 2000; Percival & Boyle 2005; Spinelli et al. 2005; Shaaban 2009; Ahmed et al. 2010; Smitley et al. 2010; Doccola & Wild 2012). Since the compound is contained within the tree, this allows increased selectivity of exposure to unwanted pathogens and insect pests, but also reduces pesticide exposure to non-target organisms, farm workers and the environment. Along with the reduced risk chemistries, which meet high environmental and human safety standards (US EPA 1997), the use of tree injection to deliver these chemistries could allow achieving a higher level of sustainability in agriculture, healthier environment and safer food supply in the future.

Trunk injection technology has been invented, developed and used for efficient tree protection and nutrition in urban and forest landscapes where topical pesticide application is not possible due to tree size or is not allowed due to vicinity of populated areas (Guillot & Bory 1997; Grosman et al. 2002; Takai et al. 2003; Takai et al. 2004; Harrell 2006; Smitley et al. 2010; Doccola et al. 2003; 2012). However, the design, implementation and use of injection technology have been limited with few key problems that could also have an enormous impact in tree-based agriculture, if used for plant protection.

The first important problem of current trunk injection devices is their inability to provide slow i.e. controlled release of injected compound over time. A slow release would provide temporally uniform distribution of the compound dose in the tree canopy and secure protection of a tree at a desirable dose and at a required time period. Previous research has shown that temporal distribution of injected compounds in the canopy is significantly non-uniform and that

compound dose is at certain times oversupplied and at others undersupplied (Shabi et al. 1974; Clifford et al. 1987; Schutte et al. 1988; Tattar et al. 1998; VanWoerkom 2012; Aćimović et al. 2014). Further evidence for this effect after injection and its potential value for tree-based agriculture is found in studies reporting one year or more of insect control after a single injection of an insecticide in forest or an agricultural tree species (J.J. Doccola et al. 2007; 2012; Smitley et al. 2010; McCullough et al. 2011; Grosman et al. 2002; Byrne et al. 2012; 2014). To find a solution for temporally non-uniform distribution of injected compound we simulated slow time-release of trunk-injected insecticide at a unique dose per tree. Our objective was to compare the schedules of time-splitting of a unique dose that could successfully simulate slow time release of an injected insecticide and which could provide its temporally uniform distribution in apple leaf canopy.

Currently we know relatively little about the comparative efficacy of various trunk injection methods. In the last two decades many drill-based and needle insertion-based trunk injection devices have been proposed, invented and used to facilitate efficient tree and grapevine protection (Düker et al. 2006; Düker & Kubiak 2009b; 2009a; Doccola et al. 2003; 2012; Shang, Liao, et al. 2011; 2011; Düker & Kubiak 2011b; 2011a; Montecchio 2011; 2013). However, there is limited research showing the efficiency of compound delivery and plant protection after use of these technologies. Since the efficiency requirements for protection in tree-based agriculture are high, we investigated whether the level of compound distribution and pest and disease control depends on used trunk injection device.

To address both objectives, besides evaluating the level of pest and disease control where needed, we utilized temporal distribution profile of injected compounds within the canopy as the main parameter for comparison of the injection devices and the treatments simulating their slow

time-release (Fuchs 1988; Düker et al. 2006). Previous research on trunk-injected imidacloprid indicated that both spatial and temporal distributions of injected compound in the tree canopy are important factors governing the efficiency of plant protection (Aćimović et al. 2014).

Our first hypothesis was that increasing dose-splitting over time, will provide more uniform temporal distribution of trunk-injected insecticide in the tree canopy. Further, we hypothesized that drill-based injection of a liquid compound formulation will provide higher residue concentrations than the needle insertion-based injection of a liquid formulation or a drill-based injection technology of a slow release, solid injection formulation. Finally, we hypothesized that higher residue concentrations of a fungicide in apple leaf canopy will be detected after trunk injection with drill-based versus the needle insertion-based injection technologies, and that significantly better apple scab control, comparable to spray standard, will be achieved with fungicide injected with drill-based versus the needle insertion-based injection technology.

We wanted to determine whether different trunk injection devices provide different compound delivery in the canopy thus affecting the efficiency of pest control, and how to achieve uniform temporal distribution of injected compound.

Materials and Methods

Simulation of slow time-release of injected compound and the comparison of three trunk injection technologies in temporal distribution of imidacloprid

Orchard experiments were conducted in 2011 at Michigan State University's Trevor Nichols Research Center (TNRC) in Fennville, MI (42º 36' 0.94" N, 86º 9' 12.02" W and 42º 36' 29.388" N, 86º 9' 19.826" W). For slow release simulation, we injected 29-yr-old apple trees of 'Mac Spur', *Malus domestica* Borkhausen, with a dose of 1 g of imidacloprid per tree (20 ml of IMA-jet™, 5% imidacloprid, Arborjet Inc., Woburn, MA). This dose corresponds to the maximum allowed seasonal rate of imidacloprid used as foliar spray per 0.405 ha with 250 planted apple trees, according to the EPA registration label in USA. We imposed time-split delivery of this dose as follows: full dose of 1 g a. i. at 0 DAI (1 x 20 ml of Imajet), half dose of 0.5 g a. i. at 0 and 14 DAI (2 x 10 ml of Imajet), and quarter dose of 0.25 g a. i. at each 0, 14, 28, and 42 DAI or on 8 and 22 June, and on 6 and 20 July, respectively (4 x 5 ml of Imajet).

For the comparison of trunk injection technologies we injected 20-yr-old apple trees of 'Golden Delicious' with the same dose of 0.2 g of imidacloprid per tree across injection devices. This dose corresponds approximately to maximum foliar rate of imidacloprid lableled for use in US apples, based on 250 trees per 0.405 ha (1 acre). To deliver this dose we used the following devices representing three trunk injection technologies in combination with their producer's respective formulation of imidcaloprid: Quik-jet® micro-injection system as a drill-based technology which delivered IMA-jet™ (ArborJet Inc, Woburn, MA), Wedgle Direct-Inject™ System® as a needle insertion-based technology which delivered Pointer™ (5% imidacloprid,

ArborSystems LLC., Omaha, NE), and MSU-1 as a novel, solid formulation for slow time-release of imidacloprid (95%, Shanghai AgroChina International Co., Ltd., China), which was inserted into the drilled injection ports.

To reduce experimental unit variability, apple trees were selected based on a uniform canopy and the trunk diameter at 30 cm height. Canopy in the slow release simulation was 3.7 m high and 3.5 m wide and in the comparison of trunk injection technologies 3.6 m high and 2.5 m wide. In the slow release simulation and the comparison of trunk injection technologies trunk diameter means with standard errors were 15.24 ± 1 cm and 29.8 ± 0.13cm, respectively.

For slow release simulation, we equally divided and delivered doses split over time and specific for treatment into 4 injection delivery ports per tree, by using Quik-jet® micro-injection system (Arborjet Inc., Woburn, MA). At each time point from 0-42 DAI, apple trees were injected into a freshly drilled set of 4 delivery ports to avoid confounding effect of former ports closure by compartmentalization (Shigo 1981; Doccola et al. 2011). Similarly, for the comparison of trunk injection technologies we divided the dose per tree into 4 injection delivery ports across all three injection technologies.

In both experiments, a 3/8" drill bit on a cordless 1500 rpm drill was used to create trunk injection ports 9.5 mm in diameter and 25.4 mm deep for trunk injection treatments (DeWalt Industrial Tool Co., Baltimore, MD). We sealed these ports with Arborplugs® no. 4 (Arborjet Inc., Woburn, MA). For Wedgle injections we created delivery ports through the bark, 4-6 mm deep and 4 mm in diameter, with WedgeCheck Puncher® and sealed them with WedgeCheck bark plugs® (ArborSystems LLC., Omaha, NE). All ports were equally distanced along the trunk circumference, according to the cardinal and intermediate directions and were positioned

approximately 30 cm above the ground surface, and vertically separated by approximately 5 cm between the opposing port pairs.

Both experiments were arranged in a completely randomized design (CRD) with 6 replicate trees per each treatment in the slow release simulation and 5 in the comparison of trunk injection technologies.

In both experiments, we collected leaf samples after each injection treatment to compose their temporal distribution profiles of imidacloprid in apple canopy. Composite leaf samples were taken from each crown within a tree replicate and each sample consisted of 40 leaves collected throughout the crown (per each cardinal direction of the crown 10 leaves was collected). We collected samples 6 times in the slow release simulation, at 1, 14, 28, 42, 56 and 70 days after the first injection (DAFI) and at 1, 7, 14, 28, 42 and 56 DAI in comparison of trunk injection technologies. All samples were stored, processed and analyzed for imidacloprid parent residues using the method and reported before (Aćimović et al. 2014).

Imidacloprid leaf residues were averaged for 6 replicate trees within each injection treatment and processed with a mixed model using the MIXED procedure in SAS 9.3 (SAS Institute, Cary, NC, 2011). In the slow release simulation, temporal distribution of imidacloprid was analyzed with repeated measures best adjusted using a first-order heterogeneous autoregressive covariance structure. In the comparison of trunk injection technologies, we log transformed imidacloprid residues data in apple leaves and temporal distribution of imidacloprid was analyzed with repeated measures best adjusted with unstructured covariance structure (α=0.05). In both experiments, a tree was used as the subject of repeated measurements over time and the analysis was conducted for only 5 out of 6 time points since no imidacloprid was detected in leaves collected at 1 DAI. When the main factor effects or their interactions were

found to be statistically significant ($P < 0.05$), slicing, i.e. examination of interactions within main effects, was performed, *F*-tests were carried out and pairwise or specific time or treatment comparisons were conducted using *t*-tests ($\alpha = 0.05$).

Comparison of trunk injection devices in temporal distribution of difenoconazole and cyprodinil in apple canopy and in control of apple scab

An orchard experiment was conducted in 2012 at Michigan State University's TNRC in Fennville, MI (GPS: N42° 36' 27.91", W86° 9' 26.54"). Using seven different trunk injection devices (Table 18, Figure 18), on 29 March (pink stage) we injected 14-yr-old apple trees of 'Red Delicious' with a combined dose of 0.59 g of difenoconazole and 1.69 g of cyprodinil per tree (7 ml of Inspire Super® EW, Syngenta, Basel, Switzerland). This dose corresponds to the maximum allowed seasonal rate of Inspire Super as a foliar spray for control of apple scab pathogen *Venturia inaequalis* per 0.405 ha with 250 planted apple trees, according to the EPA registration label in USA. In the case of Quik-jet, Viper, Tree IV, Bite and Chemjet, we added 73 ml of distilled water to the fungicide dose, while in Mauget capsules we added only 33 ml of distilled water per tree. Due to small injection volume of Wedgle per port, we were not able to add water to aid the delivery of fungicide (Figure 18G). To reduce experimental unit variability, before injection, apple trees were selected on the basis of a uniform canopy (3.6 m high and 2.5 m wide) and the trunk diameter at 30 cm height (diameter means with standard errors were 16.7 ± 0.07 cm.

We equally divided and delivered fungicide dose into 4 injection delivery ports per tree with all trunk injection devices. Drilled and Wedgle ports were created, positioned and sealed on

the trunk in the same fashion as described before. For Quik-jet, Viper and Tree IV we used ports 9.53 mm in diameter positioned perpendicular to the vertical axis of an apple tree trunk. For Chemjet and Mauget, we used ports 4.4 mm in diameter positioned perpendicular and at 45º angle to the vertical axis of an apple tree, respectively. Chemjet and Mauget ports were unsealed, while lenticular ports created by needle insertion of Bite were sealed with a silicone gasket only during the injection process. We used hand pressure for fungicide injection with Quik-jet, while with Tree IV and Viper we used 60 psi for injection. Chemjet and Mauget injections were performed under 40 and ~6 psi, respectively, using 4 units of each per tree. Bite injections were conducted via screwdriver-shaped needle (3.5×5×31.75 mm) connected to 30 ml syringe, relying upon the negative pressure generated by the sap flow in lenticular trunk port opened in wood by insertion of the needle (Venturi effect).

During injection we measured the following parameters of injection devices: time per tree needed to create 4 injection ports and use the injection device(s), and time needed to deliver injection solution into the tree with 4 injection ports. We used this to quantify and compare trunk injection devices in labor economics. The experiment was arranged in a completely randomized design (CRD) with 6 replicate trees per each injection device, except for Bite where only 4 replicate trees were injected.

To compare delivery efficiency of injection devices we collected leaf samples to compose temporal distribution profiles of difenoconazole and cyprodinil in apple canopy. Composite leaf samples were taken from each crown within a tree replicate and each sample consisted of 40 leaves collected throughout the crown (per each cardinal direction of the crown 10 leaves was collected). We collected samples 6 times at 14, 28, 42, 56, 70 and 84 DAI. Leaf samples were analyzed for difenoconazole and cyprodinil residue quantity with Waters 2695 separator module

HPLC equipped with the Waters MicroMass ZQ2000 mass spectrometer detector and a Waters X-Bridge C_{18} reversed phase column 3.0×50 mm bore, 2.5 µm particle size (Waters Corp., Milford, MA). The mobile phase, solvent A, was water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. We used solvent gradient shown in Table 19. Ion mode was ES plus and monitored ions for quantification of difenoconazole were 250.87 and 405.96 m/z (Da) and for cyprodinil were 93.14, 118.51 and 226.18 m/z (Da). The levels of quantification for difenoconazole and cyprodinil were 3.3 µg/g (ppm) and the levels of sensitivity were 0.00510 and 0.0505 ppm, respectively. Levels of detection were 0.01683 and 0.16665 ppm, respectively.

Difenoconazole and cyprodinil leaf residues were averaged for 6 replicate trees for each injection device and processed with a mixed model using the MIXED procedure in SAS 9.3 (SAS Institute, Cary, NC, 2011). Prior to statistical analysis, cyprodinil and difenoconazole residue data were log 10 and log transformed, respectively. We analyzed the temporal distribution of both active ingredients in leaves with repeated measures best adjusted using an unstructured covariance structure.

We monitored the efficiency of fungicide delivery with 7 trunk injection devices by rating apple scab control on leaves. We used previously reported method by examining 20 spurs and 20 terminal shoots per tree replicate and rated the leaf scab incidence (Ehret et al. 2010). Due to severe frosts during bloom, apple fruit crop was lost and fruit scab was not rated. The effect of injected fungicide on apple scab was compared to spray standard with 5 seasonal applications of mancozeb (Penncozeb™ 75DF, Cerexagri Inc., King of Prussia, PA) and 1 application of fenarimol (Rubigan® E.C., Dow AgroSciences LLC, Indianapolis, IN). The sprays were conducted in accordance with the alerts from apple scab forecast system through Enviro-weather station at TNRC in Fennville, MI (http://enviroweather.msu.edu).

The data on control of apple scab were analyzed with a mixed model using the MIXED procedure in SAS 9.3 (SAS Institute, Cary, NC, 2011). Prior to statistical analysis, the data on spur and shoot leaf scab incidence were cos and sqrt transformed, respectively. We analyzed both, the spur and shoot leaf scab control through time with repeated measures best adjusted using an unstructured covariance structure.

For both, apple scab control and fungicide residues in leaves, a tree was used as the subject of repeated measurements over time. When the effects of injection device and time or their interaction were found to be statistically significant ($P < 0.05$), slicing, i.e. examination of interaction within main effects, was performed, *F*-tests were carried out and pairwise or specific comparisons of injection devices were conducted using *t*-tests ($\alpha = 0.05$).

Results

Slow time-release of imidacloprid and the differences of three trunk injection technologies in temporal distribution of imidacloprid

Slow release treatment, time and their interaction affected distribution of imidacloprid. (Table 20). With more dose-splitting in injections, the uniformity in distribution of imidacloprid in apple canopy improved by showing more flat residue concentration curve, though it still changed significantly over time (Figure 19). As expected, the injection of a quarter doses showed slower and more gradual rate of increase in imidacloprid concentration.

The three trunk injection technologies, time and their interaction affected distribution of imidacloprid (Table 20). Quik-jet and Wedgle provided significantly higher concentrations of

imidacloprid compared to MSU-1 but its distribution was not uniform over time (Figure 20). MSU-1 provided statistically the most uniform distribution of imidacloprid but the lowest delivered concentrations, not significantly different from zero (Figure 20). Wedgle provided the second best uniform temporal distribution of imidacloprid.

Temporal distribution of difenoconazole and cyprodinil and apple scab control after trunk injection with different devices

The type of trunk injection device did not significantly affect temporal distribution of cyprodinil in apple leaf canopy, but it interacted with time (Table 20). The type of trunk injection device, time and their interaction affected temporal distribution of difenoconazole. Early after injection, only Bite provided significantly the highest concentrations of cyprodinil and difenoconazole in apple leaves (Table 21). Hence, Bite provided the most non-uniform temporal distribution of cyprodinil and difenoconazole in apple leaf canopy while the other devices provided much less non-uniform temporal distribution but with very low compound delivery. Regardless of the injection device type, both active ingredients were supplied to the leaves at very low concentrations close to zero (Table 21). Difenoconazole residue concentrations were overall lower than of cyprodinil (Table 21B).

The incidence of spur and shoot leaf scab varied significantly among the injection and spray treatments and the times of rating (Table 22). All the injection devices allowed the delivered fungicide to significantly reduce apple scab on spur and shoot leaves. Disease incidence was the lowest after use of Tree IV and Wedgle. Only Tree IV provided comparable

control of spur leaf scab to both spray standards (Table 22). On the shoots these comparable effects were largely absent.

Mauget and Bite required the shortest time per one tree to prepare 4 injection ports and connect injection device to the ports or the trunk (Table 23). Wedgle and Chemjet followed with almost twice longer required time, while Quikjet and Viper required approximately 3 min. The longest time required for preparation before injection was of the Tree IV (Table 23).

Wedgle required the shortest time to deliver the fungicide into the tree, while Quikjet and Viper were similar and followed with more required time (Table 24). However, in use of Wedgle, even with no addition of water to the fungicide, we encountered bark splitting while injecting the dose and the leakage of minuscule amounts of fungicide from the cracks around the injection port. The next time consuming devices were Chemjet and Tree IV and then Bite. Mauget capsules required the longest time to discharge and sometimes 1-2 capsules per tree remained partially filled with fungicide residues (Table 24). Hence, Wedgle and Mauget showed the lowest efficiency of injection due to non-complete dose delivery. Overall, the total times needed for preparation and injection of a single apple tree with 4 injection ports using needle-insertion devices like Wedgle and Bite were 2.73 and 85.05 min., respectively. The total times needed for drill-based injection with devices like Quikjet, Viper, Chemjet, Tree IV and Mauget are 5.89, 6.62, 50.15, 61.8 and >1440.75 min., respectively (Tables 23 and 24).

Table 18. Devices used for trunk injection of 'Red Delicious' apple trees with fungicide Inspire Super for apple scab control in 2012.

Trunk injection technology	Device	Manufacturer	Targeted tissue
Drill based	ChemJet Tree Injector®	Chemjet Trading Pty Ltd., Bongaree, Queensland, Australia	Xylem
	Mauget Tree Injection Capsules®	Mauget Inc., Arcadia, CA	
	Quik-jet® micro-injection system	ArborJet Inc., Woburn, MA	
	Tree I.V.® Micro-Infusion™ System		
	Viper ™ Air/hydraulic Micro-Injection System		
Needle based	Bite - Blade for Infusion in Trees	University of Padova, Italy	
	Wedgle Direct-Inject™ System®	ArborSystems LLC., Omaha, NE	Cambium

Table 19. The gradient mobile phase flow used for difenoconazole and imidacloprid HPLC/MS residue analyses.

Active ingredient	Time (min.)	Flow rate (µl/min.)	Solvent A(%)	Solvent B(%)
Difenoconazole Cyprodinil		0.30	75.0	25.0
	5.00	0.30	10.0	90.0
	7.00	0.30	10.0	90.0
	7.50	0.30	75.0	25.0
	12.00	0.30	75.0	25.0

Table 20. The effects and interactions of different dose-splitting regimes, trunk-injection devices and time on temporal distribution of injected compounds in apple leaf canopy and on apple scab control.

Experiment		Main effects	*F*	DF	*P*-value
Simulation of slow time-release of injected imidacloprid		Treatment	10.07	30.6	**≤0.0004**
		Time	29.36	22.5	**≤0.0001**
		Treatment *Time	9.71	27.4	**≤0.0001**
Comparison of three trunk injection technologies in imidacloprid distribution		Technology	24.24	12	**≤0.0001**
		Time	9.9	9	**≤0.0024**
		Technology *Time	5.61	11.1	**≤0.0051**
Comparison of seven trunk injection devices	in cyprodinil distribution	Device	1.12	33	≥0.3724
		Time	53.11	33	**≤0.0001**
		Device *Time	4.41	33	**≤0.0001**
	in difenoconazole distribution	Device	3.83	33	**≤0.0053**
		Time	10.72	33	**≤0.0001**
		Device *Time	4.94	33	**≤0.0001**
	in apple scab control on spur leaves	Device	13.47	48	**≤0.0001**
		Time	66.89	48	**≤0.0001**
		Device *Time	3.41	48	**≤0.0026**
	in apple scab control on shoot leaves	Device	35.67	48	**≤0.0001**
		Time	104.01	48	**≤0.0001**
		Device *Time	6.66	48	**≤0.0001**

Table 21. Residue profiles of cyprodinil (A) and difenoconazole (B) in apple leaves of 'Red Delicious' trees after delivery with different trunk injection devices (*t*-tests, α=0.05). [1]Means within one date followed by different upper-case letters are significantly different. [2]Means within one treatment across the six dates followed by different lower-case letters are significantly different.

Device	(A) Mean cyprodinil concentration in apple leaves (ppm)											
	12 April		26 April		10 May		23 May		6 June		20 June	
Bite	0.2540	A[1]a[2]	0.1745	Ab	0.0280	Ac	0.0058	Ad	0.0019	Ad	0.0082	Ad
Viper	0.0109	BCbc	0.0051	Bc	0.0162	Aa	0.0066	Ac	0.0095	Ab	0.0022	Ab
Quik-jet	0.0103	Bab	0.0072	Bbc	0.0161	Aa	0.0048	Acd	0.0021	Ad	0.0060	Acd
Mauget	0.0086	Ba	0.0029	Bb	0.0083	Aa	0.0051	Aab	0.0011	Ac	0.0066	Aab
Wedgle	0.0075	Bab	0.0034	Bc	0.0103	Aa	0.0070	Abc	0.0010	Ad	0.0044	Abc
ChemJet	0.0042	BDb	0.0033	Bb	0.0131	Aa	0.0039	Ab	0.0023	Ab	0.0030	Ab
Tree IV	0.0025	CDe	0.0039	Bce	0.0144	Aa	0.0078	Abd	0.0031	Ade	0.0156	Aabcd

Device	(B) Mean difenoconazole concentration in apple leaves (ppm)											
	12 April		26 April		10 May		23 May		6 June		20 June	
Bite	0.1779	Aa	0.0785	Aab	0.0118	Abcd	0.0055	Abd	0.0015	BCDc	0.0010	Ac
Quik-jet	0.0045	Bab	0.0011	Bc	0.0022	Abc	0.0024	Bb	0.0011	Dac	0.0014	Aac
Mauget	0.0044	Ba	0.0003	Ba	0.0014	Aa	0.0007	Ca	0.0012	Da	0.0010	Aa
Viper	0.0027	Bbcd	0.0017	Bbde	0.0037	Aab	0.0023	Bbd	0.0029	ABCb	0.0012	Aace
Wedgle	0.0020	Babc	0.0000	Bcd	0.0026	Aa	0.0000	Cbd	0.0014	CDac	0.0009	Aac
Tree IV	0.0000	Bc	0.0000	Bc	0.0041	Aab	0.0028	Bb	0.0035	ABb	0.0011	Aac
ChemJet	0.0000	Bc	0.0004	Bc	0.0013	Abc	0.0025	Bab	0.0052	Aa	0.0012	Ac

Table 22. Apple scab control on spur and terminal shoot leaves of 'Red Delicious' trees after trunk injection of combination of cyprodinil and difenoconazole with different trunk injection devices (*t*-tests, α=0.05). [1]WIC - water injected untreated control, SpraySTD - spray standard, Spray IS - Inspire super sprayed. [2]Means within one date or column followed by different upper-case letters are significantly different. [3]Means within one treatment across the two or four dates followed by different lower-case letters are significantly different.

Injection device	Mean apple scab incidence (%)					
	Spurs		Shoots			
	1 May	17 May	1 May	17 May	13 June	16 August
WIC[1]	78.08 A[2]a[3]	80.13 Aa	32.84 Aa	62.25 Ab	74.95 Ab	41.18 Aa
Bite	43.08 Ba	56.93 Bb	21.93 ABa	43.35 Bb	35.43 Bb	13.56 Ba
Quikjet	37.48 BCa	57.23 Bb	19.75 Ba	31.75 Bb	39.88 Bb	13.86 Ba
Mauget	35.75 BCa	57.55 Bb	18.03 Ba	30.47 Bb	37.15 Bb	16.22 Ba
Viper	32.96 BCa	51.26 BCb	12.74 BDa	28.15 Bb	34.74 Bb	14.84 Ba
Chemjet	32.88 BCa	53.72 Bb	14.47 BDa	31.94 Bb	35.62 Bb	15.57 Ba
Wedgle	29.4 BCDa	45.49 BCb	16.48 BCa	28.9 Bb	35.67 Bb	16.43 Ba
Tree IV	18.83 CDa	33 CDa	8.14 Da	25.59 Bb	35.25 Bc	15.75 Bb
SpraySTD	11.72 Da	16.09 Da	8.09 CDa	11.07 Cab	19.92 Cb	27.42 Ac
Spray IS	0.14 Da	0.0 Da	0.0 Ea	0.63 Dab	1.26 Db	11.07 Bc

Table 23. The properties of trunk injection devices used in delivery of cyprodinil and difenoconazole for apple scab control. *Applies to cases when Bite is used in combination with pressurized injection systems. **Time measurements based on unique volume of injected solution of 80 ml/tree except for Mauget where 40 ml/tree was used, and Wedgle where 7 ml/tree of fungicide was injected only. Time means based on 6 replicate trees, except for Bite with 4 replicate trees used.

Trunk injection technology	Injection device	Number of openings × their size on the injection element (mm)	Pressure used to power one unit (psi)	Time needed to prepare 4 injection ports and connect the injection device (min.)**
Drill-based injection	Mauget	1 × 3	~ 6	0.75
	Chemjet	1 × 2	20 - 40	1.95
	Quikjet	1 × 1	Hand (>50 psi)	2.99
	Viper	1 × 1	50 - 150	3.12
	Tree IV	1 × 1	60	4.00
Needle-based insertion injection	Bite	2 × (2 - 2.5 × 1.5)	None or variable*	1.05
	Wedgle	1 × 1	Hand (>50 psi)	1.83

Table 24. The comparison of seven trunk injection devices used in delivery of cyprodinil and difenoconazole for apple scab control. *The time measurements are based on 80 ml/tree volume of injection, except for Mauget where 40 ml/tree and Wedgle where 7 ml/tree of fungicide was injected only. The time does not include preparation of pesticide solutions. **Capsules remained partially filled with the fungicide residue. Time means based on 6 replicate trees, except for Bite with 4 replicate trees used.

Trunk injection technology	Targeted tree tissue for compound delivery	Injection device	Origin of used pressure force to deliver the solution	Number of trees treated simultane-ously with 1-4 device units	Number of injection units needed for 4 injection ports per tree	Diameter or width × height of drilled or insertion-created injection port (mm)	Time needed to inject one tree with 4 injection ports i.e. to discharge liquid (min.)*	Maximum reservoir volume per one injection unit (ml)	Solution volume injected per one powering of single unit (ml)
Drill based	Xylem	Mauget	Hand	1	4	4.36	>1440**	10	10
		Chemjet	Hand then metal spring				48.2	20	20
		Quikjet	Hand		1	9.53	2.9	1000	5
		Viper	Air				3.5	2000	5
		Tree IV	Air				57.8	600	600
Needle based		Bite	Tree transpiration or hand or air			3.5 x 11	84	1000	1000
	Cambium	Wedgle	Hand			0.5 x 2	0.9	120/1000	1

Figure 18. Trunk injection devices used on 'Red Delicious' apple trees to deliver fungicide Inspire Super for apple scab control. (A) Chemjet Tree Injector, (B) Mauget Tree Injection Capsules, (C) Quik-jet Micro-Injection System, (D) Viper Air/hydraulic Micro-Injection

Figure 18 (cont'd)

System, (E) Tree I.V. Micro-Infusion System, (F) Bite - Blade for Infusion in Trees, (G) Wedge Direct-Inject System.

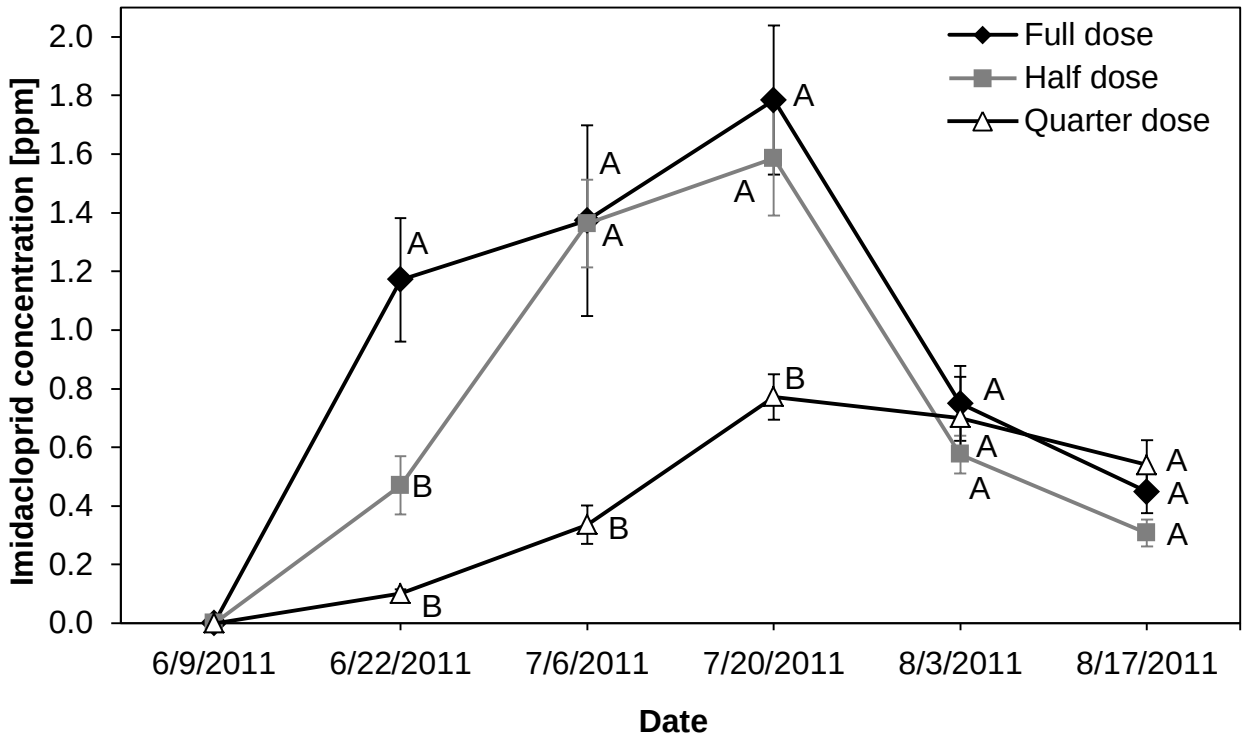

Figure 19. Imidacloprid residue profiles in the canopy of 'Mac Spur' apple trees after trunk injection. Full dose: 1 g a. i. per tree delivered on 8 June. Half dose: 2 x 0.5 g a. i. delivered on 8 and 22 June. Quarter dose: 4 x 0.25 g a. i. delivered on 8 and 22 June and on 6 and 20 July. Concentration means within one date followed by different letters are significantly different (*t*-tests, $p<0.05$). Error bars represent standard error of the mean (SEM).

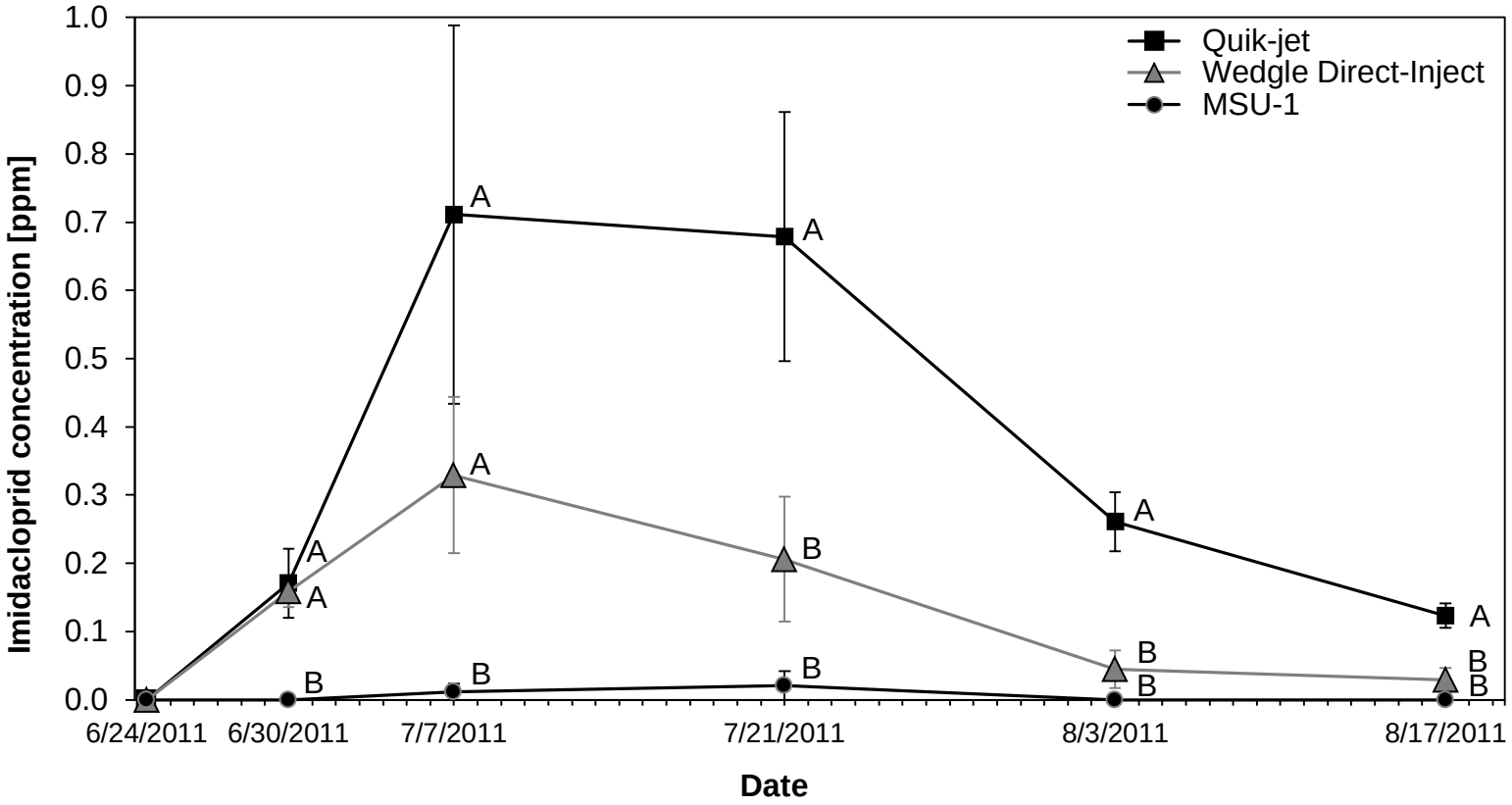

Figure 20. Imidacloprid residue profiles in 'Golden Delicious' apple leaves after trunk injection with different technologies. Quik-jet: drill based micro-injection system. Wedge Direct-Inject: needle-insertion based system. MSU-1: novel slow release imidacloprid formulation TEC2012-0014 delivered through drilled ports. Concentration means within one date followed by different letters are significantly different (*t*-tests, $p<0.05$). Error bars represent standard error of the mean (SEM).

Discussion

This study provides broad-spectrum comparative analysis of different trunk injection technologies in the efficiency of compound delivery over time, labor economics and the disease control on apple trees. The results indicate that temporally uniform compound delivery in the tree canopy can be achieved by split-dose delivery over time. Trunk injection devices provide variable temporal distribution profiles of the injected compound and the levels of disease control but in some cases only over short period of time. Further, the injection devices varied widely in the time required for preparation before the injection and the time required for delivering of the compound. This study emphasizes important quantitative parameters for consideration in use of different injection tools and serves as a basis for further evaluation of existing and the invention of new trunk injection technologies.

Temporal distribution of trunk-injected imidacloprid can be uniform

The simulation of slow time-release indicates that new trunk injection methods should achieve uniform temporal distribution of imidacloprid in the canopy by releasing dose increments at 4 times, 14 days apart. Equal emission of the injected compound to the canopy lowers the concentration footprint in the crown and secures its long-lasting activity for season-long protection of trees. With less dose splitting over time, imidacloprid is oversupplied to the canopy. The excess concentration, surpassing the canopy residues of quarter-split dose, is unnecessary expenditure over short period of time. The compound waste is especially high when pest control is achieved with lower doses. Besides slow dose release after injection, the seasonal

timing of insecticide injections is important to achieve maximum efficiency in control of different insect species.

Drill-based, needle-based and slow-release trunk injection technologies differ in temporal distribution of imidacloprid

Drill-based injection technology in combination with the liquid imidacloprid formulation provided significantly higher concentrations in apple leaf canopy versus the needle-insertion injection technology and the slow-release formulation. These differences can be assigned to the differences in injection method, the targeted tissue for delivery and the formulation. Quik-jet provided the highest concentration in leaves because it delivered liquid formulation into the xylem through a large size trunk injection port. Drilled ports secure good exposure of xylem to the injected compound.

Wedgle provided lower concentration in leaves because it delivers liquid formulation of imidacloprid to the cambium meristem tissue, below the bark. Since cambium is not sap conducive and contains only few layers of cells, imidacloprid translocation was weaker. The other possibility is that imidacloprid was not transported via cambium, or it was but only partially, while the majority of injected dose was absorbed through the outer surface of xylem and then transported into the canopy. This hypothesis is supported by the observation that Wedgle injections lead to bark separation from the xylem surface (Smith & Lewis 2005). We noticed the same effect since the injected solution expands below the bark to create a blister. Further, the exposure of cambium to the injected compound kills this tissue by phytotoxicity

(Smith & Lewis 2005). Hence, if cambium is damaged, the injected compound accumulated in leaves could only be absorbed after transport by the xylem.

The lowest imidacloprid delivery by MSU-1 can be assigned to the inability of this solid formulation to release imidacloprid from its components. Strong binding of this compound to the formulation prevented its release by the dissolving effect of water from the sap flow. It is also possible that the amount of transported water was insufficient to abundantly dissolve imidacloprid from the formulation to readily move in xylem. Water uptake is driven by the rate of daily transpiration of a tree and varies across species (Davies & Lakso 1979; Cohen et al. 1981; Brough et al. 1986). A mature apple tree, depending on weather conditions, age and health uses between 15-50 gallons of water per day (Vossen & Silver 2000). Many forest tree species translocate between 100-200 gallons of water per day or more (Kozlowski 1976; Owens & Moore 2007; Holbrook & Zwieniecki 2008). This implies that adding more water during the injection could increase the release of imidacloprid from MSU-1. Further, this formulation could be changed to bind imidacloprid weakly and readily release it. The design of formulation for injection is important since it secures fast uptake and translocation in xylem to the canopy (J.J. Doccola et al. 2007; 2008; 2012; Doccola & Wild 2012; Smitley et al. 2010; Byrne et al. 2012).

Trunk injection devices differ in temporal distribution of difenoconazole and cyprodinil and in the apple scab control

Drill-based injection devices did not provide higher residue concentrations of the injected fungicides in apple leaf canopy when compared to Bite, while Wedgle was similar to them. Since Bite provided the highest cyprodinil and difenoconazole residues in apple leaves, this indicates

that the properties of lenticular injection port allow fast absorption and uptake of the compounds into the crown. Since no trunk tissue is removed in creation of lenticular injection ports, the trunk wounding is minimal (Montecchio 2013) and the port closure is faster than on the 9.5 mm drilled ports. The injected fungicides accumulated weakly in the canopy most likely because they were rapidly immobilized in xylem due to strong binding to the organic compounds released from the parenchyma cells after port drilling. In Wedgle injections, this process could have occurred with the exposure of fungicides to cambium and phloem, also rich in organic compounds. The decline of residues after Bite injection most likely occurred due to delayed fungicide interaction and binding with the structural and sap-transported organic compounds of symplast and apoplast (Doccola et al. 2012)

Both cyprodinil and difenoconazole have a high affinity for sorption to organic compounds as their organic carbon-water partitioning coefficients (Koc) are 1550-2030 ml/g and 3495 ml/g, respectively (Serafini 2003; 2009; PPDB 2013). Further, water solubilities of cyprodinil and difenoconazole are extremely low with 13 and 15 mg/L, respectively (Serafini 2003; Mensink 2008). These properties align well with the poor accumulation of these fungicides in apple leaves and support slightly higher residue profile of cyprodinil compared to difenoconazole. In contrast, imidacloprid has medium-low Koc of ~350 (Cox et al. 1997) and low water solubility of 510 mg/L (Mulye 1995). Besides the Koc of injected compound, the plant metabolic processes and the pH changes in sap after trunk injection also shape the residue profiles (Lindquist 1965; Norris 1965; Pinkas et al. 1973; Shabi et al. 1974; Smalley 1977; Campana et al. 1979; Perry et al. 1991; Montecchio 2013).

Overall, the comparison of injection devices and technologies implies that chemical properties of the injected compound play a crucial role in its temporal distribution. Imidacloprid

showed to be a good model compound for comparing the trunk injection technologies in provided temporal distribution, while cyprodinil and difenoconazole were not.

Even with weak accumulation in leaves, injected cyprodinil and difenoconazole significantly reduced apple scab on spurs and terminal shoots. This effect is supported by *in vitro* studies on inhibition of *V. inaequalis* growth, which show that cyprodinil has EC_{50} of less than 0.05 mg/L (Kunz et al. 1998; Küng et al. 1999; Köller et al. 2005; Aleksić et al. 2012; Larsen et al. 2013). Similarly, difenoconazole has EC_{50} of 0.016-0.019 µg/ml (Henríquez S et al. 2011; Aleksić et al. 2012; Dahmen & Staub 1992). Even though Tree IV and Wedgle sometimes enabled significantly better apple scab reduction with the delivered fungicides, the overall variations in apple scab control were small. This shows that all injection devices were largely similar in provided apple scab control and that initially higher accumulation of cyprodinil and difenoconazole in Bite injections was not high enough to provide significantly better control.

The injected fungicide combination did not provide control of apple scab as good as the spray standards. Due to the immediate contact with leaf surface, foliar sprayed cyprodinil and difenoconazloe gave better disease control. In contrary, the translocation and accumulation of injected compounds is time consuming process due to their exposure to internal resistance points, naturally present due to the plant tissues (Shinozaki et al. 1964a; 1964b; Waring et al. 1982; Mäkelä 1986; McCulloh et al. 2003; Coomes 2006).

Overall, the more additional tools and steps were involved in the preparation for trunk injection with the device, the more time was required to conduct it. Further, the shortest time for solution discharge during the injection was required by the devices which used either more than 6 psi pressure to inject and which injected the lowest dose volume per tree. Both parameters are

important since less work time in the process before and during the injection would reduce the labor price of injection.

Bite and Mauget required the use of least additional tools and steps before the injection, while Wedgle and Chemjet and then Quikjet, Viper AH and Tree IV, respectively, required more of these and hence took longer time to prepare for injection. On the other hand, Mauget needed the longest time to inject the fungicide into the trunk either because of the low pressure used to discharge the capsule or because the outer rings of xylem, most active in sap flow, were blocked with the wall of inserted feeder tube. Similarly, Bite was the second slowest since the fungicide solution discharge was governed solely by the negative pressure created from the transpiration stream of the tree. With the increase in utilized pressure to deliver the solution, all other devices delivered the fungicides faster into the tree. Wedgle required the shortest time for injection primarily because no water was amended to the fungicide dose.

In conclusion, all the injection devices variably required the use of additional tools and steps to install, thus influencing different time for preparation of trunk injection. Use of pressure or low volume for injection quickened the time of fungicide delivery into the tree. Nevertheless, all the used devices shared two common traits of low throughput of injection element i.e. low volume per one powering of the device and high labor input for the injection of single tree under largely unified parameters. Further, none of the currently available injection devices allows simultaneous treatment of two or more trees (Düker et al. 2006). Future research should aim to increase the efficiency of injection process by allowing the injection of more trees at the same time and by inventing a system for remote control of an equal volume delivery per each tree.

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CHAPTER 7. COMPARISON OF DRILL- AND NEEDLE-BASED TREE INJECTION TECHNOLOGIES IN HEALING OF TRUNK INJECTION PORTS ON APPLES

Abstract

Potential use of trunk injection technology for plant protection in agriculture has been limited by tree wounding after creation of trunk injection ports. This study focused on the timeline of trunk injection port healing in apple trees and monitored parameters such as diameters of callus tissue, bark cracking sizes, and visible and covered port depths. We compared drilled ports from 11/64" and 3/8" drill bits and lenticular port from double-edged blade. Depending on the port size and type, the port closure with callus lasted for 1 to 1.3 and more than 2 years. Port closure was faster on the ports with the smaller diameters. Bark cracking due to frost events was higher in vertical direction of the trunk, around all injection port types. Due to protection by a plastic-silicone plug (Arborplug) on the sealed port from 3/8" drill bit, there was less bark cracking from frost. The visible port depth declined faster on port from 11/64" drill bit and on the lenticular injection port from blade, versus the unsealed port from 3/8" drill bit. When the port from 3/8" drill bit was sealed with Arborplug, visible and covered port depths significantly increased in time due to callus formation on top and laterally, around the plug. The list from least to most injurious trunk injection ports is as follows: 11/64" drill bit port, blade i.e. lenticular port, 3/8" drill bit port sealed with an Arborplug, and the unsealed 3/8" drill bit port.

Introduction

The methods for pesticide application in agriculture have changed little in last 100 years. Although providing adequate pest control with frequent seasonal treatments, technologies designed for topical pesticide delivery carry excessive costs due to product losses in the environment from spray drift. Less than 0.1% of the applied pesticide for plant protection contacts the target pest (Pimentel & Levitan 1986; Pimentel et al. 1992; Pimentel & Lehman 1993; Pimentel 1995; 2005; Shaaban 2009). Air-blast sprayers used in tree-based agriculture are inefficient means of delivering pesticides to their target, with only 29 to 56% of the applied spray solution being deposited on the tree canopy (Steiner 1969; Reichard et al. 1979; Perry 1998; Zhu et al. 2006). In order to achieve efficient and more target-precise delivery of active compounds to tree canopies, current technologies need to significantly improve, new approaches and technologies be explored and invented, and technology transfers from other branches of plant-care science need to occur (Holownicki et al. 2000; Torii 2000; Takai et al. 2003; Düker et al. 2006; Llorens et al. 2010; Shang, Liao, et al. 2011; 2011).

Trunk injection as an *in planta* delivery method for pesticides could be adapted from landscape tree care industry for use in tree-based agriculture. This approach allows precise and confined pesticide and nutrient delivery to trees (Wilson 1979; Barney et al. 1984; Barney et al. 1985; Navarro et al. 1992; Fernández-Escobar et al. 1993; Guillot & Bory 1997; Sánchez-Zamora & Fernández-Escobar 2000; Percival & Boyle 2005; Spinelli et al. 2005; Shaaban 2009; Ahmed et al. 2010). Trunk injection is an environmentally safer alternative for pesticide application which utilizes the tree's vascular system to translocate and distribute active compounds into the canopy. In this way, the compound is contained within the tree, thus

allowing increased selectivity of exposure to unwanted pathogens and insect pests. This is expected to reduce pesticide exposure to non-target organisms, farm workers and the environment. Along with reduced risk chemistries (US EPA 1997), which fulfill high environmental and human safety standards, the use of tree injection could achieve a higher level of sustainability in agriculture, healthier environment and safe food supply in the future.

Tree injection technology has been developed for the landscape tree care industry, where topical pesticide application is not allowed due to close vicinity of urban areas (Guillot & Bory 1997; Grosman et al. 2002; Takai et al. 2003; Takai et al. 2004; Harrell 2006; Smitley et al. 2010; Doccola et al. 2003; 2012). However, the design, implementation and use of trunk injection technology in tree protection have been limited by the problem of the tree wounding resulting from the creation of trunk injection ports. The injection ports allow direct access to tree's xylem and the delivery of injected compound into this tissue. There has been wide-spread concern that injection wounding could have a negative impact on tree health and longevity, especially if repeated injections are needed for sustained pest control. Recently, less-injurious delivery systems for stem injection or infusion of plant protective compounds have been developed (Düker et al. 2006; Düker & Kubiak 2009b; 2009a; Doccola et al. 2003; 2012; Shang, Liao, et al. 2011; 2011; Düker & Kubiak 2011b; 2011a; Montecchio 2011; 2013). These systems were specifically designed to impose minimal injury to trunk tissues when the injection port is created. Nevertheless, no research has shown the comparisons of the level of injury after creation of different injection ports. Therefore, the question of whether the tree wounding by injection ports leads to economically important damage to trees and impairment of tree longevity and fertility, remains unanswered.

This controversial topic of tree wounding by injection is widely discussed in tree care industry and is the most frequently hypothesized obstacle for potential implementation of trunk injection in tree-based agriculture (Shigo et al. 1977; Shigo & Marx 1977; Shigo & Service 1979; Costonis 1980; Shigo 1981; 1978; 1984; 1985; Santamour Jr 1984; 1986; Neely 1988; 1979; Perry et al. 1991; McGillivary et al. 1993; Wasniewski, Chaney & Holt 1993; Smith & Lewis 2005; Shortle et al. 2010; Doccola et al. 2011). One of the most important parameters for measuring the degree of harm from trunk injection wounds is the time needed for injection ports to heal. Only a handful of studies address some aspect of injection port healing as it related to forest tree species, peaches, apples and grapevines (Neely 1979; 1988; Costonis 1980; Wasniewski, Chaney & Holt 1993; Percival & Boyle 2005; Düker et al. 2006; Doccola et al. 2011; Smith & Lewis 2005; Cooley et al. 1992; Shigo et al. 1977; Shigo & Marx 1977). Some of these studies show frequency and differential rate of wound closure in time but not on apple trees. To address this knowledge gap, using apple tree as a model, our objective was to compare the time needed for injection port closure after creation of four different types of trunk injection ports.

Our leading hypothesis was that drilled injection ports smaller in size will close faster in time than the larger drilled injection ports and thus be less injurious to the tree. Further, we hypothesized that the injection ports created by needle insertion will heal faster than drilled injection ports, and thus be less injurious than drilled injection ports. The aim of this study was to answer the question how fast trunk injection ports close in time, and record any other parameters related to visible tree injury caused by injection ports on the trunk.

Materials and Methods

An orchard experiment was conducted during 2012 and 2013 at Michigan State University's Plant Pathology Farm in Lansing, MI (GPS: N42° 41' 34.93", W84° 29' 31.657"). On 14 April 2012, 13-yr-old 'Kit Jonathan' apple trees, *Malus domestica* Borkh., were wounded with the four most common types of trunk injection ports: 1) drilled port 4.4 mm in diameter after using 11/64" wood drill bit, 2) drilled port 9.53 mm in diameter after using 3/8" wood drill bit, 3) drilled port 9.53 mm in diameter sealed with a solid plastic-silicone plug (Arborplug® no. 4, Arborjet Inc., Woburn, MA), and 4) lenticular port 1 mm in width and 28 mm in height, after insertion of symmetrical double-edged blade 4 mm in width, 33 mm in height, 50 mm in length (Figure 21A). A similar lenticular port is created after use of needle-insertion based injection device with a screw-driver like needle, called Bite® - blade for infusion of trees (University of Padova, 2011). All injection ports were 25.4 mm deep and created by drilling into the trunk xylem with a cordless 1500 rpm drill (DeWalt Industrial Tool Co., Baltimore, MD) or by temporary insertion of a knife blade with a hammer. We inserted the blade perpendicular to the trunk axis but positioned vertically so that it separates vertically oriented wood fibers (Figure 21B). Arborplugs in the port from 3/8" drill bit were positioned 3 mm below the bark surface, so that the orifice around the silicone septum for injection on the plug is in line with the level of the bark surface. This allows an undisturbed healing process by cambium.

For each type of injection port we used three replicate trees arranged in completely randomized design (CRD). We created four injection wounds per tree replicate for each port type and deployed them on the trunk according to cardinal directions. Ports were positioned approximately 30 cm above the ground surface and vertically separated by approximately 5 cm

between opposing port pairs. In 2012, used apple trees ranged from 7.11-10.16 cm in trunk diameter at 1' height (DFH) (average 8.03 cm). In 2013, the same trees ranged from 7.75-11.43 cm in DFH (average 8.84 cm).

Each injection port was repeatedly measured on 20 July 2012 and on 14 April and 20 July 2013. We measured the following parameters for each port type (mm): 1. Horizontal and vertical diameters of callus tissue around the injection port (indicating healing), 2. Width and height of bark crack around the injection port, 3. Visible injection port depth from the bark surface (also indicating healing) and covered injection port depth from the Arborplug surface. If the entrance to port cavity was closed by the callus from the cambium, as the visible port depth from the bark surface we measured the distance from the old, raised periderm on the bark which is around the injection port, and the new periderm from callus formed from cambium which is positioned below the old periderm (Figure 23). If the entrance to the port cavity was closed by an Arborplug, as the visible port depth from the bark surface we measured the distance from the old, raised periderm on the bark and the Arborplug surface around the entrance to silicone septum for injection. For measuring the covered injection port depth from the Arborplug surface, we inserted a needle through the silicone septum for injection, then marked the spot on the needle in line with the Arborplug's surface when the needle tip reached the port cavity bottom, and measured this needle increment length. We calculated parameter means from 4 injection wounds per each tree replicate. Then, for each port type, we calculated grand parameter means from 3 replicate trees and conducted statistical comparisons.

Data were analyzed using MIXED procedure in SAS 9.3 (SAS Institute Inc., 2012). To normalize the residuals, we transformed data on horizontal diameter of callus tissue around the injection port with SQUARE ROOT (SQRT) function. For the data on height and width of bark

crack around the injection port we used the COSINE (COS) transformation. We analyzed the effects of port type and time on horizontal and vertical diameters of callus tissue, on the height and width of the bark crack, and on the injection port depth from the bark surface, by using CRD with repeated measures best adjusted with unstructured variance covariance structure. For drilled 9.53 mm port sealed with an Arborplug, we analyzed these effects by using CRD with repeated measures best adjusted with autoregressive covariance structure. We chose variance-covariance structures by lowering the AIC and BIC criterions. Tree was used as subject of repeated measurements through time.

When the effects of port type or time, or their interactions, were found to be statistically significant ($P < 0.05$), slicing, i.e. examination of interactions within main effects, was performed, *F*-tests were carried out and pairwise or specific time or port type comparisons were conducted using *t*-test ($\alpha=0.05$) and using Tukey-Kramer test ($\alpha = 0.05$).

Results

The type of injection port, time and their interaction significantly affected the rate of wound healing in both the horizontal and vertical diameter of callus tissue (Table 25). The port type did not significantly affect the width of bark crack around injection ports, but it interacted with time (Table 25). The port type only affected the height of the bark crack around the injection port (Table 25). Since the port type and time interaction did not affect the height of bark crack, we examined it with Tukey-Kramer's test. Both main factors significantly affected the visible port depth from bark surface, but also significantly interacted. Covered port depth from

the surface of the plastic-silicone plug (Arborplug) significantly changed through time in the case of sealed 3/8” drill bit port.

From 20 July 2012 onwards, both horizontal and vertical diameters of callus tissue significantly declined on all injection port types, indicating on the healing of port wounds by the activation of cambium (Figure 22). Significant differences in callus diameters of different port types, within each time point, are shown in Figure 22. Excluding 11/64” drill bit ports, the horizontal diameter closed faster in time than the vertical port diameter. The fastest healing or wounds was in 11/64” drill bit and blade ports (Figure 22).

In regards to the frequency of closed injection port wounds, none of the injection port types completely healed on 20 July 2012. On 14 April 2013, in ports from blade and 11/64 drill bit, 8 out of 12 and 11 out of 12 injection ports healed, respectively (Figure 22). On the same date, in ports from 3/8” drill bit and 3/8” drill bit sealed with Arborplug, there was 5 out of 12 and 0 out of 12 injection ports healed. Only ports from blade and 11/64” drill bit, completely healed by 20 July 2013 (Figure 22). At the end of the experiment, none of the ports from 3/8” drill bit sealed with Arborplug healed, while 9 out of 12 ports from 3/8” drill bit healed completely. The appearance of callus tissue around all the injection ports is shown in Figure 23.

The height of bark cracks around the injection ports was much larger than their width. The height of bark cracks initially increased faster through time than the width (Figure 24). From 20 July 2012 onwards, the width of the bark crack did not change significantly only around the port from 3/8 drill bit sealed with an Arborplug. In the same time period, the height of the bark crack did not change significantly around any type of the injection ports. The examination of main factors interaction showed that only on 20 July 2012, port types differed significantly and Figure 24 shows which port types led to formation of the smallest and the largest widths and

heights of bark cracks around the injection ports. The appearance of bark cracking around all the injection ports is shown in Figure 23.

Overall, in all injection port types, visible port depth from the bark surface was smaller when compared to the covered port depth from the Arborplug surface on the sealed port from 3/8" drill bit (Figure 25). For the ports from 11/64" drill bit and blade, visible port depth from the bark surface declined rapidly and significantly through time, while for the port from 3/8" drill bit this decline occurred much later. Both visible port depth from the bark surface and the covered port depth from the Arborplug surface on sealed port from 3/8" drill bit, significantly increased through time (Figure 25).

Table 25. The effects and interactions of port type and time on healing of injection port wounds on trunks of 'Kit Jonathan' apple trees in 2012 and 2013.

Monitored parameters		Main effects	*F*	DF	*p* value
Callus tissue diameter	**Horizontal**	Port type	1.12	8	**≤0.0008**
		Time	104.42	7	**≤0.0001**
		Port type*Time	10.25	7.79	**≤0.0024**
	Vertical	Port type	10.95	8	**≤0.0033**
		Time	329.22	7	**≤0.0001**
		Port type*Time	33.86	7.79	**≤0.0001**
Bark crack	**Width**	Port type	1.12	8	≥0.3977
		Time	8.31	7	**≤0.0142**
		Port type*Time	4.72	7.79	**≤0.0253**
	Height	Port type	4.48	8	**≤0.0400**
		Time	1.62	7	≥0.2644
		Port type*Time	1.66	7.79	≥0.2509
Port depth	**From bark surface**	Port type	24.06	8	**≤0.0002**
		Time	9.01	7	**≤0.0116**
		Port type*Time	4.57	7.79	**≤0.0275**
	From Arborplug surface	Port type	-	-	-
		Time	6.24	4.94	**≤0.0391**
		Port type*Time	-	-	-

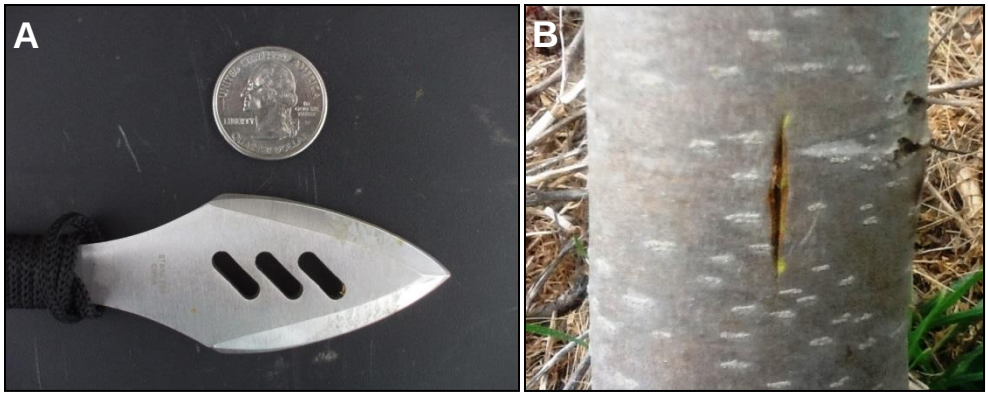

Figure 21. The shape of double-edged blade used in wound evaluation (A) and of the lenticular port created from double-edged blade insertion (B). Similar port is used to inject trees with of Bite® - blade for infusion of trees (University of Padova, 2011).

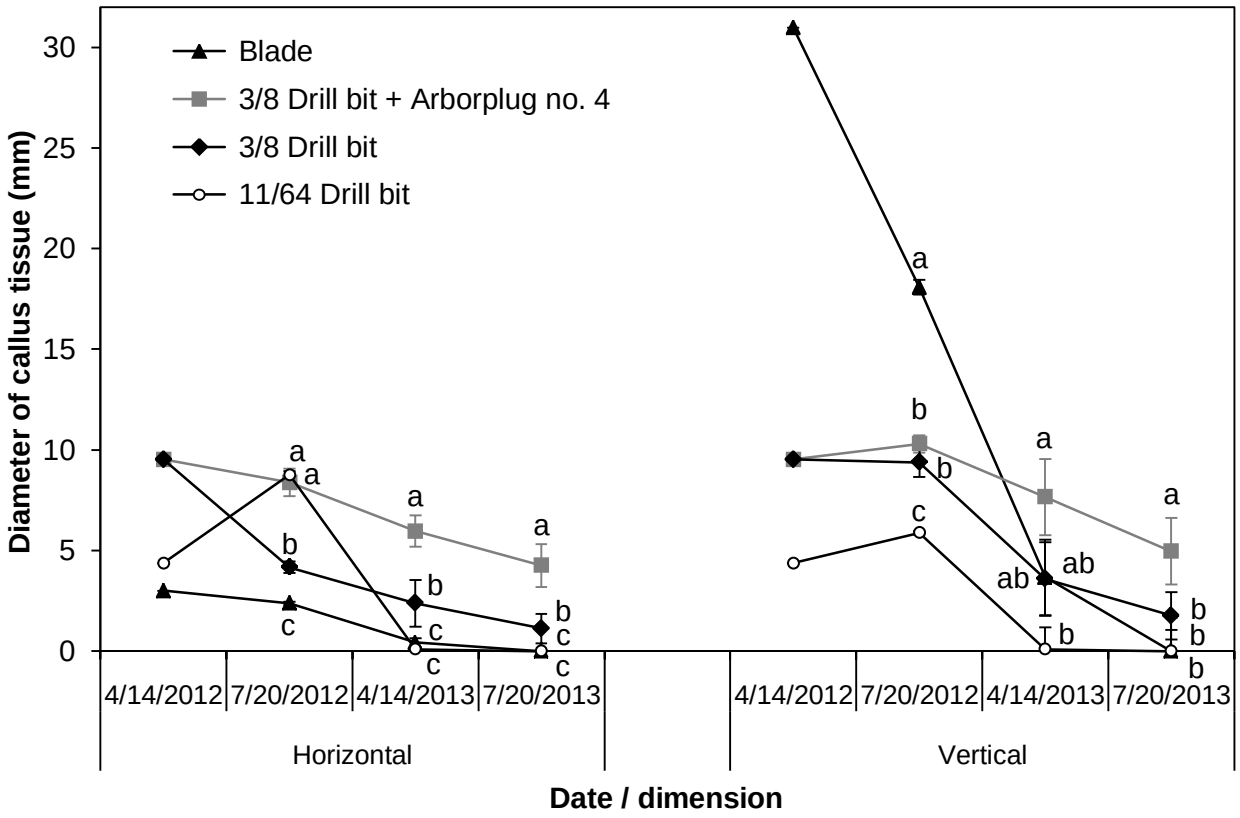

Figure 22. Diameter of callus tissue around injection port wounds during two years after creation of 4 different types of trunk injection ports. Means within one date followed by different letters are significantly different (*t*-test, α=0.05) and are based on 3 replicate trees and 4 replicate wounds per each tree. Error bars represent standard error of the mean (SEM).

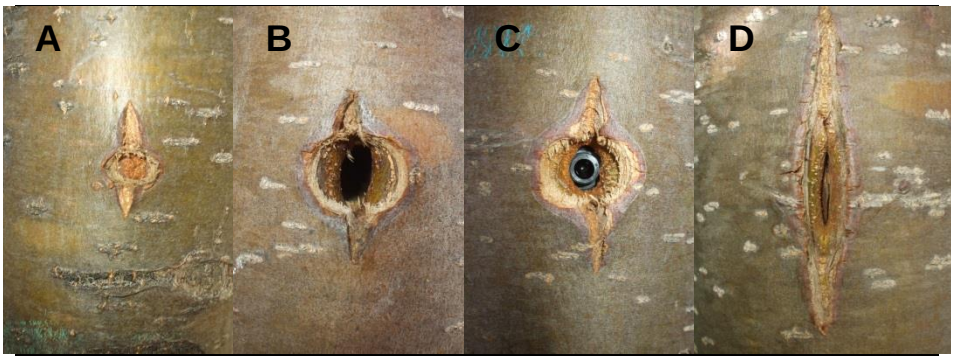

Figure 23. Four types of trunk injection port on 20 July 2012, evaluated for port healing. Drilled ports from 11/64" wood drill bit (A), 3/8" wood drill bit (B), 3/8" wood drill bit sealed with an Arborplug® no. 4 (C), and lenticular port, 1 x 28 mm, from double-edged blade (D).

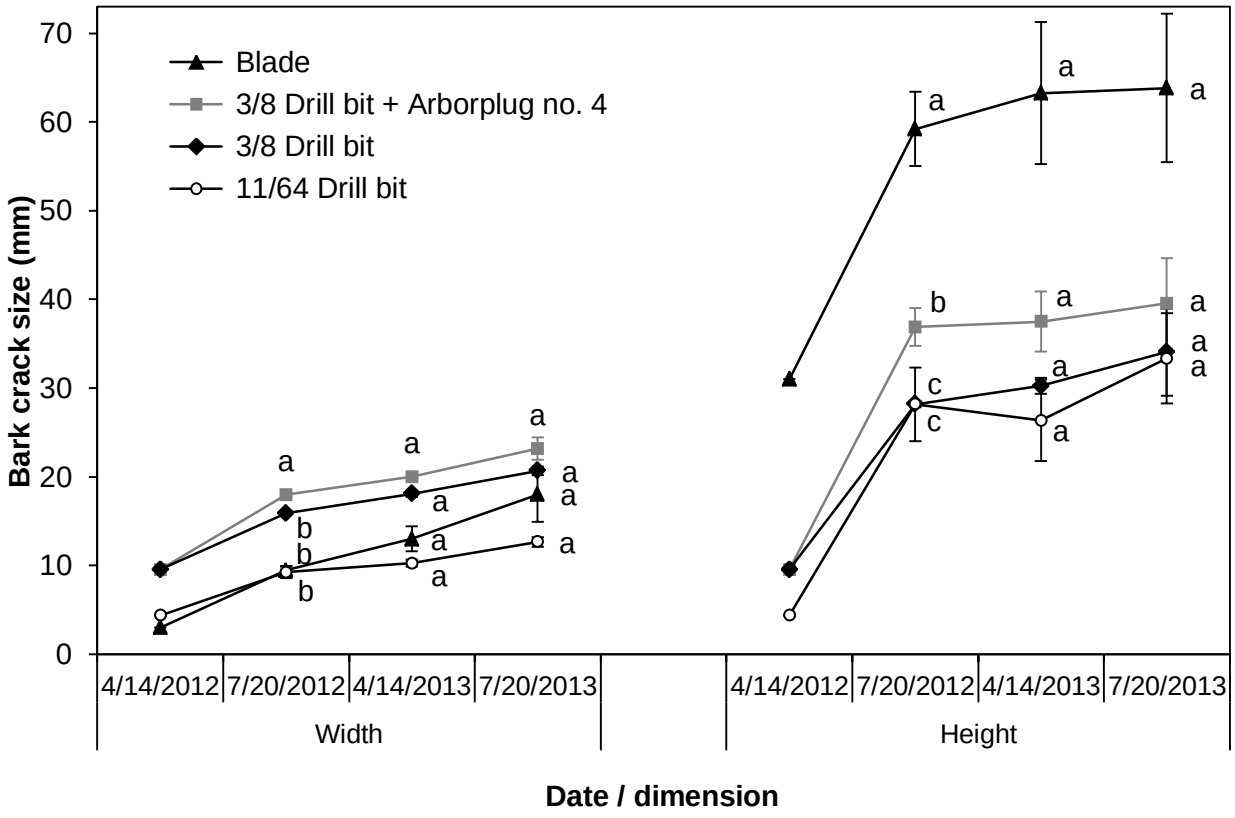

Figure 24. Size of the bark crack around injection port wounds two years after creation of 4 different types of trunk injection ports. Means within one date followed by different letters are significantly different (α=0.05) (*t*-test for width; Tukey-Kramer test for height). Means are based on 3 replicate trees and 4 replicate wounds per each tree. Error bars represent standard error of the mean (SEM).

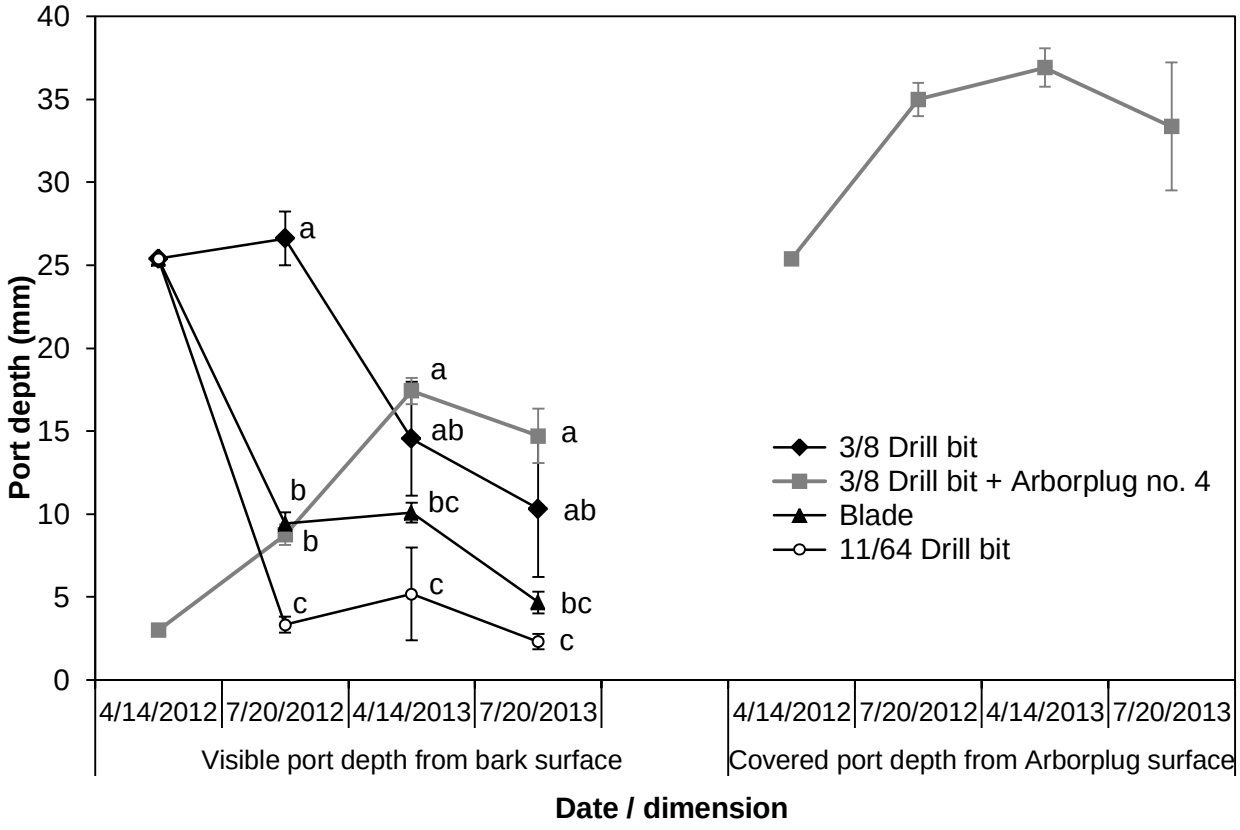

Figure 25. Injection port depth two years after creation of 4 different types of trunk injection port (*t*-test, α=0.05). Means within one date followed by different letters are significantly different (*t*-test, α=0.05) and are based on 3 replicate trees and 4 replicate wounds per each tree. Error bars represent standard error of the mean (SEM).

Figure 26. Formation of callus tissue from xylem parenchyma cells inside the port cavity on the lenticular trunk injection port from double-edged blade.

Discussion

This study brings new insights on bark wounding and rate of healing of four injection port types on apple trees. The results indicate that, depending port type and size, the process of port closure with callus can last for 1 to 1.3 or more than 2 years. We emphasize the advantage of faster healing of small drilled injection ports and the lenticular injection ports, as well as the need for sealing of the large drilled injection ports to protect the cambium. This study shows the importance of multiple quantitative parameters that need to be monitored on injection ports to precisely depict the time required for wound healing process and its port-specific expressions on

the trunk. This serves as a basis for further evaluation of the impact of injection ports on tree health, function and longevity. Our findings are directly relevant for crabapples, and other *Malus spp.* and thin-barked trees in urban landscapes.

The frequency of closed injection ports and the diameters of callus tissue around them show the variable speed of wound healing depending on the port size and type. The results demonstrate that the smaller the horizontal diameter of trunk injection port is, the faster the process of port healing i.e. closure with the callus tissue. This trend was similar also for the vertical diameter of the callus tissue, except in the case of lenticular injection port due to the inherently larger port height from the blade. Further, the healing was faster on large diameter injection ports when these were unsealed with an Arborplug. Hence, the presence of an Arborplug, as a foreign object which seals the 3/8" drill bit port, significantly increases the time needed for complete port closure by callus. This, however, does not mean that plug sealing of injection port should be avoided; on the contrary Arborplug mimics bark and more importantly creates protective compartment barrier necessary for preventing microbial infection and for complete wound closure with callus, which eventually occurs.

Similar results showing faster healing of the small injection ports and the slowest healing of the large ports, have been reported before on white ash, *Fraxinus americana* L., honey locust *Gleditsia triacanthos* L. and pin oak *Quercus palustris* Münchh., and as in the present study with apple trees, the level of wound closure with callus was correlated with cambium activity which enables radial trunk growth (Neely 1979; 1988; Doccola et al. 2011). Similar report on healing process expressed as a frequency of drilled injection ports was recorded on green ash, *F. pennsylvanica* Marsh. (Doccola et al. 2011). The present study is the first to show the timeline of injection port healing on apple as a diffuse porous hardwood, as all the above species are with

ring porous xylem. Previous research on trunk injection of apple trees for disease control, reported that all drilled injection ports, 3 mm in diameter, imposed only 5 mm deep into the xylem of root flare, were fully callused over within the same growing season (Percival & Boyle 2005). This largely agrees with the fastest callus closure of our small drilled ports, 4.37 mm in diameter, from 11/64" drill bit, within one season.

Since the height of the bark crack around all injection port types initially increased faster and was overall higher than the width, this implies that vertical reaction zone of the bark on apple trunk is much more sensitive to cracking than the horizontal reaction zone. Significant increase of the width of the outer bark crack through time, around all port types, except around the Arborplug sealed port, implies that Arborplug offers more protection from bark cracking which was most likely caused by frost damage. Namely, during frost events after rain or with high air humidity, we observed that water collects and/or condensates inside the unsealed injection ports and on top of the Arborplug. Since water freezes in the opened ports and the ice extends outside on the bark surface, bark cracking typical for frost damage occurs on the trunk (Caplan 1988; Snyder & Melo-Abreu 2005). We speculate that unsealed injection ports allow more frost damage on tree bark. The facts that bark crack height was large around all port types and that after 20 July 2012 it did not change in size support the scenario of frost damage causing cracking and indicates on short lasting of frost events. These results indicate that whichever type of injection port is used on apple trees which have thin bark there is a chance for bark damage during frost. However, this side-effect can be avoided by conducting trunk injections later in the season, when risks from frost events are minimal.

The highest width and height of bark cracking in ports from 3/8" drill bit and blade, respectively, can be attributed to the presence of an Arborplug in the drilled port and the largest port height imposed by the insertion of blade.

Bark cracking has been observed after 1-2 growing seasons on eastern hemlock, *Tsuga canadensis* (L.) Carr., around drilled injection ports sealed with an Arborplug and around lenticular injection ports, following the injection with Viper™ Air/hydraulic micro-injection system (Arborjet, Inc., Woburn, MA) and Wedgle Direct-Inject™ System® (ArborSystems LLC., Omaha, NE), respectively (Smith & Lewis 2005).

In 2012, rapid decline of visible port depth from the bark surface in all injection port types, except in the two ports from 3/8" drill bit, indicated that during radial trunk growth, callus forming from cambium covered the entrance to the port cavity. The newly proliferating tissue "stretched" over to cover the port cavity and this contributed to visible port depth decline from the initial depth of 25.4 or mm (Figures 23 and 25). Nevertheless, the cavity from removed xylem tissue below the new callus cover still remained empty in injection port from the 11/64" drill bit.

On the lenticular injection port from blade, on the exposed xylem surface we observed that from inside the port cavity there was a callus tissue proliferation from ray parenchyma cells (Figure 26). This additionally contributed to a significant decline in visible port depth from the bark surface. Callus formation from the xylem rays was probably promoted by the natural tendency of laterally separated wood fibers, to retract into the previous position and re-seal or partially close the opened triangular cavity. When trunk bark on cherry and other tree species is removed by girdling, to an extent at which cambium callus cannot "bridge over" i.e. cover the large wound, the cells of living xylem parenchyma closest to the wound transform and start

proliferating along the xylem rays (Neely 1979; Layne & Flore 1991). These new cells form an extensive wound repair callus, which starts functioning as new bark with periderm, phloem and cambium. It seems that this process happens on young apple trees but the question remains at which order of the magnitude. It is possible that this callus formation from xylem parenchyma contributed to the increase of the visible port depth in port from 3/8" drill bit sealed with an Arborplug. We could not conduct destructive autopsy dissections of apple trees to confirm the callus formation from parenchyma in this and the lenticular injection port.

In ports from the 3/8" drill bit sealed with an Arborplug, a significant increase in visible port depth from the bark surface occurred because of the callus tissue growth over the Arborplug surface. Since the plug has fixed position in sapwood, the callus "stretching" over and above the plug rises as a protrusion, thus increasing the visible port depth.

On the port from 3/8" drill bit, in July 2012, the visible port depth from the bark surface was unchanged because of the large port diameter. The amount of removed tissue in this port was too extensive to be rapidly covered with new callus tissue from cambium, within one season. However, the visible port depth declined during the next tree growth cycle in 2013. Hence, larger ports like this require more time to be covered with callus from cambium and therefore should be sealed with an Arborplug after creation.

The significant increase of covered port depth from the Arborplug surface, in the sealed port from 3/8" drill bit, leads to an opposite inference from the one reached above, that Arborplug does not have a fixed position in sapwood during port healing. It seems that during cambial growth, the callus formation pushes the Arborplug outwards to a degree, and thus contributes to the increase of the covered port depth below the Arborplug. This indicates that, along with the process of callus growth on top of the plug, callus formation from both cambium

and xylem parenchyma could act around the inserted Arborplug, thus significantly protruding it above the bark level with the mass of new proliferating cells. Nevertheless, after 20 July 2012 it seems that this outward movement of Arborplug ceased. To our knowledge, no previous research reports the change in depth of trunk injection ports through time, thus indicating on the progress of port healing.

Depending on tree species, most commonly observed side-effects of drill-based trunk injection with pesticides are tissue discoloration and dysfunctionality due to phytotoxic effects from injected chemical, trunk splitting, cambium death or its distorted growth, weeping and fluxing of sap, and wood decay and rot due to infections by microorganisms (Shabi et al. 1974; Neely 1979; 1988; Costonis 1980; 1981; Santamour Jr 1986; Perry et al. 1991; McGillivary et al. 1993; Wasniewski, Chaney & Holt 1993; Shigo 1978; Smith & Lewis 2005; Shortle et al. 2010). During our experiment on apple trees, we have not detected any symptoms of fungal or bacterial infections in all injection port types.

The degree to which injection leads to tree function impairment is hypothesized to depend on variety of factors including tree species, pesticide formulation and injection technology (Sachs et al. 1977; Santamour Jr 1984; Doccola et al. 2011; Doccola & Wild 2012; Montecchio 2013). Some vigorous forest trees have the capacity to quickly heal the ports resulting in minimal disruption of function. For example tulip poplar, *Liriodendron tulipifera* L., was able to effectively close 8 mm diameter drilled ports after 16 months (Wasniewski, Chaney & Holt 1993; 1993). Physical wounding by injection ports on large trunk diameter forest trees is often perceived as less severe than on the trees with smaller trunk diameter. In spite of all the reported observations, only few in-depth research references exist on quantification and characterization of the side-effects of injection or the timeline of port healing on trees. For

example in elms, physical injury due to drill-based injection can reflect in dead trunk bark and columns of discolored and compartmentalized (occluded) xylem, extending 15 feet upwards and downwards and leading up to 40% of functional transport disruption (Perry et al. 1991). On fruit trees, such as apple, even fewer references exist and these only mention trunk injection port closure with callus and some aspects of phyotoxicity on wood tissues or leaves (Shabi et al. 1974; Long et al. 1989; Guest et al. 1995; Percival & Boyle 2005).

Due to tissue removal, drill-based injection technologies are perceived as more damaging to the trunk. However, this technology offers an option of closing the port cavity with a plug which provides protection and creates a reservoir to which the solution is injected (Quikjet, Viper AH, Tree IV). In other methods drilled ports are not sealed after injection (Mauget, Chemjet). Nevertheless, the major advantages of drill-based injection technology are the high level of xylem exposure to the injected chemical, fast solution uptake and uniform distribution of the injected compound in the tree canopy, and lower dependence on developed leaf canopy and weather conditions. This secures high level of pest and disease control efficiency, even though the use of this technology is labor intensive.

The main advantage of needle-based trunk injection technologies which create lenticular shape of trunk injection port (Bite, Wedgle), is the low extent of tree wounding due to the insertion of screw-driver like needle into the trunk, without removing the trunk tissues (Montecchio 2011; 2013). This allows faster port healing since the vertically separated wood fibers retract, partially of fully, after injection thus easing the process of port healing (Montecchio 2011; 2013). However, the main disadvantages of this injection technology are less xylem exposure to the injected chemical, non-uniform compound distribution in the canopy,

more labor and time consumption for injection, and higher dependence on developed leaf canopy and weather conditions (Bite, Wedgle) (Aćimović et al., *unpublished*).

Conclusion

Depending on injection type and port size the time needed for port healing is variable. The smaller the diameter of trunk injection port is on a tree, the faster is the process of port healing with the callus. Further, bark cracking due to frost events can be higher in vertical direction of the trunk, around all injection port types. Due to protection from Arborplug on the sealed port, there can be less bark cracking damage from frost. Finally, the visible port depth declines faster on drilled injection port with small diameter and on the lenticular injection port, versus the unsealed drilled port with large diameter. When the large drilled ports are sealed with Arborplugs, visible and covered port depth significantly increase in time due to callus formation on top and laterally, around the plugs. Therefore, we conclude the list from least to most injurious trunk injection ports as follows: 11/64" drill bit port, blade i.e. lenticular port, 3/8" drill bit port sealed with an Arborplug, and the unsealed 3/8" drill bit port.

However, many important questions are yet to be answered. Long-term consequences of seasonally repeated creation of injection ports on apples, or any other fruit tree species, are not investigated. Since the exploitation of a modern apple orchard lasts around 15 years (Katalinić 2014), the true impact of trunk injection on tree longevity and productivity needs to be investigated within this model. After injection, nearby layers of xylem tissue get suberized and die, thus becoming permanently compartmentalized i.e. sealed off and non-conducive for sap flow (Shigo et al. 1977; Shigo & Marx 1977; Shigo & Service 1979; Shigo 1978; 1981; 1984;

1985; Perry et al. 1991; Doccola et al. 2011). In the case of trunk injection port sealed with an Arborplug, most likely the same process occurs, as it is reported after destructive autopsy of green ash, *F. pennsylvanica* Marsh., (Doccola et al. 2011). Based on our observation of sap leakage from the silicone septum on Arborplugs, in spring, 8-12 months after injection, it is not clear whether the xylem compartmentalization on apple trees completely seals-off the port and prevents sap conductivity. It is possible that this process is limited in extent on apple trees. Therefore, future experiments should measure how far from the port the surrounding xylem tissues stay conductive for sap flow and at which level. To determine the extent of internal tissue functionality or dysfunctionality around injection port, sap flow speed in the xylem could be measured through time by using previously described heat-pulse method with a pair of thermocouples inserted into trunk (Cohen et al. 1981; Smith & Allen 1996; Burgess et al. 2001). The two thermocouples, installed one above another, could be positioned at differentially distant points from and above the injection port of each type. This would undoubtedly show xylem conductance around the injection port and allow quantification of the amount of tissue which is sealed off after each new injection in the season. Further, by using the same method, with one of the thermocouples inserted into the port sealed with an Arborplug, it could be determined whether the sap flow occurs in this port or not and at which level through time. The resulting data will elucidate the impact of injection port wounding on tree health and provide basis for future research in adaptation and use of trunk injection technology in fruit orchards.

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CHAPTER 8. CONCLUSION AND FUTURE RESEARCH IN TRUNK INJECTION FOR FRUIT TREE PROTECTION

Spatial and temporal distribution of trunk-injected imidacloprid in apple tree canopy

Use of pesticides in tree-based agriculture creates extensive drift-driven pesticide losses and leads to environmental contamination and occupational exposure risks (Pimentel 1995; Pimentel 2005). Trunk injection of pesticides as a target-precise delivery system could severely reduce drift-driven pesticide losses (Düker et al. 2006). The efficiency of trunk-injected compounds has been indirectly associated with their spatial and temporal distribution within the crown (Pinkas et al. 1973; Shabi et al. 1974; Clifford et al. 1977; Pilbeam 2003; Percival & Boyle 2005; Spitko 2008; Ahmed et al. 2010; Byrne et al. 2012). However, only few studies show limited data and only on temporal distribution of pesticides in tree crown after injection (Schutte et al. 1988; Tattar et al. 1998). How, where and when trunk-injected compounds distribute in the tree canopy are the key missing components in explaining the compound efficiency in the crown of fruit trees.

In an agricultural study, first of its kind, the spatial and temporal distribution of a trunk-injected imidacloprid was quantified within apple crown. The main finding in spatial and temporal transport and distribution of imidacloprid in apple leaf canopy was that spatial distribution after trunk injection depended significantly on the number of injection ports used per tree. Patterns of imidacloprid residue concentration in the crowns of apple showed that by increasing the number of trunk injection ports per tree, imidacloprid distribution in apple leaf canopy was spatially more uniform. Imidacloprid application using 4 or 8 ports resulted in more

uniform distribution of the compound in the crown than using 1 or 2 ports. Since imidacloprid injection with 8 ports did not substantially improve spatial uniformity over the injection with 4 ports, it was concluded that there is a point of diminishing returns on the number of point sources for injection. This is the first report on cultivated tree species in agriculture which shows direct connection between the number of trunk injection delivery ports and the spatial distribution of the injected compound in the canopy. Further, it has been found that uniform and non-uniform spatial distribution patterns were expressed early at 14 days after injection (DAI) and lasted throughout the experiment. Unexpectedly, upper and lower crown positions did not significantly differ in imidacloprid concentration.

Temporal distribution of imidacloprid showed that in all injections there was no uniform distribution of imidacloprid in time. However, the level of temporal non-uniformity was the lowest in the injection with 2 ports.

These results lead to a conclusion that in the tree crown with spatially non-uniform compound distribution, low or no pest control will occur in crown directions weakly or not supplied by the compound. Further, when in this non-uniform distribution the compound is oversupplied in crown directions nearest to the injection ports, the compound excess represents wasted dose unneeded to provide required control. Based on the results, the most optimal number of injection ports that can be recommended for uniform compound distribution in apple tree crown is 4. The finding that upper and lower crown positions did not significantly differ in imidacloprid concentration has an important value for tree-based agriculture from the perspective of achieving efficient plant protection on the whole apple crown.

Future research in regards to the spatial distribution of trunk injected compounds in tree crown, needs to focus on proving whether compound distribution in apple fruits is similarly

dependent on the number of used injection ports or not, and what are the amounts of residues in fruit if this dependence exists. This research needs to answer whether the similar non-significant difference in compound distribution between upper and lower crown positions is also present in fruits as it was present in leaves. The primary reason why these questions need to be answered by experiments is the fact that fruits and leaves have variable number of stomata per area of green tissue and have different types and lengths of physiological processes taking place in them (Blanke & Lenz 1989). The latter is especially important because of transpiration. It is known that apple fruits have 10- to 100-fold lesser frequency of stomata on epidermis in comparison to the leaves (Blanke & Lenz 1989). Further, the number of stomata decreases as the fruit size increases and some are transformed into lenticels during the fruit ontogeny (Blanke & Lenz 1989). This means that apple fruit transpire at a much lesser intensity than the leaves and this could severely impact i.e. reduce the rate of accumulation of the injected compounds in apple fruits. In turn this could reduce pesticide efficiency in fruit and reduce pest control.

The results on temporal distribution of imidacloprid which show no uniform compound dose delivery in time lead to a conclusion that the excess amount of compound above the dose of efficient pest control represents unneeded and wasted product in time. Therefore, future research needs to be conducted to develop new concept and design of trunk injection system which can provide controlled and adjustable release of the efficient compound doses in time. In other words, this system needs to provide long-lasting and temporally uniform delivery of equal and efficient compound doses. Currently the only two systems available in landscape tree care and forestry, which could potentially provide these performances, are High Volume Macro-Infusion^{TM} Kit and Low Volume Macro-Infusion Kit^{TM} (Rainbow Treecare Co., Minnetonka, MN). However, research is needed to test these existing and new injection systems and to

develop injection timing schedules during the season that will take full advantage of the fundamental mechanisms at work within apples trees and allow stable, efficient and long-lasting dose delivery.

Important part of this research need to be dedicated to unrevealing the ways of manipulating the reservoir effect in the trunk generated by the interactions of injected compounds with the xylem apoplast and symplast (Doccola et al. 2012; Tanis et al. 2012). This also means that the investigation of new, injection compatible pesticide formulations has to be initiated and developed parallely (Norris 1965; Pinkas et al. 1973; Shabi et al. 1974; Campana et al. 1979; Nair 1979; Nair et al. 1981; Düker et al. 2006; Doccola et al. 2012; Doccola & Wild 2012; Montecchio 2013).

Based on imidacloprid crown concentration patterns, imidacloprid transport in xylem of apple trunk was hypothesized to occur simultaneously through radial diffusion and vertical transport with a spiral pattern. Vertical transport was proposed to have a 360º helical pathway with counterclockwise turn of the spiral. More research with extensive wood core sampling per tree and in time should definitely provide evidence for confirmation or disapproval of these hypotheses.

Control of fire blight (Erwinia amylovora) on apple using trunk injection delivery of plant resistance activators and antibiotics

Classic approach in fire blight control is burdened with two major difficulties. The first is that there are no synthetic compounds with fully systemic properties available to improve fire blight protection programs (Adaskaveg et al. 2011; Balaž et al. 2013). The second difficulty is

the occurrence and spread of *E. amylovora* resistance to copper and antibiotics (Chiou & Jones 1991; 1995; Loper et al. 1991). When non-target bacterial populations in the agro-ecosystem are exposed to bactericides after topical application, which creates drift-driven compound losses, they can acquire resistance genes and then transfer these genes to target organisms such as *E. amylovora*, thus hastening the development resistance in bacterial plant pathogens (Chiou & Jones 1993; Sundin et al. 1995). This process is not only a problem in fire blight control (Sundin et al. 1995; Sundin & Bender 1996). The fear of antibiotic resistance transfer from plant pathogenic bacteria to human pathogenic bacteria fueled the legislative bans for agricultural use of antibiotics in Europe and the efforts for severe use restrictions in the USA (McManus et al. 2002; Vidaver 2002; Kumar et al. 2005). Risk from resistance acquisition drives the efforts for rational antibiotic use and preserving the efficiency of antibiotics in human medicine, while imposing scrutiny on use of antibiotics for plant protection. One novel approach which could help reduce the level of exposure of non-target bacterial populations, serving as gene pool for resistance, is trunk injection of bactericides and SAR inducers. Trunk injection is target precise pesticide delivery system since it allows compound deployment in a contained manner, without direct pesticide losses into the environment. Further, this system could be especially effective for control of *E. amylovora* which spreads through apple xylem. However, it is not known whether trunk injection of bactericides and SAR inducers can provide efficient control of fire blight thus fulfilling the high standards of plant protection in tree-fruit industry. Further, it is also not known whether SAR inducers after trunk injection can induce PR-protein gene expression and can the effect on disease be enhanced due to trunk injection as *in planta* delivery approach.

Blossom and shoot blight control

When trunk injection of bactericides and SAR inducers was evaluated in control of *E. amylovora* infection, significant blossom blight incidence reduction was detected in 2 out of 3 years of trials. At medium level of infection of 50% in 2012, the most effective blossom blight reduction was achieved by injected Agrimycin, Phosphojet, and Actigard, all providing similar effects. Imajet failed to reduce blossom blight.

At high level of infection of 75% in 2013, all injected compounds provided significant reduction of blossom blight. The most effective blossom blight reduction was provided by antibiotic Arborbiotic. Significantly lower reduction was achieved by Kasumin, Agrimycin and Phosphojet, which were similar in effect. The weakest, but still significant reduction was provided by Copper chelate which was similar to Actigard. Phosphojet was also similar to Actigard in effect but better then Copper chelate.

When trunk injection of bactericides and SAR inducers was evaluated in control of shoot blight originating from blossom infections, significant shoot blight incidence reduction was detected in 1 out of 3 years of trials. At low level of infection of 22.3% in 2012, Phosphojet and Agrimycin provided significant shoot blight incidence reduction but only at one out of two time points. At high level of infection of 78.75% in 2013, all injected compounds provided significant reduction of shoot blight. The most effective shoot blight reduction was provided by antibiotic Arborbiotic. Significantly lower reduction was achieved by Kasumin, Agrimycin, Copper chelate, Actigard and Phosphojet. The weakest, but still significant reduction was provided by Phosphojet. Overall, reduction of fire blight on apple trees after trunk injection of bactericides and SAR inducers did not reach the acceptable standards of commercial fire blight control in

apple orchards. Therefore, it can be concluded that trunk injection does not enhance the activity of trunk injected antibiotics and SAR inducers in disease control.

Based on these results, it was concluded that time allowed for translocation of injected compound, the injected dose and a substantial pre-dilution of this dose in water are crucial factors most likely acting in concert and governing the effects on fire blight reduction in apple tree canopy. In fire blight control experiments of 2011 the time allowed for translocation of injected compound was too short, the injected compound doses were too low, and the added amounts of water to the dose were too low. These parameters were set to be much higher in the experiments conducted in 2012 and 2013, thus yielding better results. The key underlying properties of the injected a. i.-s, explaining why more time for translocation and more amended water were needed to allow their better performances in the apple canopy, are their water solubility and Koc coefficient. The higher the Koc values, the stronger is the binding of the injected compound is to xylem tissue in the tree. The lower the water solubility, the lower is the movability of the injected compound in the xylem. Therefore, more water and time were needed to increase the dilution of injected a. i.-s thus easing their translocation and allow higher compound accumulation in the crown, respectively. With more time and water negative effects of a. i. properties were most likely alleviated.

In case of injected Arborbiotic, reduction of blossom and shoot blight did not reach the acceptable standards of commercial fire blight control most likely due to the fact that this antibiotic was injected only once in 2013 and the injected dose was too low to provide substantially better fire blight control. In regards to the Arborbiotic effects, this is the first report on use of trunk-injected oxytetracycline hydrochloride in fire blight control.

PR-protein gene expression

The same trees evaluated for fire blight disease incidence after trunk injection with SAR inducers were tested for PR-protein gene expression in leaves and flowers. In 2012, significant PR-protein gene expression in apple leaves was detected only after injection of Actigard. However, in 2013 significant PR-protein gene expression in apple leaves was detected after injection of both Actigard and Phosphojet, but at different times. This is the first finding of its kind which shows that SAR induction takes place in apple trees after trunk injection of SAR inducers. Further, this is the first report of significant PR-protein gene expression achieved after use of mixture of mono- and di-potassium salts of phosphorous acid i.e. Phosphojet on fire blight infected apple trees. In 2012, only PR-8 protein gene was significantly expressed in apple flowers, while in 2013 no PR-protein genes were significantly expressed in apple flowers.

These results led to a conclusion that times of significant PR-protein gene expression have been closely dependent on translocation and accumulation of the injected SAR inducers in the apple canopy. These two processes were very likely governed by differential speeds of leaf unfolding i.e. sudden or gradual increases of transpiration of the green leaf area in 2012 and 2013, respectively. Most likely, whenever more of the injected SAR inducers were accumulated in the leaves significant PR-protein gene expression was detected. Also, differential PR-protein gene expressions in leaves and flowers and higher fire blight incidences on flowers versus shoots were hypothesized to be due to the lower rate of transpiration of apple flowers versus leaves (Blanke & Lenz 1989).

However, in order to confirm these conclusions and test the above hypotheses future research needs to encompass experiments which will show the a. i. residue analyses or the

analysis of salicylic acid levels in the canopy for SAR inducers (Hassan & Buchenauer 2007; Frías et al. 2013). The leaf samplings in these experiments, and any future PR-protein gene expression analyses, should be conducted closer in time to the date(s) of injection(s). Further, based on blossom blight experiments in 2012 and 2013, future research needs to encompass measurements of bacterial population levels on and in the flowers thus clearly showing whether the trunk-injected compounds accumulated in the canopy at a certain dose, directly reduce the epiphytic/endophytic populations of *E. amylovora*. With the compound residue data analysis from flowers, this would provide an answer on whether the accumulated doses in the flowers are insufficient or sufficient to provide blossom blight control. However, in determining the compound residues associated with flowers, it would also be needed to detect whether there is any amount of the compounds present solely on the surface of generative flower parts and how much. If present, only these compound deposits would be able to establish contact with the bacteria and prevent population build-up in the phylloplane. It especially needs to be determined whether the trunk-injected compounds accumulate in or on the nectarthodes, anthers and pistil where the pathogen most successfully multiplies and invades the plant.

Shoot blight control

In the case of control of shoot blight severity in trunk injection experiments conducted in 2012 and 2013, Arborbiotic proved to be ultimately better than injected Apogee. In fact, Apogee due to high Koc value most likely did not move after injection from the trunk or was rapidly metabolized in the trunk (Lindquist 1965; Campana et al. 1979). Similar results and assumptions after trunk injection of Apogee on apple were reported before (Spitko 2008). This led to the

supporting evidence in claiming the severe influence of chemical and physical properties of the a. i. on the movement, accumulation and the activity of injected compounds in fire blight control. Thorough investigation is needed to answer which key contents of formulation or chemical reactions can allow changing of the negative chemical properties of a. i., which hamper its movement in the trunk xylem, and turn them into more positive chemical properties suitable for its ample movement. For example, in trunk injection on apple trees, the change of a. i. which helped prohexadione-calcium to become effective in control of *E. amylovora* on blossoms, was the use of prohexadione carboxylic acid (PCA) i.e. the free acid of prohexadione-calcium instead of this calcium salt (Düker & Kubiak 2011a). Most likely the prime property allowing this acid to move upward in xylem was higher water solubility and/or low Koc value. Based on this example, it can be concluded that plant protective compounds in their original molecular state or formulation for topical application cannot and should not be used *ad hoc* for trunk injection (Montecchio 2013). Some of the other properties determining a. i. adequateness for trunk injection are neutral pH of a final solution, high water solubility, low Koc values and potentially low molecular weight (small effective molecular radius). However, for each individual compound and formulation it is not known which property has the primate in governing its behavior in xylem and why.

Arborbiotic reached the point of substantial accumulation of effective doses which provided a certain degree of curative control of *E. amylovora* in 2013. Further, trunk injection significantly enhanced the activity of trunk-injected oxytetracycline in fire blight control. This is primarily because the efficiency after topical application is usually not satisfactory because the levels of antibiotic decline on plant surfaces due to short half-life and bacterial populations build-

up again (Johnson & Stockwell 1998; McManus et al. 2002). This did not happen after trunk injection of Arborbiotic.

Future trunk injection research on fire blight severity control on shoots needs to confirm the above conclusions and assumptions by accompanying the control effects with the residue analyses of prohexadione-calcium and oxytetracycline from shoots and leaves and from the wood core samples in the trunk. If possible, the same residues should be collected from the sprayed trees and compared, along with the control effects. Further, the pathogen epidemiology studies need to be conveyed to answer whether bacterial populations in shoots are declining accordingly with the increases in accumulation of trunk injection compounds in shoots and leaves through time. Finally, the most important big research steps in shoot blight control via trunk injection should be 1) testing of design(s) and invention of new antibiotic formulations compatible for trunk injection and 2) providing measurable proof that trunk injection as target-precise delivery method for antibiotics reduces the risks of *E. amylovora* resistance acquisition through minimizing the exposure of non-target bacterial populations as gene pools of resistance. The latter research goal would be very hard to reach, but if the question behind it is answered it could lead to significant reduction of the risks for resistance transfer from bacterial pathogens to human pathogens (Nair 1979; Hiruki 1988).

Control of apple scab (Venturia inaequalis) using trunk injection delivery of bio-pesticides and fungicides

Fungicide application in apple orchards, primarily for control of apple scab fungus (*V. inaequalis*, is one of the largest production costs of this industry in humid continental climate.

Air-blast sprayers widely used in tree-based agriculture for pesticide application allow substantial drift-driven pesticide losses into the environment, thus increasing the costs due to wasted active ingredients and their negative impacts on human health and environment (Pimentel 2005; Rawn et al. 2007; Greenburg et al. 2008; Hines et al. 2008). Trunk injection as a novel and more target-precise pesticide delivery approach has been investigated to determine whether commercially acceptable control of apple scab can be achieved with single or multiple injections of fungicides and biopesticides. Along with the effect on disease, pesticide residue levels in leaves and fruits were quantified to connect the achieved results with the levels of disease control.

Control of apple scab using trunk injection of biopesticides based on phosphorus acid salts in 2012 and continuation of apple scab control in 2013

In 2012 experiment with four trunk injections of five biopesticides on 'Red Delicious' apple trees, only Phosphojet and Prophyte based on salts of phosphorous acid (phosphites) significantly reduced apple scab incidence on spur leaves. Their effects were were not comparable to Spray standard and did not provide commercially acceptable apple scab control on spur leaves. It can be concluded that trunk injection cannot enhance the activity of phosphites to provide good control of spur leaf scab, most likely since spur leaves are the first green tissue to develop in the season when transpiration in trees is low (Chaney 1979; 1986) and the apple scab infections are intensive. At this time injected biopesticides were not able to translocate fast enough via transpiration stream and accumulate at amounts which can significantly control apple scab.

On shoot leaves, significant apple scab reduction was provided by trunk-injected Oxidate, Phosphojet 1 and 2, Prophyt and Nutrol and these effects were comparable effect provided by Spray standard. Only Milstop failed to reduce apple scab on shoots. These good effects were attributed to the combined influence of intensive shoot growth providing new healthy leaves on tips and higher transpiration of shoots versus spurs driving better compound accumulation in leaves. It can be concluded that the activity of most of the trunk-injected biopesticides was enhanced in control of apple scab on shoot leaves.

When in the same experiment apple scab on spur leaves was rated again in 2013, a year after trunk injections, significant and commercially acceptable apple scab control was achieved by Phosphojet and Prophyt. However, control with Spray standard was significantly better. Alamo (propiconazole) failed to control apple scab on spur leaves in 2013. Thus, one year after injections in 2012, trunk-injected compounds showed continued effect on disease incidence, which is in accordance with previous trunk injection research with fungicides on apple (Long et al. 1989; Percival & Boyle 2005). This leads to a conclusion that during one year as a substantial amount of time, compound translocated and accumulated in the crown substantially to control apple scab. Thus, spur leaves in 2013 were protected from primary scab infections. Hence, trunk injection in concert with enough time for translocation and accumulation can enhance the activity of injected compounds to provide good control of apple scab. Similar conclusions were reached also when the shoot leaf scab incidences were analyzed in 2013. However, it was noticed that with intensive shoot growth in 2013 there was weakening of the apple scab reduction effect probably due to the dose “dilution” by new leaf growth.

Phytotoxicity effects detected on leaves of ‘Red Delicious’ trees after four injections of Phosphojet were caused most likely because no water was amended with the overall high

injected total dose. On the other hand, Alamo caused pytotoxicity on branches due to high injected dose amended with substantial amount of water allowing good compound translocation.

Future research in control of apple scab via trunk injected plant protective compounds should encompass long-term collection of leaf samples from spurs and shoots, separately, so that the residue levels can be determined in them thus fully supporting above explanations. Further, on shoots, leaf residue samples should be taken at several times from different levels i.e. strata of leaves along the shoot height. Probably three levels such as basal, middle and top shoot section should be the most intuitive. This would allow answer to the question to which degree and at which time the effect of accumulated biopesticides in shoots contributes to the apple scab incidence reduction and at which shoot height level. Further, this would also provide answer to question to which degree intensively developing new leaf mass on shoots contributes to the apple scab incidence "dilution" effect.

Optimization of trunk injection timing for control of apple scab in 2012 and 2013

Significant reduction of spur leaf scab on 'Mac Spur' apple trees in 2012, after four cross-seasonal trunk injections of all doses of Alamo and Phosphojet, reinforces and confirms previous conclusions and explanations reached in control of apple scab using trunk injection of biopesticides in 2012. However, as the main purpose of this study, it was determined that in all treatments the injection conducted first in fall 2011, then followed by the three injections in spring 2012, slightly improved the reduction of spur leaf scab incidence in comparison to when all four injections are conducted in spring (trunk injection of biopesticides in 2012). Still, the long time for compound translocation and accumulation in spur leaves was likely insufficient to

provide compound levels as efficient in control of apple scab as the Spray standard. This was primarily because in apple and other forest trees there is a seasonal decline in water use after mid-July (Dragoni et al. 2005; Harrell 2006). This means that in fall 2011, this weakening in transpiration hampered uptake of injected compounds into the canopy, at higher concentrations. But due to somewhat improved reduction of leaf scab on spurs by fall injection it seems that this slow transpiration still allowed some compound uptake but at a very low concentration or not all the way up into the leaves. If the compound was only partially translocated up to a certain height in trunk and branches, without reaching the leaves and buds, it would still be contributing to the advantageous improvement in reduction of leaf scab on spurs by fall injection because it is closer to its final destination. When intensive transpiration reestablishes in spring, these compound accumulations from fall would reach the leaves much sooner than after the newly conducted trunk-injections in current season. However, besides weak transpiration in fall, also plant metabolic processes and environmental factors could have influenced on the reduction of effective doses thus contributing to the weaker apple scab control compare to Spray standard.

Since in 2012, after injection of Phosphojet, there was a delay in severe onset of apple scab on shoots of ‘Mac Spur’ versus on the shoots of ‘Red Delicious’ in biopesticides experiment, it can be concluded that first trunk injection in fall 2011 contributed significantly to the overall reduction of leaf scab incidence on shoots in 2012. Thus, fall injection improved the effect of trunk injected Phosphojet on apple scab and it makes sense that this improvement is more easily seen on shoots due to their natural higher transpiration versus spurs.

On both spur and shoot leaves, apple scab reduction effects were not fine-tuned by different doses of Alamo and Phosphojet and it remains to be determined why. It is possible that, no matter which dose is injected in the tree, xylem capacity for translocation is the same and

limits the amount of uptake of a compound according to this unique capacity. Therefore if the high dose is injected only a certain part of it will be translocated and the other is kept in xylem tissue. When the low dose is injected, either the whole dose is translocated, if the xylem capacity can handle it, or also a certain part of it is translocated and the other is kept in xylem tissue. Further, it is unknown whether the injected compound doses were too low or chemical properties of the a. i. and formulation were unfavorable thus reducing the activity in apple scab control (Kondo 1978; Montecchio 2013). Future research needs to address there unknowns to improve the use of trunk inejction in tree-based agriculture.

The temporal residue profiles of propiconazole and phosphorous acid in 2012 are more-less well correlated with the effects of injected Phosphojet and Alamo on apple scab on both spurs and shoots. All previous observations and conclusions in disease control become substantiated and supported by the detected compound residues. When more propiconazole or phosphorous acid were accumulated in leaves during the time of the experiment, but the apple scab levels were significantly rising on shoots, it was concluded that either dose accumulated was insufficient to control the disease, or *V. inaequalis* is resistant to propiconazole, or the dose was reduced by the "dilution" effect from new shoot growth or metabolic degradation (Lindquist 1965; Campana et al. 1979; Stanis & Jones 1985; Köller et al. 1997). Early residue profile differences between low and high dose injections of Phosphojet indicated that after injection of lower dose there was an easier translocation of injected compound into the canopy from the trunk. In the case of higher injected dose, the excess amount which was not translocated immediately was stored in trunk tissues until later translocation. Since phosphorous acid was accumulated in 10- to 50-fold higher concentrations compared to propiconazole it was concluded that this was primarily due to different chemical properties of these compounds. Propiconazole

has moderate to high level of Koc around 382 - 1817 ml/g depending the organic carbon content in the environment (BCP Council 2011) and a moderate to low level of water solubility of 100-150 mg/L at 20ºC (pH 5.2 or 6.9, respectively) (Herner & Acock 2003). Therefore it was poorly accumulated in leaves and mostly bound in the trunk xylem.

Failure of majority of 1-2 cross-seasonal or seasonal trunk injections of Inspire super to significantly reduce apple scab on spur leaves in 2013 were assigned to unfavorable chemical and physical properties of difenoconazole and cyprodinil which severely hampered their movement in the tree xylem and translocation into the canopy. Both difenoconazole and cyprodinil have Koc values of 3495 ml/g (Serafini 2009) and 1550-2030 ml/g (Serafini 2003) and which is designated as very low movability in soil (PPDB 2013). At 25ºC, water solubility of difenoconazole is 15 mg/L (pH 7.2) (Mensink 2008) and of cyprodinil is 13 mg/L (pH 7.0) (Serafini 2003), which is very low. Hence, both fungicides were most likely strongly bound to the organic components of symplast and apoplast in xylem, thus allowing slow emission of small compound amounts into the canopy which were not effective in apple scab control. It can be concluded that Inspire Super is not a good model fungicide for investigation of apple scab control using trunk injection.

All 1-2 cross-seasonal or seasonal injections with Phosphojet showed significant reduction of spur leaf scab. Single Phosphojet injections in fall 2012 or spring 2013 were statistically similar to the significant effect of total of nine spray applications of Agrifos. This for the first time shows that trunk injection can significantly enhance the activity of fungicidal compounds such as phosphites to control *V. inaequalis*. Therefore future research need to find even better optimal time schedules of injection which can provide commercially acceptable levels of apple scab control. When Phosphojet was injected twice during the spring 2013 or the

first time in fall 2012 and then the second time in spring 2013, similar significant effects are achieved indicating that fall injection does not significantly improve the control of spur leaf scab with injection of phosphites. However, since the injection in fall 2012 and then in spring 2013 was statistically similar to Inspire super spray treatment, fall injection did significantly contribute to better control of apple scab on spurs. Since significantly better efficiency of double Phosphojet injections is detected versus single injections, more than just one injection is definitely needed to provide control of *V. inaequalis*. Higher total injected doses per tree secured higher efficiency of double Phosphojet injections in apple scab reduction on spur leaves. These results are achieved because of the favorable chemical properties of phosphites and phosphorous acid. Water solubility of phosphites mixture is 500 g/L, and of phosphorous acid is 3100 g/L (Sigma-Aldrich 2012). Koc values for potassium phosphites are 228-587 ml/g (EFSA 2012). This suggests very good mobility of Phosphojet in apple trunk xylem and accumulation in leaves, leading to significant apple scab reduction.

On terminal shoot leaves in 2013, significant apple scab reduction achieved with single injections of 3.5 and 7 ml of Inspire super in spring and with two injections of 7 ml in spring, can be attributed to the higher transpiration of shoots versus the spurs. This allowed accumulation of effective amounts of difenoconazole and cyprodinil in shoots. Further, significant reduction was not fine-tuned by the higher or lower injected doses. All the single injection treatments of 3.5 and 7 ml in fall 2012 and the two injection treatment with 3.5 ml in fall 2012 and spring 2013 failed to significantly reduce apple scab on shoots. Cause of this failure could be dose decline due to plant metabolic or environmentally triggered degradation during fall 2012-spring 2013 (Lindquist 1965; Norris 1965; Campana et al. 1979). However, most likely it is the low intensity

of green tissue transpiration in fall and much higher transpiration in the spring and summer that caused this difference between two groups of treatments.

Significant reduction of leaf scab on terminal shoots with all injection treatments of Phosphojet clearly confirmed better accumualtion of phosphorous acid in the apple canopy. However, significant differences between Phosphojet injection treatments support conclusions on differences in transpiration from the trees in fall versus in the spring and on dose reducing influence of plant metabolic processes or cold winter temperatures. Based on results in 2013 and opposite or the data on 2012 it seems that that after two injections of Phosphojet, fall injection does not contribute to better control of apple scab on terminal shoot leaves. However, these opposing conclusions could be due to the differential times of severe apple infection onset in 2012 (early) and 2013 (late) and the times of injection in relation to them. Similar as in spurs, similar effects of Agrifos and double injection of Posphojet in spring 2013 show that trunk injection can significantly enhance the activity of phosphites in control of *V. inaequalis* on shoots. However, higher dose per tree was delivered through double trunk injection of Phosphojet than with Agrifos sprays. Hence, number of injected doses per season, the total dose per season, and the time of injection need to be further investigated before trunk injection for apple scab control can be improved to meet the commercial standards acceptable in orchards.

Residue profiles of difenoconazole and cyprodinil on leaves in 2013 showed that Inspire super spray provided several fold higher fungicide residue levels versus injection. This is because sprayed fungicides immediately reach plant surface while injected fungicides require time to translocate into canopy. However, the residues after topical application are exposed to environmental and plant metabolic processes, as well as new tissue growth which reduce the

fungicide levels on and in leaves. We found correlation of the spray deposited levels of Inspire super with the levels of apple scab control on leaves.

Since residues of fungicides in leaves were 10- to 100-fold lower after trunk injection it was inferred that translocation of injected compounds is a complex process where the final residues in leaves depend on the influence of plant physiology and fungicide chemistry. Leaf residues collected in fall after fall injection finally confirmed previous claim that injected compounds do not reach leaves probably because of decline in water use of apple trees after mid-July (Dragoni et al. 2005). Low residues in leaves also confirm that due to unfavorable chemical properties cyprodinil and difenoconazole have been bound to tissues in xylem and this limited their substantial accumulation in the canopy. Therefore, due to low levels of cyprodinil and difenoconazole in leaves there was very poor control of apple leaf scab.

There was no good correlation of the differentially accumulated cyprodinil and difenoconazole in apple leaves with the effects on apple leaf scab, primarily because the accumulated levels were very low to express any effect on disease. However, residue data clearly showed that the injections of Inspire super in fall 2012 improved accumulation of cyprodinil and difenoconazole apple in leaves. More time for translocation in these treatments versus less time after spring injections of Inspire super allowed significantly higher fungicides residues in apple leaves. However, since no compounds were detected on 25 October 2012 and 21 April 2013 in leaves and buds, respectively, it can be concluded that due to very weak tree water use, translocation in xylem might have happened only up to a certain distance within the tree trunk and branches, but without reaching the leaves and buds. Then, in late spring, when intensive sap flow is widely reestablished in xylem, since these fungicides were previously positioned closer in distance to the leaves they were translocated much sooner and in higher quantities into them.

Residue profile analysis for phosphorous acid from leaves showed that total of nine Agrifos sprays provided similar or sometimes lower residues than after all injection treatments. It is possible that after spraying environmental factors rapidly reduce Agrifos levels on leaves, before they are absorbed, and thus become similar in quantity to the residues after trunk injection. On the other hand it is possible that trunk injection is a superior delivery for phosphites which provides significantly better accumulation of phosphorous acid in apple canopy, without the dose reduction effects from environmental factors. In conclusion, 1-2 trunk injections can substitute numerous sprays of phosphites to achieve the same effect in residue accumulation.

Declining residues in leaves collected at the end of experiment indicated that doses of Phosphojet after fall injections become depleted or metabolized earlier that in spring injection treatments where there was continued accumulation of the phosphorous acid to the canopy.

The accumulated phosphorous acid residues after injection(s) of Phosphojet and sprays of Agrifos correlated perfectly with the significant effects of apple scab reduction on spur and shoot leaves. This was pronounced on apple shoot leaves. Based on similarity of residue profiles after different Phosphojet injection treatments it appears that fall injection does not improve the accumulation phosphorous acid in leaves.

Since in 2013 there was no control of fruit scab provided by any of the injection treatments with Inspire super, it can be concluded that both difenoconazole and cyprodinil were not accumulated in fruits or their accumulated amounts were negligible, thus not affecting *V. inaequalis* infections. Indeed, residue profiles of cyprodinil and difenoconazole in apple fruit confirmed this conclusion. Previously stated unfavorable chemical properties such as very low water solubility and high Koc values prevented their movement from the trunk and subsequent

activity in fruits. Further, fruits transpire much less than leaves further limiting compound inflow with the water.

Completely opposite, Phosphojet in 2013 reduced fruit apple scab on injected trees which confirms that due to excellent chemical properties phosphorous acid accumulated in fruits. Fruit residue profiles confirmed this conclusion. Double injections of Phosphojet and a single injection of Phosphojet in spring achieved significantly better apple scab reduction then other Phosphojet treatments. It can be concluded that higher total dose of Posphojet injected per tree or injection in spring alone, significantly improve control of *V. inaequalis* on fruits.

Activity of injected fungicides in fruit depends on the chemical and physical interactions of a. i. with the tissues or sap of the plant and its ability to readily move or not as an outcome of these interactions. Future research needs to focus on finding was how to manipulate these interactions by designing pesticide formulations compatible for trunk injection (Smalley 1977; Kondo 1978; Montecchio 2013). All chemical and physical properties of the injected compounds must be considered and further investigated as interdependent. They all act simultaneously and pinpointing the key properties which hamper or improve the best performance of *in planta* delivered compound in pest control, must be crucial part of pesticide formulation design for trunk injection (Campana et al. 1979; Montecchio 2013).

Residue profiles in 2013 for difenoconazole, cyprodinil and phosphorous acid in fruits support the lack of effectiveness of Inspire super and good apple scab reduction provided by Phosphojet.

Somewhat higher concentrations of cyprodinil detected in apple fruit confirmed its systemic properties. Very low concentrations of difenoconazole in apple fruit confirmed its local systemic properties (Knauf-Beiter et al. 1995; Serafini 2003; Mensink 2008; Serafini 2009;

PPDB 2013). After inejction, both fungicides were exposed to aforementioned chemical and physical interactions in xylem and due to their low water solubility and high Koc values were largely stored in the trunk. Only small portion was translocated into fruit and thus showed much weaker accumulation of residues in apple fruit versus the sprayed Inspire super. By topical application of fungicides the complex and time governed a. i. interactions in the tissues are avoided. In the early stages of apple fruit development detected residue profiles of trunk-injected cyprodinil and difenoconazole showed significant accumulation. It can be concluded that this accumulation was driven by high transpiration of small fruits since it has been reported before that stomata on small fruits are as functional as on leaves (Blanke & Lenz 1989).

After trunk injection and topical application of Inspire super, fungicide residues on and in apple fruits declined in concentration probably because of the fruit size increase, environmental factors, metabolic processes and ontogenic development. Due to intensive increase in size of the fruits, fungicides in and on the fruit get diluted. Further, environmental influences also reduce their deposits. Towards the end of the season fruit xylem gradually becomes dysfunctional (Dražeta et al. 2004). Next, frequency of stomata on outer fruit epidermis is lower in comparison to epidermis of leaf in *Malus* species (Blanke & Lenz 1989). With fruit development, due to stomata conversion into lenticels transpiration and photosynthesis of apple fruit decline (Blanke & Lenz 1989). In case of trunk-injected inspire super all these processes reduced influx of fungicides in the fruit and let to residue decline in fruits.

Residue data indicate that fall injection of Inspire super, alone or in combination with the spring injection, improved the accumulation of cyprodinil and difenoconazole in apple fruits, but only very early when the fruits are small in size. This improvement is also contributed by more time allowed for translocation and accumulation of injected compound into the canopy.

In general, cyprodinil and difenoconazole fruit residues after trunk injection were far below the tolerances and MRL's set by US EPA and Codex Alimentarius. Fungicide application by trunk injection allows discriminatory distribution within apple tree and the vast majority of fungicide ends up in foliage versus fruit.

Phosphorous acid accumulated in fruits at a much higher concentrations than cyprodinil and difenoconazole, primarily because it is a systemic fungicide which moves through plant easily due to high water solubility and medium low to medium Koc values (Cohen & Coffey 1986; Jackson et al. 2000; Hardy et al. 2001; Reuveni et al. 2003). Binding of this compound to trunk tissues was much weaker and therefore required less time to be translocated into the canopy. In general, residue levels accumulated in apple fruits after injection treatment were comparable and sometimes higher than after Agrifos sprays. Thus, it can be concluded that trunk injection is successful in delivering substantial and effective phosphorous acid residue levels into the fruit.

In double spring injection treatment of Phosphojet, residue levels in fruits showed early decline in concentration but residue levels later significantly increased. In contrary, single Phosphojet injections in spring and fall, and a double injection in fall and then spring, showed significant increase in phosphorous acid concentration right after the time of early decline in double spring injection of Phosphojet. These patterns could be assigned to the influences of physiological and metabolical changes in fruit development or due to different times of conducted injections. Namely, two doses in double injection treatment in spring, very close in time, could have overwhelmed the conductive capacity of xylem and led to temporary slow conductance and low concentration translocation into the fruits. Later, residues in fruit increased in concentration since the capacity of xylem conductance was restored. In the case of single

Phosphojet injections in spring and fall, and a double injection in fall and then spring, the above mentioned increase in phosphorous acid concentration could have happened because there was no effect of xylem overwhelming with two doses of Phosphojet injected close in time. In these treatments xylem was functioning at an optimal conductive capacity, primarily because the doses were too far apart in time or there was only one dose unable to overwhelm the capacity.

There was a good correlation of phosphorous acid residues with apple scab control on fruits. The comparisons of phosphorous acid residues led to a conclusion that fall injection of Phosphojet alone or in combination with the spring injection does not improve the accumulation of this compound in apple fruits.

Since phosphorous acid and its sodium and potassium salts are exempt from food tolerances and since there are no maximum residue levels (MRLs) established for these compounds (US EPA 2006) future research their trunk injection needs to be continued. The most important next experiment needs to be attempting the development of time-wise and dose-wise trunk injection schedule which will yield in commercially acceptable disease control on apple trees during the whole season.

In summary, apple scab experiments in 2012 and 2013 proved that significant control of apple scab cannot be achieved with 1-4 cross-seasonal of within seasonal trunk injections of maximum seasonally allowed or lower doses of biopesticides and fungicides. However, in some experiments, apple scab control effects after trunk injection of biopesticides were comparable to spray standards. The results also proved that, at current state of technology and knowledge, trunk injection cannot yet significantly enhance the activity of potassium phosphites to provide significant control of *V. inaequalis*. However, in key experiments trunk injections of phosphites provided better apple scab reduction effects than their topical spray application.

Trunk injection technology simulations and comparisons

Conventional technologies for topical pesticide delivery in tree-based agriculture have changed relatively little in last 100 years and carry exorbitant costs due severe losses in wasted product (Steiner 1969; Pimentel & Levitan 1986; Pimentel et al. 1992; Pimentel & Lehman 1993; Pimentel 1995; 2005; Shaaban 2009). There are very few new technologies that enable more target precise and efficient delivery of active compounds to fruit trees. Recent inventions and technology transfers from urban tree care are delivery systems and formulations for trunk injection and infusion of plant protective compounds to grapevines and fruit trees (Doccola et al. 2003; 2012; Düker et al. 2006; Montecchio 2011; 2013; Shang, Liao, et al. 2011; 2011; Düker & Kubiak 2009b; 2009a; 2011b; 2011a).

However, before trunk injection could potentially be used in tree-based agriculture the problems of temporally non-uniform pesticide distribution in the crown, lack of performance evaluation of trunk injection tools, and the unknown impact of trunk injection ports on tree damage, need to be addressed.

Simulation of slow compound release

When slow compound release, which would secure temporally uniform compound distribution in the crown, was simulated by trunk injection of unique dose split in time, the results showed that the most uniform imidacloprid delivery through time in the apple tree canopy was achieved only by four splits of a unique dose delivered 14 days apart. However, the residue concentration profile was the lowest. In contrary, dose injected at once or split injected twice,

and 14 days apart, showed temporally non-uniform imidacloprid distribution through time but with much higher residue concentration profile. These two modes of delivery showed that excessive amount of the compound supplied in apple leaves above the one provided with four split injections, is unnecessary and wasted in short amount of time. This leads to a conclusion that in fruit trees where temporally precise pesticide dose delivery in time and space is crucial, trunk injection can be adjusted to meet these requirements. However, research on new concepts and designs of trunk injection systems which could provide controlled and adjustable release of the efficient compound doses in time is limited (Düker et al. 2006; Düker & Kubiak 2009b; 2009a). These systems need to provide long-lasting and temporally uniform delivery of equal and efficient compound doses. Currently, the only two trunk injection devices in landscape tree care and forestry which could potentially provide these performances, are High Volume Macro-Infusion^{TM} Kit and Low Volume Macro-Infusion Kit^{TM} (Rainbow Treecare Co., Minnetonka, MN). These systems need to be tested and potentially improved or new injection systems which would allow stable, efficient and long-lasting dose delivery should be designed. Further, if temporally uniform dose delivery is to be achieved in fruit trees then injection timing schedules during the season that will take full advantage of the fundamental mechanisms at work within apples trees, need to be developed. As indicated and showed in previous research, the important part of future investigations needs to be the evaluation of the longest time that injection elements of a system can be left within the trunk before the process of injection port healing starts by callus formation (Düker et al. 2006). If multiple injections per season are needed to achieve temporally uniform emission of the pesticide in the tree canopy, knowing this time would maybe allow reduction of labor or mechanical force and associated costs needed to conduct the injection

process. Since in fruit trees process of injection port healing is highly desirable it must not be disturbed by the injection elements left in the trunk.

Comparison of the three main trunk injection technologies in compound delivery

Besides other parameters, temporal distribution of trunk-injected compounds in tree canopy serves as the best parameter for comparison of trunk injection technology differences in performance. This is because the efficiency of plant protection achieved in time depends heavily on temporal compound distribution in tree canopy. Comparison was conducted between temporal residue profiles in apple leaf canopy provided by drill-based injection technology in combination with liquid imidacloprid formulation (Quikjet), needle insertion injection technology in combination with liquid imidacloprid formulation (Wedgle), and drill-based injection technology in combination with slow release, solid injection formulation of imidacloprid (MSU-1). The results showed that the highest concentration of imidacloprid in apple canopy was provided by Quikjet. This technology was followed by Wedgle which provided significantly lower concentration of imidacloprid in the canopy. The lowest, concentration of imidacloprid was provided by MSU-1.

The results led to the conclusion that trunk injection with Quikjet was the best since this injection system targets actively conducting xylem tissue and since imidacloprid was delivered in a prediluted liquid formulation with 5% of a. i. specifically designed for trunk injection. As stated before, xylem and injection compatible formulations are crucial for fast and easier uptake of injected compounds into the canopy. The injection with Wedgle was less efficient in providing imidacloprid to apple canopy because this technology targets cambium meristem

tissue which is not vascular tissue and is far less conductive in upward imidacloprid translocation after injection, that the xylem. The injection with MSU-1 showed to provide the lowest compound concentration most likely due to solid instead of a liquid formulation design of imidacloprid. MSU-1 solid formulation allowed slow release of imidacloprid in time but at too low concentrations. This led to a conclusion that there was a high dependence of solid formulation in xylem on the water in sap to dissolve i.e. liberate imidacloprid from it and then translocate it upward into the canopy. Most likely this amount of water was too low to dissolve imidacloprid sufficiently or the solid formulation MSU-1 was binding imidacloprid too strongly to allow release of larger concentrations into the canopy. In general, the results prove that due to variability in delivery performances injection technology has a significant impact on distribution and most likely efficiency of injected compounds in apple trees.

Future research should include also the comparison of these temporal residue profiles of imidacloprid with the needle insertion injection technology in combination with liquid imidacloprid formulation but with xylem as a target for delivery instead of cambium (Bite-infusion® powered by Venturi effect i.e. sap flow generated negative pressure driving infusion or by hand/air/hydraulic pressure (patent of University of Padova, Italy). There are indications that this system is similar to Quikjet of even better in imidacloprid delivery after trunk injection in apples (Wise et al. 2013, unpublished). The next step in research of slow-release solid formulations such as MSU-1 could be changing the contents of the formulation to allow easier compound release from it, when relying solely on amount of water naturally translocated in apple trees. Or the water could be supplied externally through the injection port to ease the imidacloprid release from the formulation.

Further, this technology comparison research would be more complete if it was accompanied with a set of data showing the effect of imidacloprid on at least one insect pest, with green apple aphid *Aphis pomi* DeGeer (*Homoptera: Aphidida*e) being the most suitable due to its specific lifestyle of feeding on young shoots and leaves.

Comparison of seven trunk injection tools in compound delivery and disease control

After use of seven trunk injection tools i.e. Bite, Wedgle, Chemjet, Mauget, Quikjet, Viper AH and Tree IV, only Bite provided significantly the highest concentrations of cyprodinil and difenoconazole in apple leaves. Probable explanation for this Bite performance is that lenticular shape of injection port allows faster absorption and uptake of the injected solution in comparison to drilled injection ports and insertion port of Wedgle. Better absorption and uptake in lenticular port occurs most likely because the tissue injury and the contact with the injection element are minimal and the exposed tissue surface is higher than in other trunk injection ports. Low concentration footprints of the two fungicides facilitated by other trunk injection tools could have been due to the higher level of xylem tissue injury in ports, low amount of amended water in injection solution, and the fact that Wedgle targets non-vascular tissue for injection i.e. cambium.

In time, the decline of cyprodinil and difenoconazole concentrations in residue profile provided with Bite occurred likely due to binding of these fungicides in apple xylem as rich carbon environment due to their high Koc values. This process might have happened faster in drilled trunk injection ports. However, it is also possible that parent compounds were metabolized in the apple tree and thus reduced in content. Due to high Koc values cyprodinil and

difenoconazole were immobilized in the trunk and thus were not good model fungicide combination for the investigation of a. i. temporal distribution in apple leaves.

Very low cyprodinil and difenoconazole residues accumulated in apple leaves were able to significantly reduce apple scab on spurs and terminal shoots. High activity of low concentrations has been reported before for these fungicides (Dahmen & Staub 1992; Kunz et al. 1998; Küng et al. 1999; Köller et al. 2005; Henríquez S et al. 2011; Aleksić et al. 2012; Larsen et al. 2013). The best apple scab control by injection of Inspire super was provided after delivery with Tree IV. Wedgle, Viper AH and Chemjet allowed Inspire super to only significantly reduce disease incidence. However, completely opposite to the initially the highest cyprodinil and difenoconazole residues in leaves, injected Inspire super with Bite provided very weak apple scab reduction similar to majority of other trunk injection tools. It has been concluded that lenticular injection port and the construction of injection element (needle) in injection with Bite does not allow substantial radial diffusion of the injected compound in the trunk, thus leading to non-uniform spatial distribution of Inspire super in the apple tree crown. Due to this non-uniform spatial distribution of fungicide, heavily infected patchily zones intercepted with healthy zones of leaves were observed in the crown. Sampling of 40 leaves per tree replicate for fungicides residues was of low resolution to equalize the differences in high and low residue concentrations present in leaves well protected from scab and in leaves unprotected from scab, respectively.

Overall, the apple scab incidence reduction by two injected fungicides was insufficient to provide commercially acceptable control of *V. inaequalis*. It seemed that trunk injection allowed better activity i.e. improved the effect of cyprodinil and difenoconazole at detected low concentrations in the leaves, since if the same concentrations were applied topically they might have resulted in higher apple scab incidence. In topical Inspire super application fungicide

residues are high after spraying and have immediate contact with leaf surface providing better disease control. In trunk injection fungicide residues are low due to long time needed for compound translocation and accumulation in apple leaves, thus providing poor disease control. If the same high fungicide residue levels detected after spraying were accumulated i.e. present in leaves after trunk injection, the activity against *V. inaequalis* would be much better and might lead to good apple scab control.

When times in installation of seven trunk injection tools until injection were measured, Bite and Mauget took shortest time since they require manual use of fewer additional equipment and tools. Less work i.e. time spent in the process up until the injection starts, severely reduces labor price. Since also requiring use of fewer additional equipment and tools, Wedgle and Chemjet were the second best, with slightly more time spent in installing until injection. Longer time needed to prepare use of Quikjet and Viper AH was due to more used equipment, more operations conducted in making and sealing of drilled port, and connection of injection needle to the ports. Tree IV took the longest time for installation due to the fact that after drilling and sealing of injection ports, additional time was used to prime the device for injection, connect the needles to ports, and initiate the solution release from the device.

The times of injection solution discharge from Mauget, Chemjet, Tree IV and especially Bite, is highly dependent on the speed of sap flow in the trunk xylem, which is controlled primarily by the rate of daily transpiration of a tree. Further, the discharge times of tools depended on the conceptual construction and position of injection elements while connected to the injection ports, the amount of hydraulic pressure provided by powering the tools, and the amount of water added or not to the solution. From longest to shortest time of discharge of

injection solution, the tools follow the order: Mauget, Bite, Tree IV, Chemjet, Quikjet, Viper and Wedgle.

Future research needs to yield in development of trunk injection systems which are fully automated and do not require use of additional tools and long times spent in installation and solution discharge. These injection tools/systems need to facilitate simultaneous and automated or semi-automated treatment of two or more trees in per acre basis (Düker et al. 2006). More importantly, these systems should facilitate automated control of the delivery of an equal solution volume in per tree basis, and of the insertion or retraction of injection elements in and out of the injection ports.

Comparison of trunk injection elements in injection port wound healing

After injection, bark cracking has been reported on other tree species (Smith & Lewis 2005). On apples, there was a significant increase in width of the outer bark crack in time, around all the injection port wounds except in the treatment 3/8 Drill bit sealed with Arborplugs no. 4. This led to a conclusion that bark cracking typical for frost damage occurs due to water freezing in unsealed ports. In case of larger port sizes, there was larger frost damage as width of the outer bark crack.

Vertical dimension of the bark crack i.e. its height did not change in time within evaluated injection port wounds. Height of bark cracking was significantly higher in treatments Blade and 3/8 Drill bit sealed with Arborplugs no. 4 because of the initially larger size of the port height in Blade treatment and because of the sealing of 3/8 Drill bit port with plug. During frost conditions ice formation on top of the plug has been detected and most likely caused vertical

bark cracking. Statistical similarities in height of the outer bark crack between treatments in specific dates indicate that whichever injection port is inflicted there is a chance for equal bark damage. However, in average throughout two years, 11/64 Drill bit treatment showed to be least injurious since it had the smallest height of the outer bark crack.

Since horizontal and vertical diameter of outer callus tissue significantly declined in time, around all trunk injection ports, the process of wound healing takes place by production of callus tissue from active cambium that enables radial trunk growth. The fastest port healing i.e. port closure with bark was in treatment 11/64 Drill bit and then in Blade, 3/8 Drill bit, and 3/8 Drill bit sealed with Arborplugs no. 4, respectively. Due to Arborplug in sealed 3/8 Drill bit treatment the time of wound healing was the slowest.

Port depth from bark level significantly changed in time in both sealed and non-sealed 3/8 Drill bit ports. This occurred because during radial trunk growth and in the process of healing, callus is produced from cambium and this tissue sooner or later rises like a mild protrusion above the normal level of the bark. Later, callus grows laterally, from left and right sides, to close the port cavity below and its tissue “stretches” over the port. Therefore, port depth significantly rises and then declines. In Blade and 11/64 Drill bit ports there was no significant change in the port depth since callus covered the port openings very fast and no protrusions formed on the sides and above the ports.

Port depth from the Arborplug surface in sealed 3/8 Drill bit port, showed significant increase through time. This led to a conclusion that callus tissue produced by cambium moves the Arborplug slightly outwards, to the bark edge. Later, this outward movement of Arborplug did not continue since port depth did not change anymore.

Overall, injection port wound closure data show that depending wound type process of callus healing facilitated by cambium lasts 1-2 years. Further, it was confirmed that needle-based trunk injection technologies impose lower extent of tree wounding due to direct needle insertion in the trunk, without removing the tissue to create reservoir for the injected solution (Montecchio 2011; 2013). The main disadvantage of needle-based trunk injection technologies observed in our experiments on apple is spatially non-uniform distribution of the compound in the crown. Drill-based injection technologies inflict more extensive physical damage to the trunk tissues since parts of wood tissues are removed by drilling. The main advantage of this technology is more extensive xylem tissue exposure to injected chemical and its more uniform distribution in the tree canopy.

Future research should encompass testing of conductive functionality of xylem tissues around the injection ports. If trunk injection ports are not sealed, nearest layers of xylem tissue die off and become sealed-off from the living tissue in the process compartmentalization (Shigo et al. 1977; Shigo & Marx 1977; Shigo 1978; Shigo & Service 1979; Shigo 1981; Shigo 1984; Shigo 1985; Perry et al. 1991; Doccola et al. 2011). These tissues stay permanently dysfunctional. If the port is sealed with a Arborplug the same process occurs in different forest tree species (Doccola et al. 2011). However, in apple trees leaking of water from Arborplugs occurs 8-12 months after injection and it needs to be tested whether compartmentalization of xylem around injection port is complete or limited, thus making these xylem tissue layers dysfunctional or still functional. Different tree species react differently to trunk injection injuries and the most common reported symptoms are tissue dysfunctionality, phytotoxicity, discoloration, microorganism infections through injuries, trunk splitting, distorted cambium growth, weeping and fluxing of sap, decay, killed bark, and other (Shabi et al. 1974; Neely 1979;

1988; Costonis 1980; 1981; Santamour Jr 1986; Perry et al. 1991; McGillivary et al. 1993; Wasniewski, Chaney & Holt 1993; Shigo 1978; Smith & Lewis 2005; Shortle et al. 2010). The extent of these symptoms depends also on cultivar and injection port type (Sachs et al. 1977; Santamour Jr 1984; Santamour Jr 1986). Therefore, it is important that next research steps test also the extent of internal tissue functionality/dysfunctionality around injection port so that true impact on tree longevity can be measured. For example, sap flow speed could be measured in time with pair of inserted thermocouples by previously described heat-pulse method (Cohen et al. 1981; Smith & Allen 1996; Burgess et al. 2001). The two thermocouples, one above another, could be strategically positioned at points around different injection delivery ports such as 5 cm below and above the port and 3 cm left and right form the port. Further, at different times during the season, sap-flow measurements should be conducted by inserting one of the thermocouples in the center of previously imposed injection port of each type, while the other should be inserted below the first. The resulting data should show the true impact of injection port wounding on xylem functionality around injection ports.

Closing comments

It has been defined previously in literature, that the concept of precision horticulture is based on knowledge achieved in site-specific farming. In fruit production the goal is to optimize production processes by means of adapted i.e. specific treatment of individual trees (Zude et al. 2012). Therefore, the approach of trunk injection is in accordance with the concept of precise tree-based agriculture since it allows adapted i.e. specific treatment of individual trees or rows of trees. Further, if there was a way to map the spatial deployment of resistance in pest populations

in orchard i.e. to visualize its local areal of spread, there could be a possibility to selectively manage resistant population zones with different but still effective pesticides and biopesticides, while in the other zones with no resistance, previously known to be effective pesticides could still be used. This is one of the examples where trunk injection could facilitate tree selective treatments and aid in management of pest resistance besides the existing strategies. However, a lot more needs to be done through scientific research and technology development before this could be possible even experimentally. Future research in trunk injection needs to address many obstacles through experiments and generate new knowledge and technology solutions that could enable ultimately target-precise plant protection in tree-based agriculture.

Srđan Goran Aćimović, July 2014

APPENDICES

APPENDIX 1. Preliminary research in 2011 and 2012 on control of fire blight using trunk injection of protective compounds

Material and methods

Blossom blight and shoot blight control experiments in 2011

Chemical materials. Orchard experiment was conducted during 2011 at Michigan State University's Horticulture Teaching and Research Center in Holt, MI (GPS: N42° 40' 22.47", W84° 28' 39.36"). Mature, 10-yr-old apple trees of cv. 'Gala', *Malus domestica* Borkhausen, Geneva 16 rootstock, were trunk injected with plant SAR inducer acibenzolar-S-methyl (Actigard 50WG, Syngenta AG, Basel, Switzerland) and antibiotic streptomycin sulfate (Agrimycin® 17, Nufarm Limited, Melbourne, Australia) both intended for blossom blight control. Injected doses per tree were 0.2 g of Actigard and 0.9 g of Agrimycin. Each dose per tree was amended and delivered with 20 ml of distilled water per tree. Untreated control trees were injected solely with 20 ml of distilled water each. Used doses were adjusted according to the registered label recommendations for one spraying treatment with each respective product (Wise et al. 2010). Namely, recommended spray dose of 226.8 g/378.5 L of water per 0.405 ha of Agrimycin (100 ppm) was divided by 250, representing a standard number of planted apple trees per acre in Michigan, to get the dose of 0.9 g received per tree in one spray application. Similarly, recommended spray dose of 56.7g/378.5 L of water per 0.405 ha of Actigard was divided by 250 to get the dose of 0.2 g received per tree in one spray application. Water volumes in the treatments were adjusted according to the injection capacity and reservoir volume capabilities of Quik-jet® micro-injection system for tree injection (Arborjet Inc., Woburn, MA).

Trunk injection. All treatments were applied on 9 May 2011 or 5 days instead of 7 expected days before 80% bloom stage in 'Gala' apple trees. Trunk injections were performed by drilling four delivery ports per tree, 25.4 mm deep and 9.53 mm in diameter, into the trunk xylem tissue. Drilling was imposed by a cordless 1500 rpm drill (DeWalt Industrial Tool Co., Baltimore, MD). Ports were sealed with Arborplugs® no. 4 (Arborjet Inc., Woburn, MA), using screwdriver-like plug tapper and hammer, and with plug positioned just below the bark level to allow cambium port healing (Arborjet Inc., Woburn, MA). Total plant protective solution volume of 20 ml per tree was divided equally among the four ports and increments of 5 ml were injected per port. Injections were conducted using Quik-jet micro-injection system (Arborjet Inc., Woburn, MA) inserted through the one-way valve silicone septum on Arborplugs, into the four freshly drilled xylem port reservoirs. Delivery ports were strategically oriented below the four main scaffold branches radially deployed around the trunk and positioned approximately 15 cm above the ground level. The ports were vertically separated by approximately 5 cm in distance between opposing port pairs. Diameter at half foot or 15.24 cm of trunk height (DHFH) was measured for each tree as an experimental unit. Each treatment consisted of 4 replicate trees arranged in a completely randomized design (CRD). DHFH of trunk injected 'Gala' apple trees as experimental units ranged from 5.84-8.38 cm (average 6.63 cm).

Inoculation. Five days after the injection treatments, on 14 May 2011, all 'Gala' apple tree blossoms were spray inoculated with distilled water suspension of *Erwinia amylovora* strain Ea110 (5×10^5 CFU/ml) cultured in liquid LB medium (Appendix 3). Serial dilutions of bacterial suspension were conducted in 0.5X PBS buffer and plate counted on LB agar medium. Blossom

blight incidence range intended to be reached with this rate of inoculum was 10-30% with goal not to overwhelm the SAR response in the plant. Inoculation of blossoms was conducted at 80% bloom to expose the maximum number of blossoms to the infection. Inoculation was performed using hand-sprayer (Solo® 457 handheld sprayer with 3 US gal. or 11.36 L tank, Solo Inc., Newport News, VA) during the evening to ensure good conditions for bacterial survival.

Disease evaluation. Blossom blight incidence per tree was evaluated 4 times in 7 day intervals on 6, 13, 20 and 27 June 2011. After counting total numbers of diseased and healthy blossom clusters on spurs per tree, blossom blight incidence was calculated as blossom blight percent in per tree basis. Since the disease was successfully spreading from the infected blossoms onto the intensively growing shoots, blossom blight driven shoot blight was also evaluated at the same time points when blossom blight. After counting total numbers of blighted and healthy shoots per tree, shoot blight incidence was calculated as percent of blighted shoots in per tree basis. For each treatment blossom and shoot blight incidence means were calculated from 4 replicate trees.

PR protein gene expression in apple leaves in 2011

Sample collection. From the same trunk injected 'Gala' apple trees rated for blossom blight control in 2011, total of 5 leaves per tree replicate were taken as samples for pathogenesis related (PR) protein gene expression i.e. quantification analysis. Samples were collected three times on 20 and 31 May and 21 June 2011. Collected samples were placed in paper bags and kept in a cooler with ice packs until transported to the laboratory. Samples were then placed in plastic bags and sent without ice packs overnight to Oregon State University, Corvallis, for processing and PR protein expression analysis. The RNA extraction has been conducted according to the previously reported method (Maxson-Stein et al. 2002).

RNA extraction. Individual leaves were ground using a mortar and pestle in extraction buffer (200 mM Tris base, 300 mM lithium Chloride, 1.5% lithium dodecylsulphate, 10 mM EDTA, 1% sodium deoxycholate, and 1.43% tergitol NP-40) plus 1% β-mercaptoethanol to disrupt cells followed by treatment with 6M potassium acetate to precipitate polysaccharides. RNA precipitation using isopropanol was carried out at for 15 min. at -80ºC. RNA was bound to acidified silica and washed 3 times using a solution of 10 mM Tris-HCl, 0.5mM EDTA, and 5M NaCl followed by 100% ethanol rinse to remove phenolic compounds. RNA was eluted in 0.01 M Tris-HCl, pH 8.5. Isolated RNA was DNase treated for removal of DNA contamination. RNA was visualized on a 1% agarose gel (70 V for 45 minutes) and concentration was determined using a NanoDrop Spectrophotometer for RNA-40 (NanoDrop products, Thermo Fisher Scientific Inc., Wilmington, DE).

cDNA library synthesis. 200 ng per ul of RNA was used to synthesize cDNA using the superscript III first strand synthesis kit (Invitrogen™, Life Technologies Corp. Corp., Carlsbad, CA). RNA was incubated with random hexamers and dNTP's for 5 min at 65ºC, cooled on ice for 1 min., then incubated with the synthesis mix (5X reaction buffer, 2.5 mM MgCl2, 0.01 M DTT, 40 U RNaseOUT, and 200 U superscript III reverse transcriptase) at 25ºC for 10 min., 50ºC for 50 min., and 85ºC for 5 min. Residual RNA was eliminated using RNase H by incubating at 37ºC for 20 min. First strand components were cleaned from the cDNA using a wash in Buffer QG and Buffer PE (Qiagen N.V., Hilden, Germany) on a mini-elute column (Epoch Biolabs Inc., Sugar Land, TX).

Measuring of PR-protein gene expression. A 10-fold dilution of cDNA was mixed with IqSYBR green supermix (Bio-Rad Laboratories Inc., Hercules, CA) and with pear primers Ef1-alpha (elongation factor alpha housekeeping gene), PR-1 (unknown function), PR-2 (β-1,3-glucanase), and PR-8 (type III chitinase). Each leaf had 3 technical repetitions for each primer set. qPCR was carried out in the Bio-Rad CFX96 real time PCR machine. Amplification protocol was 90ºC for 5 min., then 39 cycles of 90ºC 10 sec., and 58ºC for 30 sec., followed by melt curve analysis (95ºC 10 sec., 65ºC 5 sec., 95ºC 50 sec.) to verify single gene amplification. A subset of real time PCR products were sequenced and verified as the target genes.

Gene expression data analysis. Average C_t values for each treatment were calculated by the Bio-Rad CFX96 real time PCR machine and copied into and tabulated in a Microsoft excel file. For comparison of gene expression levels the Pfaffl equation was used:

$$\text{Ratio} = (E_{target})^{\Delta Ct \text{ target (control-treated)}} / (E_{reference})^{\Delta Ct \text{ reference (control-treated)}}$$

Where C_t is defined as point at which fluorescence crosses the threshold. E_{target} is defined as the primer efficiency for PR1, PR2, PR5 or PR8. $E_{reference}$ is defined as the primer efficiency for actin or elongation factor alpha. Control is defined as water injected untreated control treatment. Treated is defined as Agrimycin injection and Actigard injection. ΔC_t is the difference in C_t value of the gene being measured for the control minus the gene being measured for a treatment. Gene expression was normalized to the reference gene actin or elongation factor alpha (housekeeping gene).

Linear relative quantification expression levels were calculated for each individual leaf and the average and standard error of the mean was calculated for the three technical repetitions. Next, an average and standard error of the four leaves per treatment were calculated. Log_{10} expression values were determined by transforming ΔC_t value ratios, and the average and standard error of the mean were calculated. Pooled data were the average of gene expression values over the time course of the experiment for each individual location. Averages and the standard deviation were calculated."

Shoot blight control in 2011

Chemical materials. Orchard experiment was conducted during 2011 at Michigan State University's Plant Pathology Research Center in Lansing, MI (GPS: N42° 41' 34.71", W84° 29' 32.43"). Mature, 11-years-old apple trees of cv. 'Kit Jonathan', *Malus domestica* Borkhausen, on M9 rootstock, were trunk injected with plant SAR inducer acibenzolar-S-methyl (Actigard) and sprayed with plant resistance inducer prohexadione-calcium (Apogee® BASF Corp., Research Triangle Park, NC) both intended for shoot blight control. Injected dose of Actigard was 0.2 g per tree amended with 20 ml of distilled water. Sprayed dose of Apogee was 340.2 g of Apogee per 378.5 L of water which is equivalent to 1360.8 g per 0.405 ha. Apogee was mixed with nonionic surfactant adjuvant or spreader-activator Regulaid® at a dose of 125 ml/100 L of water (mixture of 2-butoxyethanol, poloxalene, and monopropylene glycol 90.6%, Kalo Inc., Overland Park, KS). Regulaid improves the effectiveness of foliar applied compounds by allowing superior wetting with the spray solution, uniform spray coverage and improved foliar

penetration. Untreated control trees were injected solely with 20 ml of distilled water each. Used doses were adjusted according to the registered label recommendations for one spraying treatment with each respective product (Wise et al. 2010). Water volumes in the treatments were adjusted according to the injection capacity and reservoir volume capabilities of Quik-jet micro-injection system in Actigard treatment, and label recommendations for one spraying treatment for Apogee treatment.

Trunk injection. Treatments were applied on 24 May 2011 in petal fall growth stage of 'Kit Jonathan' apple trees. This was 10 days after the trees reached 80% bloom or when the onset of intensive shoot growth stage is taking place. Trunk injections and DFHF measurements were conducted using the same methodology described before for the blossom blight control experiment in 2011. Each treatment consisted of 4 replicate trees arranged in a completely randomized design (CRD). DHFH of trunk injected 'Kit Jonathan' trees apple trees as experimental units ranged from 6.6-9.14 cm (average 7.59 cm).

Inoculation. Shoot inoculations with suspension of *E. amylovora* (5×10^5 CFU/ml) were conducted on 10 June 2011 or 17 days after the application of treatments. Shoot blight severity range intended to be reached with this rate of inoculum was 10-30% with goal not to overwhelm the SAR response in the plant. Total of 10 randomly chosen shoots per tree were inoculated while at the same time additional 10 randomly chosen shoots on the same tree replicate were inoculated with distilled water as a negative control. Inoculations were conducted with sterile scissors dipped in bacterial suspension and by cutting off the upper third of the leaf blade of the second or the third youngest leaf on the shoot tip (Koczan et al. 2009). (Koczan et al. 2009)Inoculation of negative control shoots was conducted with distilled water using the same inoculation method with autoclaved scissors.

Disease evaluation. Percent of shoot blight severity was calculated from the ratio of measured necrotized shoot section length and total shoot length, both in [cm], for each inoculated shoot. Only total shoot length in [cm] was measured on negative control shoots since no shoot blight infections were detected on them during the experiment. First measurement of total shoot lengths was taken on 10 June 2011 shortly before inoculations were imposed. Subsequent shoot and necrosis measurements were conducted 4 more times in 7 day intervals on 21, 28 June and on 5 and 12 July 2011. Shoot blight severity and total shoot lengths were measured until the onset of terminal bud set stage at which the shoot growth ceases and shoot maturation starts. For each treatment shoot blight severity percent mean per tree was calculated from 10 shoot replicates per tree and then grand average shoot blight severity percent was calculated from 4 replicate trees. For each treatment, mean total shoot length per tree was calculated from 10 negative control shoot replicates per tree and then grand average total shoot length was calculated from 4 replicate trees.

Shoot blight control in 2012

Chemical materials. Orchard experiment was conducted during 2012 at Michigan State University's Plant Pathology Research Center in Lansing, MI (GPS: N42° 41' 34.71", W84° 29' 32.43"). Mature 14-years-old apple trees of cv. 'Gala', *Malus domestica* Borkhausen, were trunk injected on 23 April 2012 (petal fall) with plant resistance inducer Apogee. The dose of 11.23 g per tree was derived from dividing the maximum allowed seasonal spray dose of this product of 2,806.65 g/0.405 ha on 250 apple trees planted per one acre (0.405 ha) in Michigan. Apogee injection dose per tree was amended and delivered with 520 ml of water.

Trunk injection. Trunk injections were conducted at the onset of intensive shoot growth (petal fall). Trunk injections and DFH measurements for each tree as an experimental unit were conducted using the same methodology described before for shoot blight control experiment in 2011. 'Gala' apple trees as experimental units injected with Apogee ranged from 9.65-13.72 cm (average 11.87 cm) in diameter at one foot or 30.48 cm of trunk height (DFH). Apogee treatment consisted of 4 replicate trees arranged in completely randomized design (CRD)

Inoculation. Shoot inoculations with suspension of *E. amylovora* (4.7×10^7 CFU/ml) were conducted on 7 May 2012 or 14 DAI. Total of 10 randomly chosen shoots per each 'Gala' tree were inoculated with the pathogen, while at the same time additional 10 randomly chosen shoots on the same tree replicate were inoculated with distilled water as a negative control inoculation. Inoculations were conducted using autoclaved scissors with tip dipped in bacterial suspension and by cutting off the upper third of the leaf blade of second or the third youngest leaf on the shoot tip (Koczan et al. 2009). (Koczan et al. 2009)Inoculation of negative control shoots was conducted with distilled water using the same inoculation method with autoclaved scissors.

Disease evaluation. Percent of shoot blight severity was calculated from the ratio of measured necrotized shoot section length and total shoot length, both in [cm], for each inoculated shoot. Only total shoot length in [cm] was measured on negative control shoots since no shoot blight infections were detected on them during the experiment. First measurement of total shoot lengths was taken on 6 May 2012 a day before inoculations were imposed. Subsequent shoot and necrosis measurements were conducted 6 more times in 7 day intervals on 14, 21 and 28 May and on 4, 11 and 18 June 2012. Shoot blight severity and total shoot lengths were measured until the onset of terminal bud set stage at which the shoot growth ceases and shoot maturation starts. For each treatment shoot blight severity percent mean per tree was calculated from 10 shoot replicates per tree and then grand average shoot blight severity percent was calculated from 4 replicate trees. For each treatment, mean total shoot length per tree was calculated from 10 negative control shoot replicates per tree and then grand average total shoot length was calculated from 4 replicate trees.

Statistical analysis for fire blight control in 2011 and 2012

Total shoot length, blossom blight, blossom blight driven shoot blight and shoot blight control data in 2011 an 2012 were analyzed using mixed models and executed with either GLIMMIX or MIXED procedures in SAS 9.3 (SAS Institute, 2012).

The main effects of treatment and time on blossom blight control in 2011 were analyzed using CRD with repeated measures (α=0.05) best adjusted using heterogeneous autoregressive variance covariance structure of first order (blossom blight) and unstructured variance covariance structure (blossom blight driven shoot blight).

Main effect of treatment and time on shoot blight control in 2011 were analyzed using CRD with repeated measures (α=0.05) best adjusted using heterogeneous autoregressive variance covariance structure of first order. Main effect of treatment and time on total shoot length of pathogen inoculated and negative control shoots in 2011 was analyzed using CRD with repeated measures best adjusted using spatial power variance covariance structure (α=0.05).

The main effect of Apogee and time on shoot blight control in 2012 were analyzed using CRD with repeated measures best adjusted using unstructured variance covariance structure (α=0.05). Total shoot lengths data on 'Gala' apple trees treated with Apogee were transformed using SINE (SIN) transformation to normalize the residuals and equalize their variances. Main

effect of Apogee and time on total shoot lengths of pathogen inoculated and negative control shoots on 'Gala' apple trees was analyzed using CRD with repeated measures best adjusted using spatial power variance covariance structure (α=0.05).

Variance-covariance structures in both fire blight experiments and for all monitored parameters were decided by lowering the AIC and BIC fit statistics values after model fitting with different suitable structures. Tree was used as subject of repeated measurements through time. When the main effects or their interactions were found to be statistically significant ($p \leq 0.05$), main effect examination and interactions slicing examination by main effects was performed, tested with F-tests (α=0.05), and pairwise or specific time or treatment comparisons were conducted using *t*-tests (α=0.05).

Results

Blossom blight and shoot blight control experiments in 2011

Weather conditions were not conducive for infection and the blossom blight incidence was very low, ranging from 1.97-10.75%. The main effect of treatment on blossom blight percent was not significant (F=0.11, df=9.02, $p \geq 0.8964$). Further the interaction of treatment and time effects was also not significant (F=0.42, df=14.3, $p \geq 0.8508$). However, the main effect of time was significant indicating that at least in one time point across treatments, disease on inoculated blossoms significantly changed in magnitude during the time of the experiment (F=3.99, df=12.3, $p \leq 0.0340$).

The disease has spread from infected blossoms onto the intensively growing shoots. The blossom blight driven shoot blight was also relatively low with incidence ranging from 4.39-19.08%. However, the main effect of treatment on blossom blight driven shoot blight was found not to be significant (F=0.41, df=9, $p \geq 0.6738$). The interaction of treatment and time effects was also not significant (F=0.34, df=8.09, $p \geq 0.8982$). The main effect of time was significant indicating that at least in one time point across treatments disease onset and development on shoots was successful and significantly changed in magnitude during the time of the experiment (F=4.38, df=7, $p \leq 0.0493$).

PR protein gene expression in apple leaves in 2011

Overall, PR-protein gene expression after Actigard injection was low. Only PR-1 protein gene and only on 21 June 2011 (43 DAI), was found to be 7.8-fold of the expression in water injected untreated control (Figure 27).

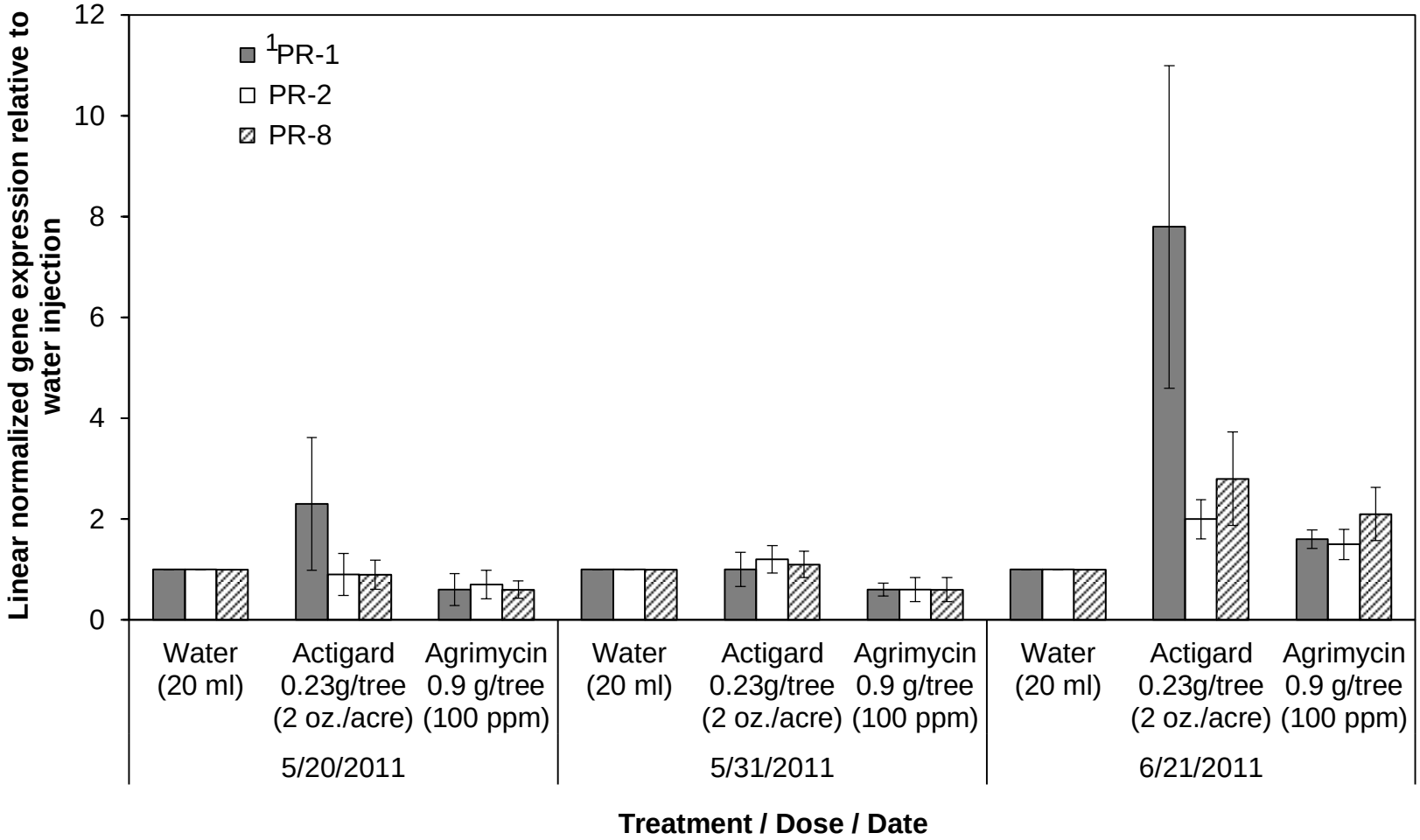

Figure 27. Relative gene expression in leaves as a linear format after trunk injection of 'Gala' apple trees with acibenzolar-S-methyl (Actigard), streptomycin sulfate (Agrimycin) and water on 9 May 2011 (5 days before 80% bloom). Product doses equivalent to dose per tree received with one spray application per one acre of apple trees in Michigan (250 trees per acre /0.405 ha/). [1]PR - pathogenesis related proteins. Average linear gene expression value is relative to water injected untreated control and normalized to actin housekeeping gene. Error bars represent standard error of the mean (SEM).

Apogee effect on shoot blight in 2011

The main effect of Apogee on shoot blight severity was not significant (F=2.51, df=9, p≥0.1364). Even though shoot blight severity in Actigard and Apogee treatments was only numerically much lower from the water injected untreated control, both were strikingly similar in provided effect (Figure 28). However, the main effect of time was significant indicating that that at least in one time point across treatments disease significantly changed in magnitude during the time of the experiment (F=167.5, df=27, p≤0.0001). Further the interaction of treatment and time effects was not significant (F=0.87, df=27, p≥0.5313).

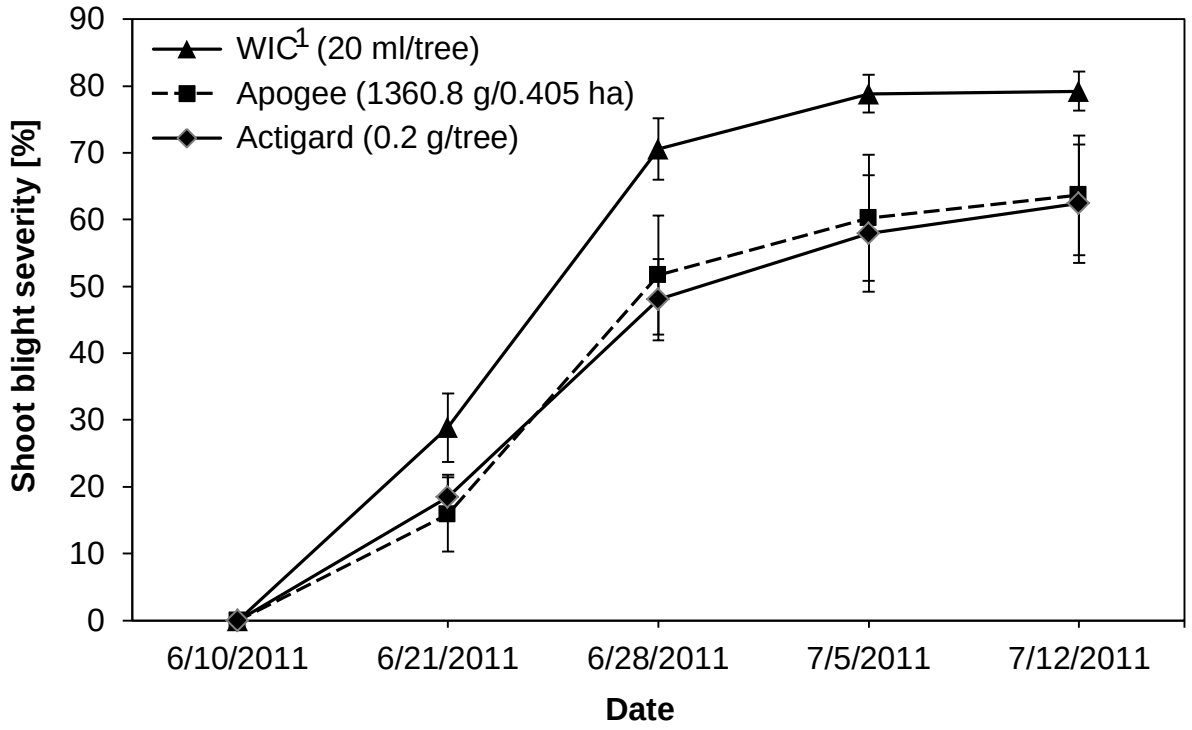

Figure 28. Apogee effect on shoot blight in 2011 after trunk injection of 'Gala' apple trees with acibenzolar-S-methyl (Actigard) and water, and spray treatment with prohexadione-calcium (Apogee) on 24 May 2011 (petal fall). Product doses are equivalent to dose per tree received in one spray application per one acre (0.405 ha) with 250 planted apple trees according to standards in Michigan. [1]WIC - water injected untreated control. Means are based on 10 replicate shoots per tree and 4 replicate trees. Error bars represent standard error of the mean (SEM).

The main effects of Apogee and inoculation (with two levels: inoculated, non-inoculated) on total shoot length of inoculated and negative control shoots were found not to be significant (F=2.3, df=9.082, p≥0.1554; F=0.06, df=9.256, p≥0.8069). Interactions of treatment and inoculation, and treatment, time and inoculation were also not significant (F=1.75, df=9.256, p≥0.2271; F=1.63, df=927.4, p≥0.1112). However, the effect of time and interactions of time and treatment, and time and inoculation, were all found to be significant (F=150.19, df=926.9, p≤0.0001; F=2.52, df=927.4, p≤0.0104; F=17.97, df=926.9, p≤0.0001). The significant effect of

time on total shoot length indicates that in all the treatments, regardless whether the shoots were inoculated or non-inoculated, shoot growth did significantly change in magnitude during the time of the experiment. Treatment and time interaction slicing examination by the main effect of time showed that only on 21 and 28 June the total shoot length in Apogee treatment, regardless whether the shoots were inoculated or non-inoculated, was significantly lower compared to water injected untreated control (t= −2.27, df=9.466, p≤0.0483; t= −2.2, df=9.446, p≤0.0540) (Figure 29). Time and inoculation interaction slicing examination by the main effect of time showed that only on 10 June there was very weak but significant difference (α=0.1) in the total shoot size between inoculated and non-inoculated shoots, regardless of the treatments (F=3.62, df=10.35, p≤0.0853). However, at all other four time points there was no significant difference (α=0.1) in the total shoot size between these shoot categories (F=3.1, df=10.35, p≥0.1076; F=0.12, df=10.35, p≥ 7327; F=1.29, df=10.37, p≤0.2823; F=2.74, df=10.42, p≥0.1278). All in all, there was no significant effect of either Actigard or Apogee on overall shoot growth regardless of inoculation with *E. amylovora* or not (Figure 29).

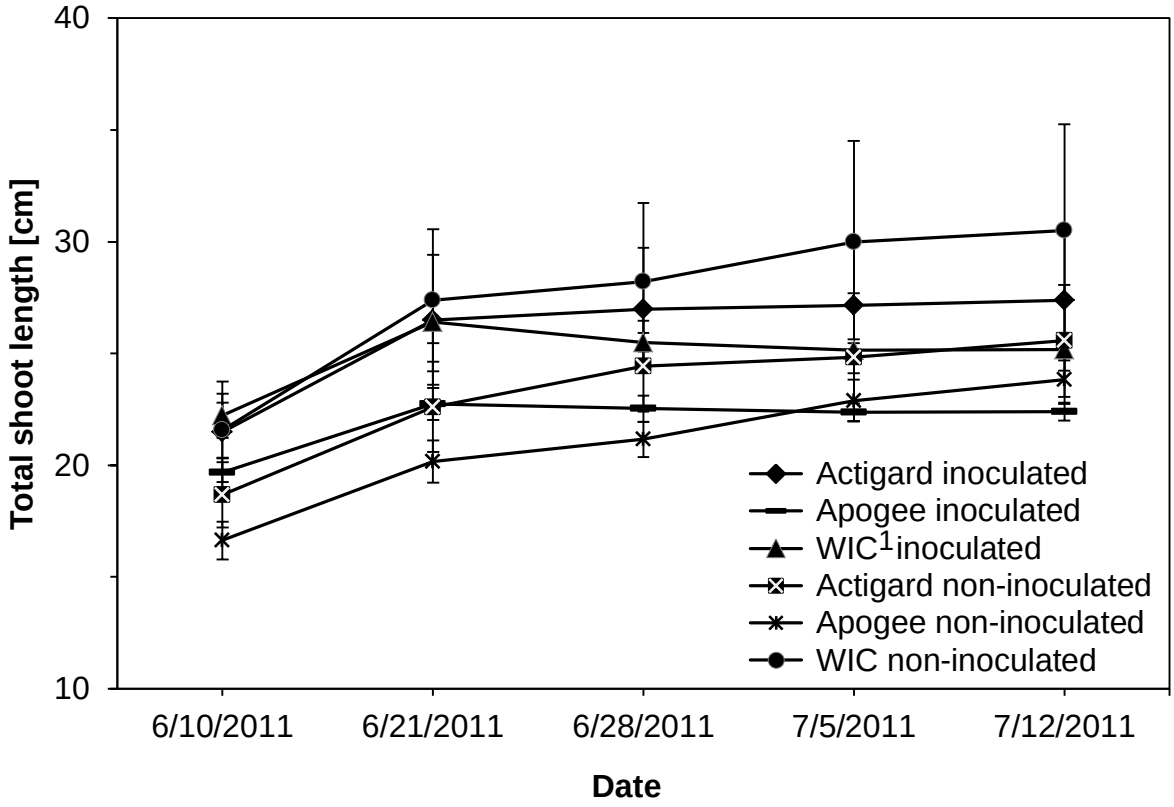

Figure 29. The effect of spray treatment with prohexadione-calcium (Apogee) and trunk injection treatments with acibenzolar-S-methyl (Actigard) and with water on 24 May 2011 (petal fall) on total length of inoculated and non-inoculated shoots on 'Gala' apple trees in 2011. [1]WIC - water injected untreated control. Means are based on 10 replicate shoots per tree and 4 replicate trees. Error bars represent standard error of the mean (SEM).

Apogee effect on shoot blight in 2012

On apple cv. 'Gala' the main effect of trunk injected Apogee on shoot blight severity was not significant (F=0.02, df=6, p≥0.9044). There was no significant difference in comparison to water injected untreated control (Figure 30). However, the main effect of time was significant indicating that at least in one time point across treatments disease onset and development on shoots was successful and significantly changed in magnitude during the time of the experiment (F=23.27, df=3, p≤0.0132). Further, the interaction of treatment and time effects was also not significant (F=0.25, df=3, p≥0.8908).

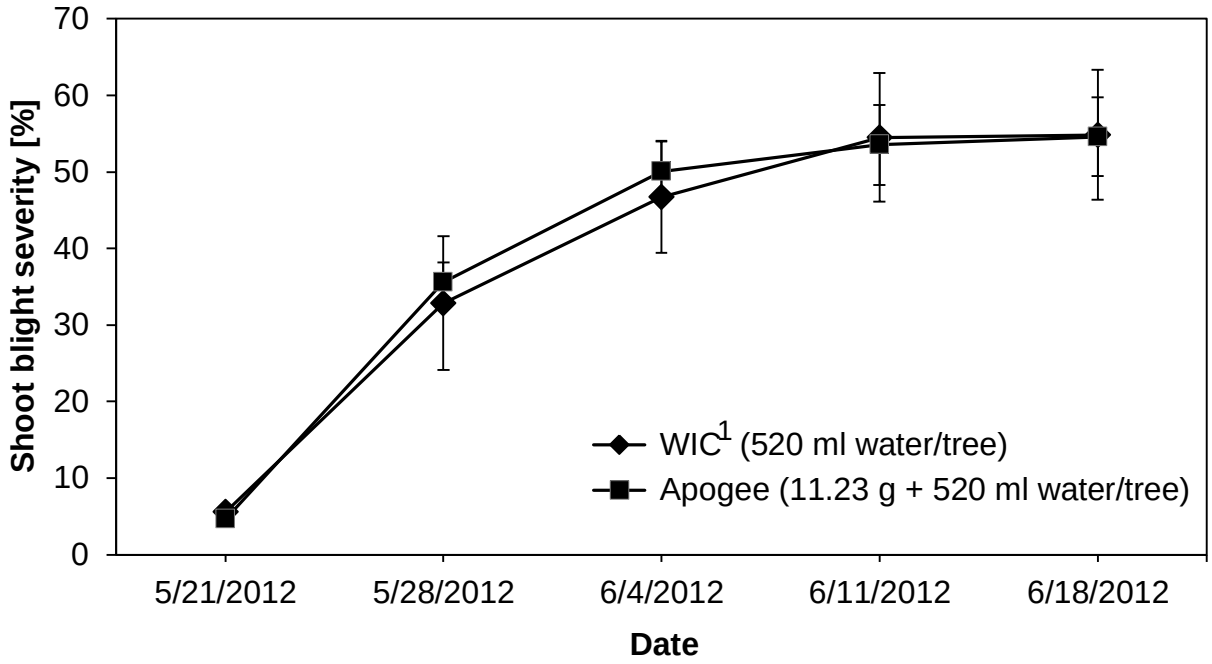

Figure 30. Shoot blight control in 2012 after trunk injection of 'Gala' apple trees with prohexadione-calcium (Apogee) on 23 April 2012 (petal fall). Apogee dose is equivalent to maximum allowed seasonal spray dose of this product per one acre (0.405 ha) with 250 planted apple trees according to standards in Michigan. [1]WIC - water injected untreated control. Means are based on 10 replicate shoots per tree and 4 replicate trees. Error bars represent standard error of the mean (SEM).

The main effects of treatment and inoculation (with two levels: inoculated, non-inoculated) on total shoot length of inoculated and negative control shoots were found not to be significant (F=1, df=6.25, p≥0.3535; F=0.78, df=160, p≥0.3775). Interactions of treatment and inoculation, treatment and time, and treatment, time and inoculation were all found not to be significant (F=0.03, df=160, p≥0.8697; F=1.5, df=929, p≥0.1762; F=0.8, df=929, p≥0.5715). However, the effect of time and interaction of time and inoculation were found to be significant (F=268.95, df=929, p≤0.0001; F=10.68, df=929, p≤0.0001) (Figure 31). Significant effect of time indicates that all shoots, regardless of the treatment and of inoculation or not, did change in total length and proves their increased growth through time. The slicing examination of the significant time and inoculation interaction showed that on 6, 21 and 28 May there was no

significant differences between the total shoot lengths of all inoculated and all non-inoculated shoots regardless of the treatments (F=2.24, df=228.5, p≥0.1362; F=2.02, df=228.5, p≥0.1563; F=1.87, df=228.5, p≥0.1730). However, significant differences between the total shoot lengths of all inoculated and all non-inoculated shoots, regardless of the treatment, were present on 14 May and 4, 11 and 18 June (F=4.4, df=228.5, p≤0.0370; F=5.78, df=228.5, p≤0.0170; F=10.85, df=228.5, p≤0.0011; F=12.61, df=228.5, p≤0.0005). On 14 May all inoculated shoots were with significantly higher total shoot length compared to all non-inoculated shoots, regardless of the treatment. However, from 4-18 June due to the pathogen advancement in infection all inoculated shoots, regardless of the treatment, were with significantly lower total shoot length compared to non-inoculated shoots. This shows that inoculation with the pathogen was successful but once more confirms Apogee did not provide both significant control of shoot blight and shoot growth reduction. All in all, there was no significant effect of Apogee on overall shoot growth regardless of inoculation with *E. amylovora* or not (Figure 31).

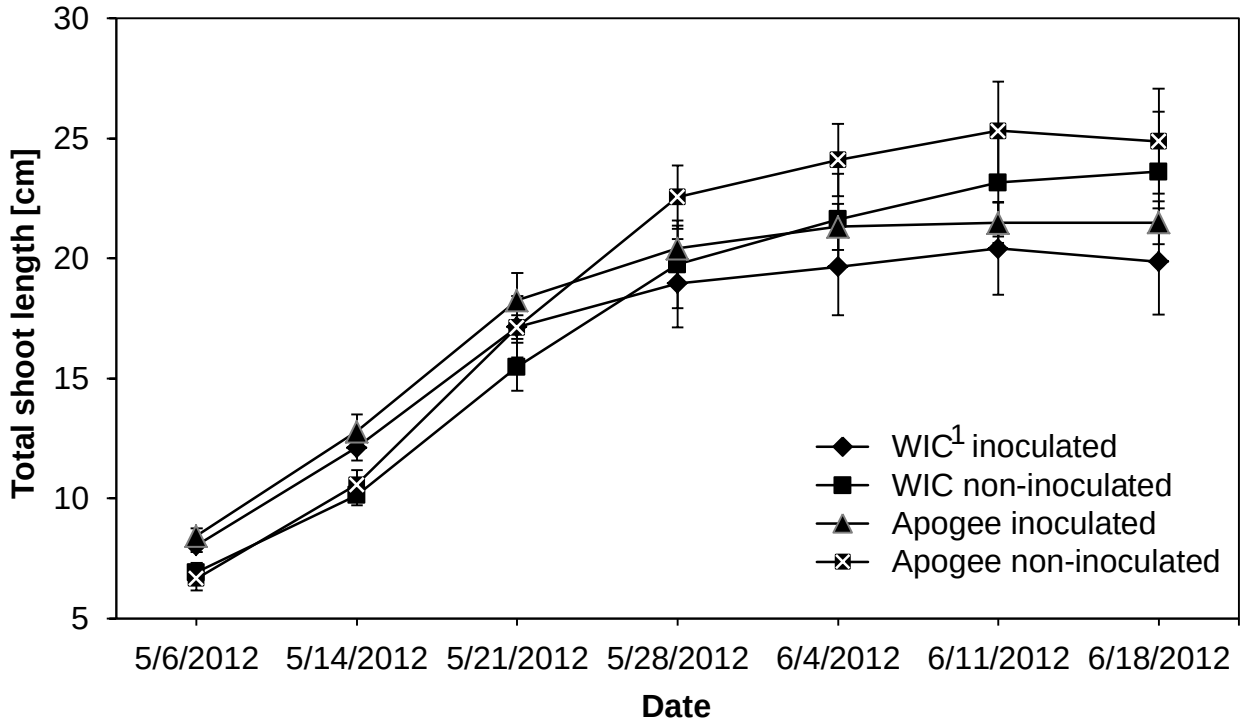

Figure 31. The effect of trunk injections with prohexadione-calcium (Apogee) and with water on 23 April 2012 (petal fall) on total length of inoculated and non-inoculated shoots on 'Gala' apple trees in 2012. [1]WIC - water injected untreated control. Means are based on 10 replicate shoots per tree and 4 replicate trees. Error bars represent standard error of the mean (SEM).

Discussion

Blossom blight and shoot blight control in 2011

In trunk injection experiments of 2011, weather conditions in the spring overall were not favorable for the establishment of *E. amylovora* infections on 'Gala' blossoms. This severely hampered the onset of blossom blight, thus yielding very low and statistically not different disease incidences detected among all the treatments and rating times. However, the few established blossom blight infections in the spring allowed limited increase in pathogen populations and a limited spreading of infections onto the surrounding flowers, fruitlets and succulent shoots. This was indicated by the significant changes in disease incidence through time on blossom/fruitlet clusters and shoots in all the treatments. Limited fire blight spreading was supported by the oncoming warm, rainy and humid days from mid-June 2011 onwards.

Since in 2011 trunk-injected compounds did not show any significant effects in control of blossom and shoot blight, several conclusions can be drawn to explain these results. The first is that time of 5 days between trunk injection and pathogen inoculation at 80% bloom, was most likely insufficient i.e. too short for substantial translocation of the effective doses of injected compounds into the apple tree crown. Expected full dose of the compound reaching the points of infection and the places of SAR induction, after more time, would surely secure the effect on the pathogen. Second, it can be concluded that the amount of 20 ml of water used to deliver the dose in each injected tree was probably insufficient to provide substantial dilution of the compound and its easier translocation through the xylem and into the crown. Finally, it is also possible that the used doses of Actigard and Agrimycin, derived from a single spray treatment per 0.405 ha, were unsuitable i.e. too low to provide significant reduction of fire blight on apple trees. However, insufficient time, weak dilution and potentially low a. i. dose might have worked in concert thus leading to no efficiency in blossom blight control in 2011 experiments.

The structural and chemical properties of xylem and physical and chemical properties of the compound and its formulation play a critical role in the pattern and time-course of movement of trunk injected compounds throughout the tree (Smalley 1977; Kondo 1978; Percival & Boyle 2005; Doccola et al. 2012; Montecchio 2013). The properties of a specific xylem type in different tree species, such as the level of compound flow sectoriality versus the level of integrated compound flow, the density of vessels, the lateral pitting of vessel element walls, the pit size and the density of intervessel pits, are all influencing the type and speed of compound movement in the trunk and distribution in the tree crown (Orians et al. 2004; Orians 2005; Tanis et al. 2012). It has been experimentally proven before that in ash trees (*Fraxinus*) trunk tissues act like temporal reservoir of trunk-injected compound (Tanis 2008; Tanis et al. 2009; Mota-Sanchez et al. 2009; Tanis et al. 2012). The mechanisms behind the reservoir effect are associated with the carbon adsorption coefficient of the a. i. or Koc i.e. Organic Carbon-Water Partitioning Coefficient (ml/g) (Doccola et al. 2012; Doccola & Wild 2012). Koc is defined as the ratio of the mass of a chemical that is adsorbed in the certain environment per unit mass of organic carbon in that environment per the equilibrium chemical concentration in solution. It expresses the level of adhesion of the a. i. to the carbon rich compounds within structures of the xylem. We further extend the importance of Koc to the components of compound formulation. In topical application inert components of the formulation are intended to reduce surface tension of the solution and adhere the active compound to the plant surface. In trunk injection these properties can lead to clogging of the xylem vessels or slow movement of the a. i. into the

canopy. Both Actigard and Agrymycin used in injection are not formulated for trunk injection but for foliar application. Also, the level of temporal distribution and activity of trunk-injected compounds in the tree canopy highly depends on water solubility of the compound (Pinkas et al. 1973; Shabi et al. 1974; Marsh 1977; Smalley 1977; Nair 1979; Nair et al. 1981; Tattar et al. 1998; Young 2002; Percival & Boyle 2005). Thus, the interaction or non-interaction of the injected compound and its formulation with woody tissues most likely govern the extent and time of release and movement of trunk injected products. This was most likely the prime reason behind the absence of significant effect of trunk injected Actigard and Agrimycin on reduction of blossom blight in 2011. It can be concluded that trunk-injected compounds require more time to be translocated and accumulated into the canopy in comparison to the immediate contact of topically applied compounds.

Compound transport through the xylem and distribution within the canopy is also dependent on daily water uptake of a tree. Water uptake is driven by the rate of daily transpiration (Davies & Lakso 1979; Cohen et al. 1981; Brough et al. 1986). Depending on tree species, age, size, health and the weather conditions, forest trees absorb and translocate between 100-200 gallons of water and in extreme cases even 300 gallons of water per day (Kozlowski 1976; Owens & Moore 2007; Holbrook & Zwieniecki 2008). On the contrary, a mature apple tree depending on weather conditions and age uses only around 15-50 gallons of water (Vossen & Silver 2000). This implies that in trunk-injection of fruit trees more water should be added to the compound to increase its dilution and ease the transport through the xylem and into the canopy. Variable rootstocks with different water uptake properties might be an important consideration when trunk injection is investigated on fruit trees in agriculture. Also, the standard doses used in landscape tree care can be unsuitable and damaging to fruit trees due to the differences in daily water uptake. The dose of injected compound needs to be carefully chosen and balanced with the most important physiological parameters of the tree.

Determination of compound doses for trunk injection is difficult. In tree-based agriculture trunk injection is novel approach and spatial and temporal distributions of trunk injected compounds in tree canopy, which secure the efficiency, are not well investigated to provide basis for this task (Eisenback 2008). Hence, the compound doses and timing of applications used in topical disease control might not be suitable i.e. efficient and readily transferrable for use in trunk injection of fruit trees. For some tree-pathogen systems, such as *Oomycetes* on apple and nematodes on pine, research has shown that single trunk injection of pesticides formulated for trunk injection can provide from 15 months to 2-3 years of protection, respectively (Long et al. 1989; Guest et al. 1995; Takai et al. 2003; Takai et al. 2004). Doses designed for use in topical spray treatments can be problematic when used in trunk injection. Excessive amount of the a. i. and/or inert components of pesticide formulation can also lead to xylem clogging and slow ascent into the canopy. This, in turn, can have severe impact on disease control due to limited amount of a. i. reaching the canopy (Smalley 1977). The excessive amount of injected a. i. can also lead to direct phytotoxicity expressed on trunk and branch tissues (Campana et al. 1979; Long et al. 1989; Perry et al. 1991; Guest et al. 1995; Montecchio 2013).

In landscape tree care, compound doses for tree injection are designed according to DBH of a tree trunk, which correlates with the overall tree size. In agriculture, individual tree doses determined according to tree DFH might not be suitable for plant protection due to constantly maintained tree sizes, primarily expressed in the uniform size of crown canopy shaped every year by pruning. In comparison to forest trees, this imposes disproportion between the crown size, which is maintained more-less similar for years, and the trunk DFH which slowly increases

in size every year. Due to controlled tree size uniformity, in orchards dose is delivered in per acre basis and compound dosing according to trunk DFH might be too high and lead to phytotoxic effect in the canopy. This side effect after of trunk injection of different compounds has been reported before for different plant pathogen systems (Campana et al. 1979).

Finally, the designed dose for topical use, besides being efficient in disease control for specific time interval, accounts for pesticide losses due to drift, run-off, UV-degradation and other dose reducing ecological and biological factors acting through time. Canopy surface being covered is much bigger in comparison to the surface of the internal vascular tissues. Therefore, dose intended for spraying might be too high for trunk injection and lead to detrimental i.e. phytotoxic effects. Phytotoxicity after of trunk injection of different compounds has been reported before for different plant pathogen systems (Campana et al. 1979). All this imposes a hypothesis that dose intended for topical application in plants might be as different from the dose needed for trunk injection as the dose of medical drug for an adult human is crucially different in amount and effect from a dose intended for an infant.

The only recent trunk injection research conducted on apple trees for control of *E. amylovora* was the investigation of the effect of prohexadione-carboxylic acid (PCA) in control of blight blossom infections (Düker & Kubiak 2011a). When the free acid of prohexadione-calcium (Apogee) or PCA was trunk-injected for blossom blight control on apple cv. 'Weisser Klarapfel' and cv. 'Gala Must', with inoculation at full bloom or 8 and 30 days after trunk injection, blossom blight incidence was significantly controlled when compared to water injected untreated control (Düker & Kubiak 2011a). On both cultivars this level of control was not significantly different from the standard streptomycin sprays (Düker & Kubiak 2011a). However, in both cultivars the stunting effect of trunk-injected PCA on longitudinal shoot growth led to significant fruit yield losses (Düker & Kubiak 2011a). Since, previous research with topically applied prohexadione-calcium showed insufficient effectiveness in blossom blight control (Bazzi et al. 2003), trunk injection of PCA could serve as a potential alternative to the topical application of streptomycin (Düker & Kubiak 2011a).

PR-protein gene expression in 2011

After trunk injection of Actigard on 9 May 2011, late detected 7.8-fold induction of PR-1 protein gene on 21 June was most likely the result of sufficient time of 43 DAI allowed for this compound to translocate into the apple leaf canopy and induce the SAR effect. However, the magnitude of this induction and SAR effect was not sufficient to affect the fire blight pathogen and significantly reduce the blossom blight incidence. This might be assigned to the ineffective dose of 0.23 g of Actigard per tree or the inadequately low amount of water amended with this dose, thus limiting its substantial dilution and hampering its subsequent translocation in the xylem and accumulation in the crown. In 2011 the conditions for fire blight infection were not favorable, driving the overall infection levels very low in all the treatments. Thus, if the injected Actigard did not significantly reduce even these low infection levels then the above stated reasons for its ineffectiveness are supported. Nevertheless, the induction of PR-1 protein gene, a marker of SAR response in plants, was an incentive to initiate new and design improved fire blight control experiments in 2012 and 2013.

Apogee effect on shoot blight in 2011 and 2012

Previous research in control of shoot blight severity by trunk injected compounds in general is limited. Arborfos and Apogee formulated for microinjection and trunk-injected on susceptible apple cv. 'Paula Red', on M7 rootstock, showed that only Arborfos provided good control by significantly reducing shoot blight severity for 67% compared to the untreated control (Spitko 2008). The effect of Apogee on shoot growth was not detected in this study since there were no significant differences after total of four shoot length measurements were taken pre-injection and post-injection (Spitko 2008). Based on absence of the effect on shoot growth authors emphasize that there is a strong indication that prohexadione-calcium probably did not translocate through the tree tissues after it was trunk-injected and thus was not able to distribute.

The efficiency of topical application of Apogee in control of shoot blight incidence and severity has also been investigated. On cv. 'Idared', Apogee applied topically at two times, 13 and 20 days before inoculation with *E. amylovora*, led to significant control of shoot blight severity by reducing it for 86% when compared to the untreated control (Momol et al. 1998). Further, when compared to Agrimycin, with shoot blight severity reduction of only 39%, the effect of Apogee on shoot blight severity was far greater. Apogee also provided significant reduction of mean shoot length (Momol et al. 1998).

In another experiment on apple cultivars 'Rome Beauty', on MM106 rootstock, 'Golden Delicious', on M7 rootstock, and 'Law Rome', on MM111 rootstock, Apogee topically applied twice at bloom showed significant reduction of shoot blight severity when inoculations were conducted 10 days after the second treatment (Yoder et al. 1999). In the same and the following studies, Apogee also significantly reduced mean total shoot length, as expected, but also significantly reduced the shoot blight incidence for 40-58% besides the significant reduction of shoot blight severity (Yoder 2001). Besides, significantly reducing the mean total shoot length, in two year trials on container-grown apple trees of cv. 'Idared' and cv. 'Freedom', both on M9 rootstock, spray treatments with Apogee at 13, 14 or 15 days before inoculation showed significant reduction of shoot blight severity at 7, 14 and 21 days after the inoculation (Bubán et al. 2004). When preventively applied acylcyclohexanediones such as prohexadione-calcium and trinexapac-ethyl were evaluated for their ability to reduce fire blight infection on apple flowers, a reduction of blossom blight up to 50-67% was recorded on differentially later inoculated plants (Spinelli et al. 2007).

In comparison to our results with trunk-injected Apogee, where no effect has been recorded on both shoot blight severity and mean shoot length, it can be concluded that Apogee most certainly did not reach the shoots in apple tree canopy, thus not exerting its stunting effect on their growth and the fire blight reduction effect. This demonstrates an extreme case in which the substantial time for compound translocation after trunk-injection was not playing a crucial role in the absence of, or weak fire blight severity control. In this extreme case the a. i. was most likely stored i.e. bound or rapidly metabolized in the trunk (Lindquist 1965; Campana et al. 1979). The long-term storage of Apogee in the trunk makes sense since the carbon adsorption coefficient or Koc (Organic Carbon-Water Partitioning Coefficient) values for this compound in sand, clay, chloroform fumigated loamy sand, and loamy soils are 173, 155, 1428 and 421 ml/g respectively (Serafini 2001). This means that prohexadione-calcium has a low mobility in loamy sand soil and loamy soil, which contain 1.1% or more of organic matter, while it is very highly mobile in sandy soil with 0.5% of organic matter (Serafini 2001). Therefore, if prohexadione-calcium is strongly bound to the organic matter in loamy soil, it is highly likely that after trunk

injection this strong binding also happened in the xylem of an apple tree, where the organic carbon content is even higher within the structures of this tissue. This indicates that if a compound with high Koc value is trunk-injected into an organic carbon rich environment, it will most likely be released and translocated very slowly or will not move at all into the canopy. Hence, there will be weak or no effect expressed in plant protection. Prohexadione-calcium has a water solubility of 174 mg/L at 25ºC (Serafini 2001), which in the case of trunk injection would be of a secondary importance for the compound movement in xylem due to the high Koc value.

In our shoot blight severity control experiments in 2011, the lack of significant effect of trunk-injected Actigard can be most certainly assigned to low injected dose, insufficient to exert the reduction effect on the pathogen, or the low amount of water used to dilute the compound. Insufficient amount of water per tree did not ease Actigard translocation into the canopy and thus failed to aid its effect on the reduction of shoot blight severity.

APPENDIX 2. Figures and tables including complete PR-protein gene expression analyses for fire blight control, treatments evaluated for apple scab control and fungicide residues

FOR CHAPTER 3

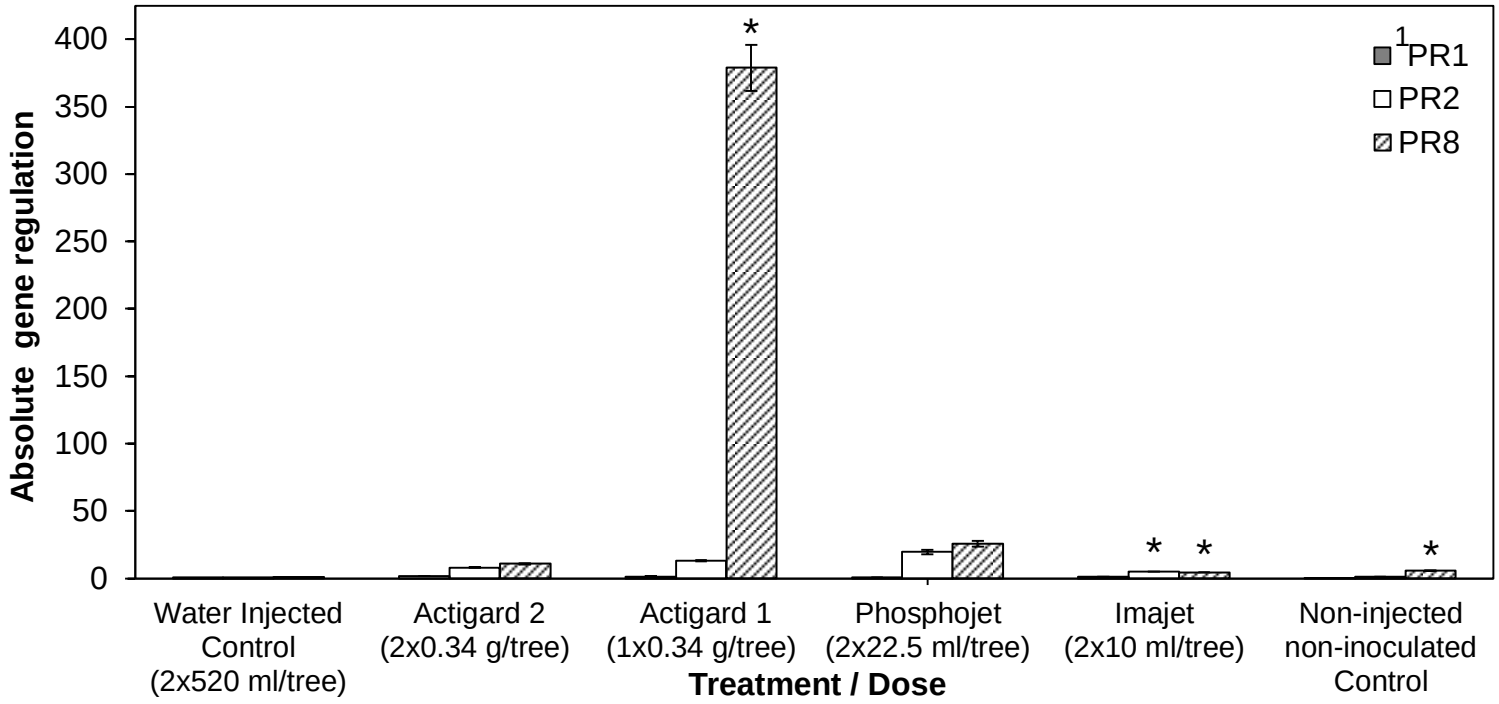

Figure 32. Gene expression in leaves on 2 July 2012 after two trunk injections of 'Gala' apple trees with acibenzolar-S-methyl (Actigard), potassium salts of phosphorous acid (Phosphojet), imidacloprid (Imajet) and water on 26 March (tight cluster) and 23 April 2012 (petal fall). [1]PR - pathogenesis related proteins. *Mean gene expression followed by an asterisk is significantly upregulated relative to water injected untreated control and normalized to actin housekeeping gene (Pair Wise Fixed Reallocation Randomization test, α=0.05). Error bars represent standard error of the mean (SEM).

FOR CHAPTER 4

Table 26. Apple scab control in 2012 on spur leaves and terminal shoot leaves of 'Red Delicious' after fungicide trunk injections on 21 March, 20 April, 25 May and 22 June (*t*-tests, α=0.05). [1]WIC - water injected untreated control, SpraySTD - spray standard. [2]Treatment means within one date or column followed by different upper-case letters are significantly different. [3]Means within one treatment across the four dates followed by different lower-case letters are significantly different. [4]Apple scab ratings not conducted due to phytotoxicity caused by injected propiconazole (Alamo).

Treatment	Mean apple leaf scab incidence [%]				
	Spurs	Shoots			
	Mean for 4 and 18 May	4 May	18 May	14 June	17 August
WIC[1]	81.93 A	35.89 A[2]a[3]	68.91 Ab	74.56 Ab	24.23 ABc
Milstop	72.36 AB	26.69 ABa	39.74 Bb	62.11 ABc	17.5 BCd
Oxidate	61.1 BC	11.41 DEa	38.57 Bb	45.26 Bb	21.01 ABc
Phosphojet 1	60.82 BC	21.65 BCa	32.37 Bb	24.18 Cab	13.04 CDc
Nutrol	60.42 BC	16.79 BCDa	32.82 Bb	45.91 Bc	16.81 BCa
Prophyt	53.49 C	12.72 CDEa	26.12 Bb	22.59 Cb	12.35 CDa
Phosphojet 2	47.67 C	16.33 CDEa	28.11 Bb	20.38 Ca	9.23 Dc
Alamo	-[4]	-	-	29.95 Ca	15.11 BCb
SpraySTD	16.68 D	9.5 E	14.06 Cb	28.19 Ccd	30.78 Ad

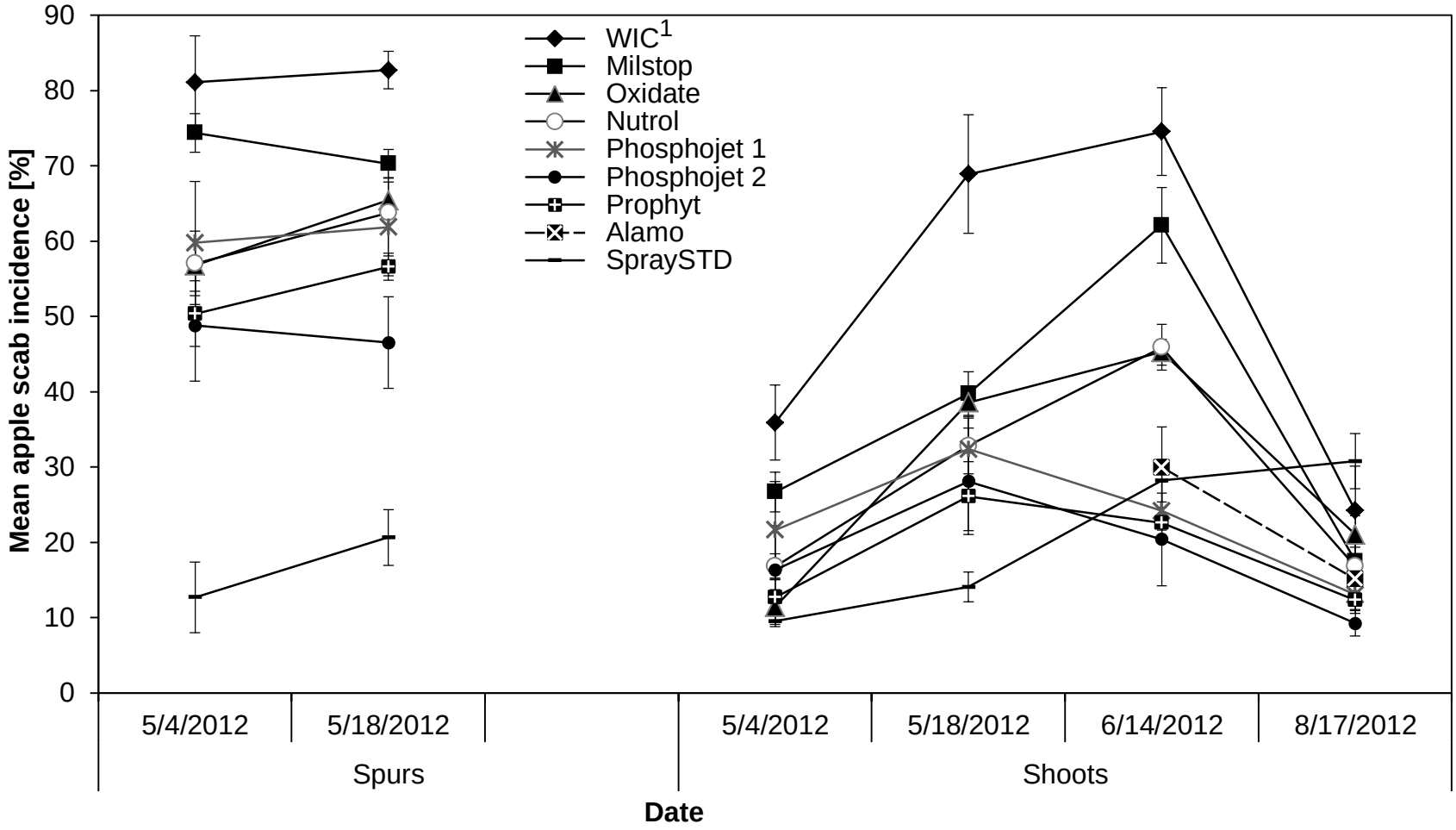

Figure 33. Apple scab control in 2012 on spur leaves and terminal shoot leaves of 'Red Delicious' after fungicide trunk injections on 21 March, 20 April, 25 May and 22 June. [1]WIC - water injected untreated control, SpraySTD - spray standard. Means are based on 20 replicate spurs or shoots per tree and 4 replicate trees. Error bars represent standard error of the mean (SEM).

FOR CHAPTER 5

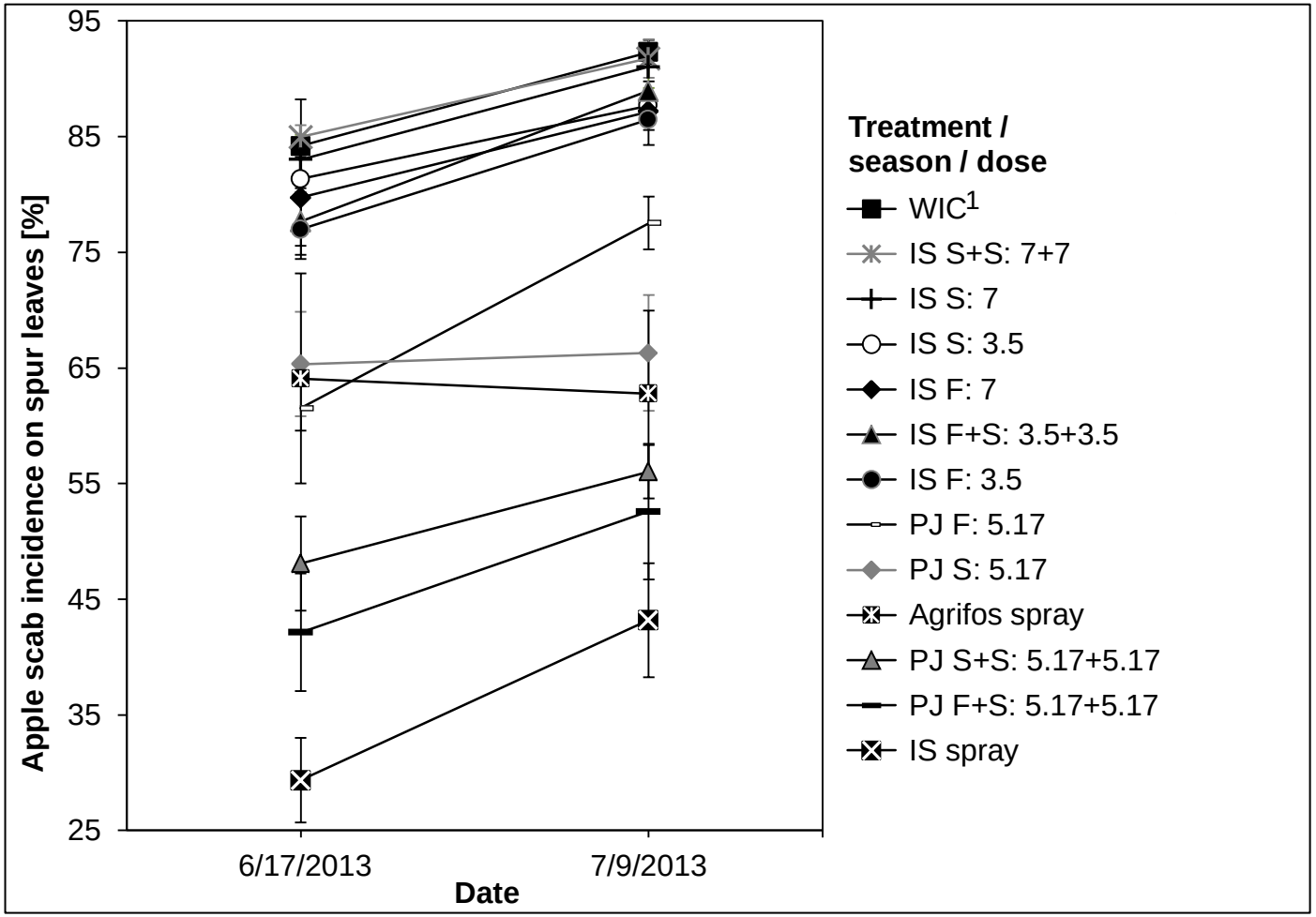

Figure 34. Apple scab control on spur leaves in 2013 after trunk injection of 'Mac Spur' trees with difenoconazole +cyprodinil (IS) and potassium salts of phosphorous acid (PJ) at different dates in 2012 and 2013. [1]WIC - water injected untreated control, IS - doses in ml/tree, PJ - doses in ml/25.4 mm DFH, F - fall 2012 injection, S - spring 2013 injection(s). Error bars represent standard error of the mean (SEM).

Table 27. Apple scab control on shoot leaves in 2013 after trunk injection of 'Mac Spur' trees with difenoconazole +cyprodinil (IS) and potassium salts of phosphorous acid (PJ) at different dates in 2012 and 2013 (*t*-tests, α=0.05). [1]DFH - tree trunk diameter at one foot height (30.48 cm). [2]WIC - water injected untreated control. [3]Treatment means within one date followed by different upper-case letters are significantly different. [4]Means within one treatment across the four dates followed by different lower-case letters are significantly different.

Treatment/ Season/ Dose [ml] per tree (IS) or per 25.4 mm of trunk [1]DFH (PJ)	Mean apple leaf scab incidence [%]			
	Shoots			
	17 June	9 July	30 July	30 August
WIC[2]	82.95 AB[3] a[4]	97.09 Ab	98.1 Ab	99.49 Ab
IS Fall 7	82.97 ABa	95.48 Ab	98.67 Ab	98.89 Ab
IS Fall+Spring 3.5+3.5	76.45 ABCa	95.71 Ab	96.7 Ab	99.36 Ab
IS Fall 3.5	85.1 Aa	93.58 Ab	94.61 ABb	99.13 Ac
IS Spring+Spring 7+7	75.27 BCa	80.67 BCa	88.12 BCDb	92.55 ABc
IS Spring 3.5	73.07 Ca	81.65 Bb	85.88 CDbc	88.08 BCc
IS Spring 7	69.04 Ca	75.56 BCa	82.98 Db	84.38 CDb
PJ Fall 5.17	56.62 Da	73.68 CDb	71.71 Eb	79.85 Dc
PJ Spring 5.17	48.18 DEa	44.49 Fa	47.98 Fa	58.18 Eb
PJ Fall+Spring 5.17+5.17	38.23 EFa	56.96 Eb	38.71 FGa	55.43 Eb
Agrifos Spray	45.43 DEc	29.56 Ga	33.95 Gab	38.8 Fbc
PJ Spring+Spring 5.17+5.17	35.96 EFa	32.32 Ga	23.16 Ga	34.92 Fa
IS spray	27.52 Fa	66.4 Db	91.69 ABCc	97.16 Ad

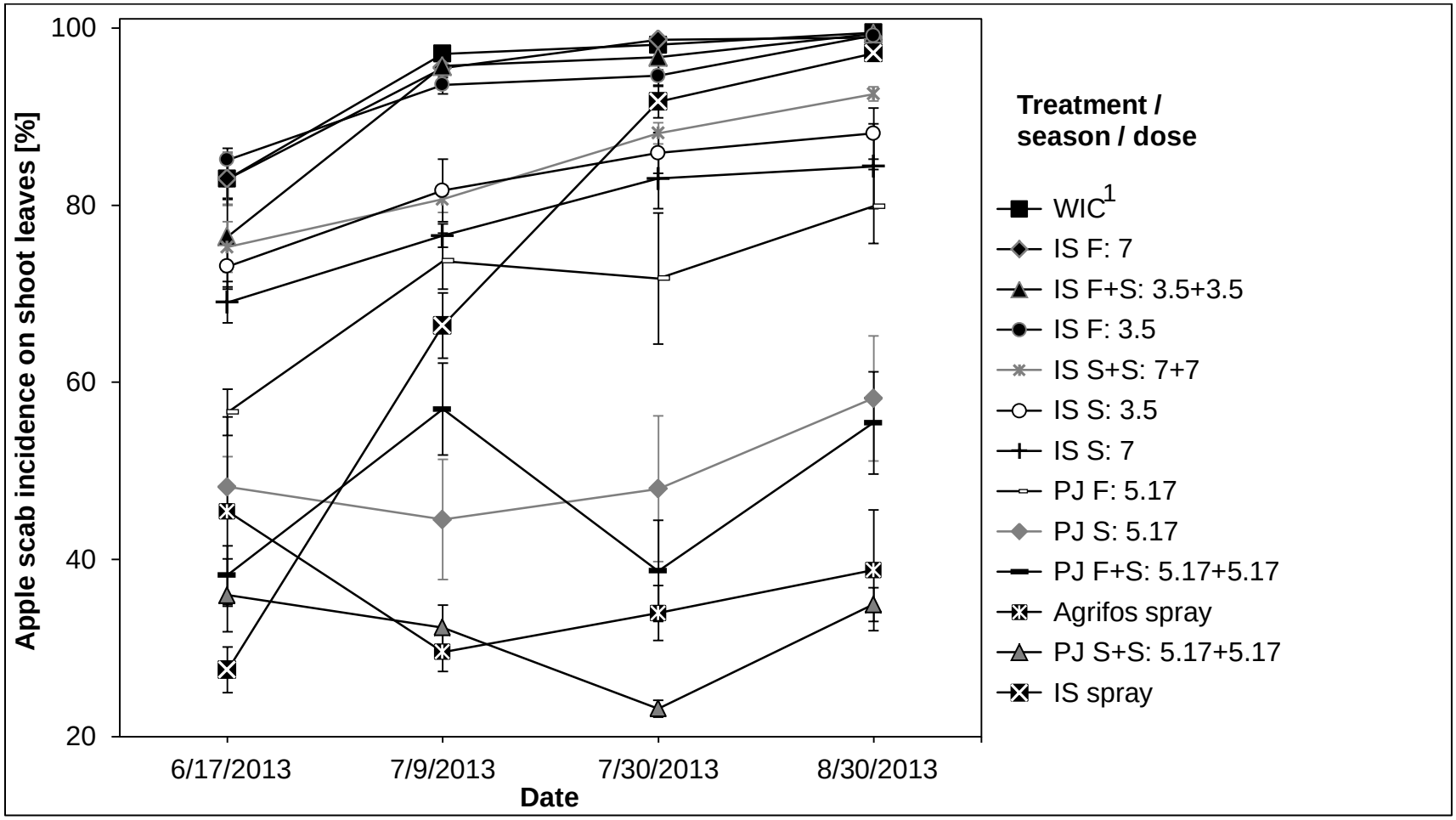

Figure 35. Apple scab control on shoot leaves in 2013 after trunk injection of 'Mac Spur' trees with difenoconazole +cyprodinil (IS) and potassium salts of phosphorous acid (PJ) at different dates in 2012 and 2013. [1]WIC - water injected untreated control, IS - doses in ml/tree, PJ - doses in ml/25.4 mm DFH, F - fall 2012 injection, S - spring 2013 injection(s). Error bars represent standard error of the mean (SEM).

Table 28. Apple scab control on fruits in 2013 after trunk injection of 'Mac Spur' trees with difenoconazole+cyprodinil (IS) and potassium salts of phosphorous acid (PJ) at different dates in 2012 and 2013. (*t*-tests, α=0.05). [1]DFH - tree trunk diameter at one foot height (30.48 cm). [2]WIC - water injected untreated control. [3]Treatment means within one date followed by different upper-case letters are significantly different. [4]Means within one treatment across the four dates followed by different lower-case letters are significantly different.

Treatment/ Season/ Dose [ml] per tree (IS) or per 25.4 mm of trunk [1]DFH (PJ)	Mean apple leaf scab incidence [%]			
	Fruit			
	17 June	9 July	30 July	30 August
IS Spring+Spring 7+7	90.56 A[3] a[4]	100 Aa	96.67 ABa	100 Aa
IS Fall 7	88.95 Aa	98.17 Aa	97.92 ABa	99.5 Aa
IS Fall 3.5	87.83 Aa	98.5 Aa	99.3 ABa	99.67 Aa
IS Spring 7	87.29 Aa	87.5 Aa	83.33 ABCDa	100 Aa
IS Fall+Spring 3.5+3.5	85.83 Aa	96.65 Aa	99.5 Ba	100 Aa
WIC[2]	84.6 Aa	98.04 Ab	99.24 ABb	100 Ab
IS Spring 3.5	81.09 ABa	100 Aa	100 ABa	100 Aa
Agrifos Spray	60.48 BCab	60.13 Ba	72.45 Dbc	78.64 BCc
PJ Fall 5.17	53.6 CDa	77 Bb	93.4 ABCc	87.5 ABc
IS spray	36.14 Da	61.95 Bb	82.49 ACDc	89.19 ABc
PJ Spring+Spring 5.17+5.17	28.89 CDa	22.91 Ca	30.63 Ea	33.29 Ea
PJ Spring 5.17	27.41 Da	26.65 Ca	29.55 Ea	58.49 CDb
PJ Fall+Spring 5.17+5.17	17.22 Da	25.67 Ca	47.14 Eb	45.28 DEb

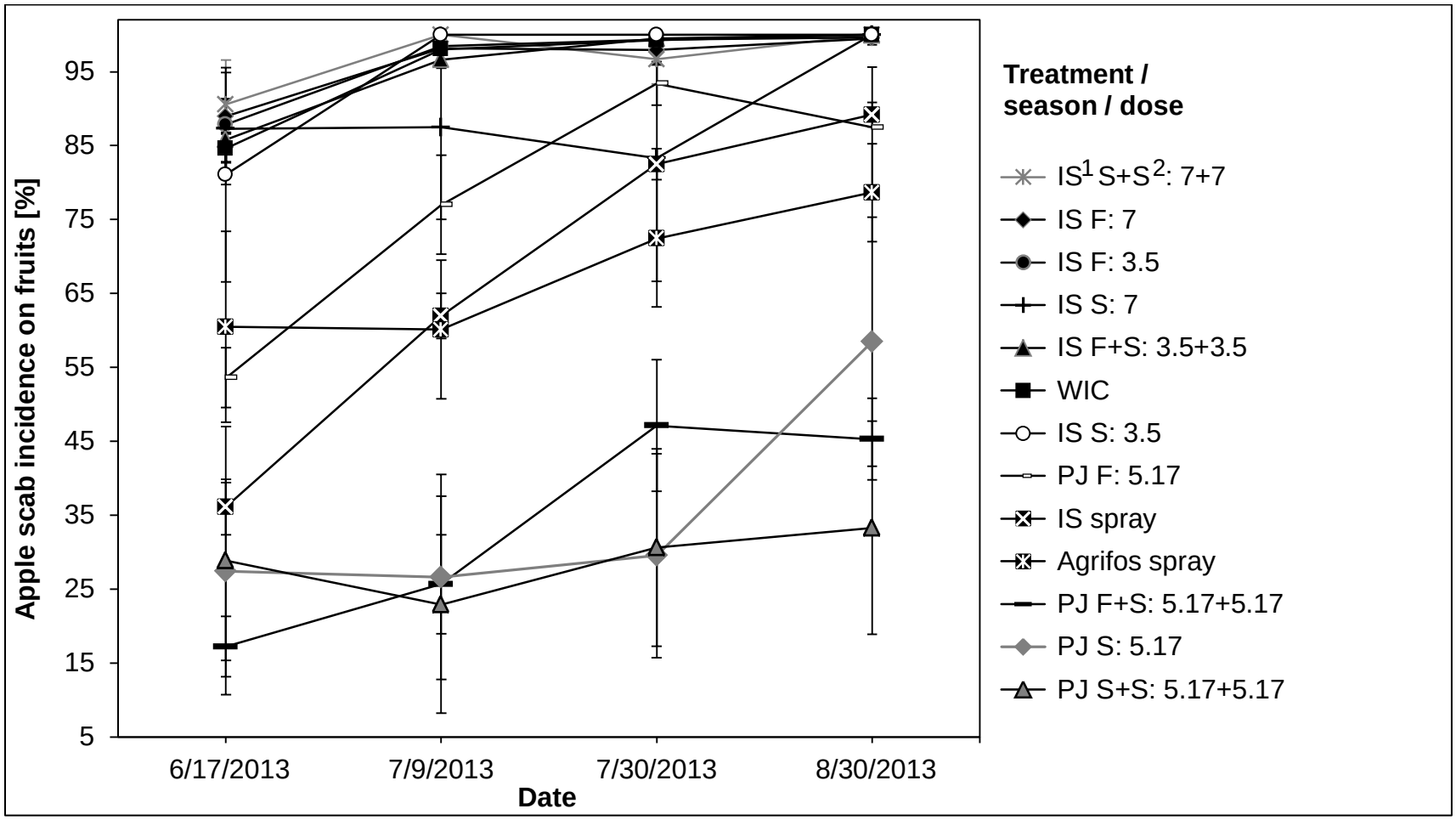

Figure 36. Apple scab control on fruits in 2013 after trunk injection of 'Mac Spur' trees with difenoconazole + cyprodinil ([1]IS) and potassium salts of phosphorous acid (PJ) at different dates in 2012 and 2013. WIC - water injected untreated control. IS - doses in ml/tree, PJ - doses in ml/25.4 mm DFH. [2]S - spring 2013 injection(s), F - fall 2012 injection. Error bars represent standard error of the mean (SEM).

Table 29. Fungicide residues in apple canopy during 2013 after trunk injection of 'Mac Spur' trees with difenoconazole + cyprodinil ([1]IS) at different dates in 2012 and 2013 (*t*-tests, α=0.05). Spring/Fall - seasons of injection in 2012 and 2013, respectively, followed by doses of IS in ml. [2]The treatment was not applied before these dates. [3]Treatment means within one date or column followed by different upper-case letters are significantly different. [4]Means within one treatment across the six or eight dates followed by different lower-case letters are significantly different.

Treatment/ Season/ Dose per tree [ml]	Mean cyprodinil concentration [ppm]							
	Buds and leaves							
	25 October 2012	21 April 2013	1 May 2013	15 May 2013	29 May 2013	12 June 2013	26 June 2013	10 July 2013
IS[1] Spray cyprodinil	-[2]	-	0.33800 Ab	0.39472 Aa	0.14815 Ac	0.14490 Ac	0.13546 Ac	0.13239 Ac
IS Fall 7	0.00044 A[3]d[4]	0.00023 Ad	0.00318 Bd	0.08041 Bb	0.14077 Aa	0.13351 Aa	0.05276 Bb	0.02183 Bc
IS Spring 7	-	-	0.00269 Bb	0.00827 Db	0.05711 Ca	0.04632 Ca	0.01156 Db	0.00698 Cb
IS Spring+Spring 7+7	-	-	0.00201 Bd	0.02657 Cc	0.04266 Cab	0.04319 Cac	0.01084 Dd	0.00679 Cd
IS Spring 3.5	-	-	0.00188 Bc	0.02117 CDb	0.04287 Ca	0.04104 Ca	0.00817 Dc	0.00652 Cc
IS Fall+Spring 3.5+3.5	0.00120 Ad	0.00023 Ad	0.00187 Bd	0.03508 Cc	0.14301 Aa	0.13101 ABa	0.06236 Bb	0.02387 Bc
IS Fall 3.5	0.00014 Ae	0.00020 Ae	0.00167 Bde	0.02110 CDbc	0.09424 Ba	0.10218 Ba	0.03003 Cb	0.01378 BCcd

Treatment/ Season/ Dose per tree [ml]	Mean difenoconazole concentration [ppm]							
	Buds and leaves							
	25 October 2012	21 April 2013	1 May 2013	15 May 2013	29 May 2013	12 June 2013	26 June 2013	10 July 2013
IS[1] Spring 7	-[2]	-	0.00200 A[3]c[4]	0.00238 Cc	0.01234 Da	0.00460 Db	0.00042 Dd	0.00018 Ce
IS Spring 3.5	-	-	0.00142 ABc	0.00573 Bab	0.00882 Da	0.00470 Db	0.00055 Dc	0.00020 Cd
IS Fall 3.5	0.00000[3]	0.00000	0.00105 ABc	0.00004 Ed	0.02194 Ca	0.01907 Ca	0.00552 Cb	0.00225 Bc
IS Fall+Spring 3.5+3.5	0.00000	0.00000	0.00103 Bd	0.00009 DEe	0.04748 Ba	0.05836 Ba	0.01323 Bb	0.00442 Bc
IS Spray difenoconaz.	-	-	0.00099 ABd	2.47867 Aa	0.46944 Ab	0.46192 Ab	0.41346 Abc	0.26265 Ac
IS Fall 7	0.00000	0.00000	0.00097 Bd	0.00041 Dd	0.03732 BCa	0.03580 Ca	0.01121 Bb	0.00315 Bc
IS Spring+Spring 7+7	-	-	0.00081 ABc	0.00818 Bab	0.01115 Da	0.00395 Db	0.00074 Dc	0.00034 Cd

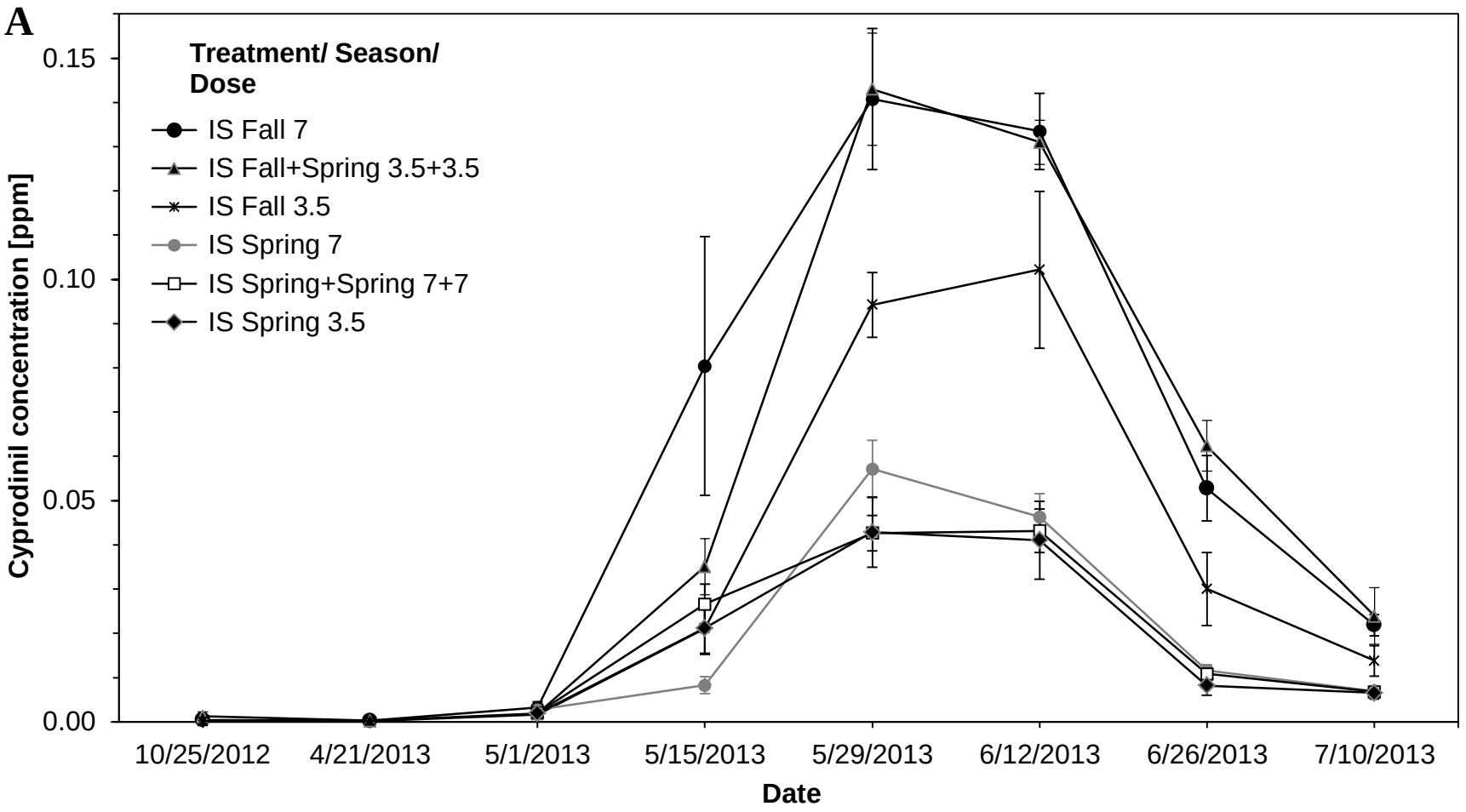

Figure 37. Fungicide residues of cyprodinil (A) and difenoconazole (B) in buds and leaves in 2013 after trunk injection of 'Mac Spur' apple trees with difenoconazole + cyprodinil ([1]IS) at different dates in 2012 and 2013. IS - Inspire super, Fall / Spring - seasons of injection in 2012 and 2013, respectively, followed by doses of Inspire super in ml. Error bars represent standard error of the mean (SEM).

Figure 37 (cont'd)

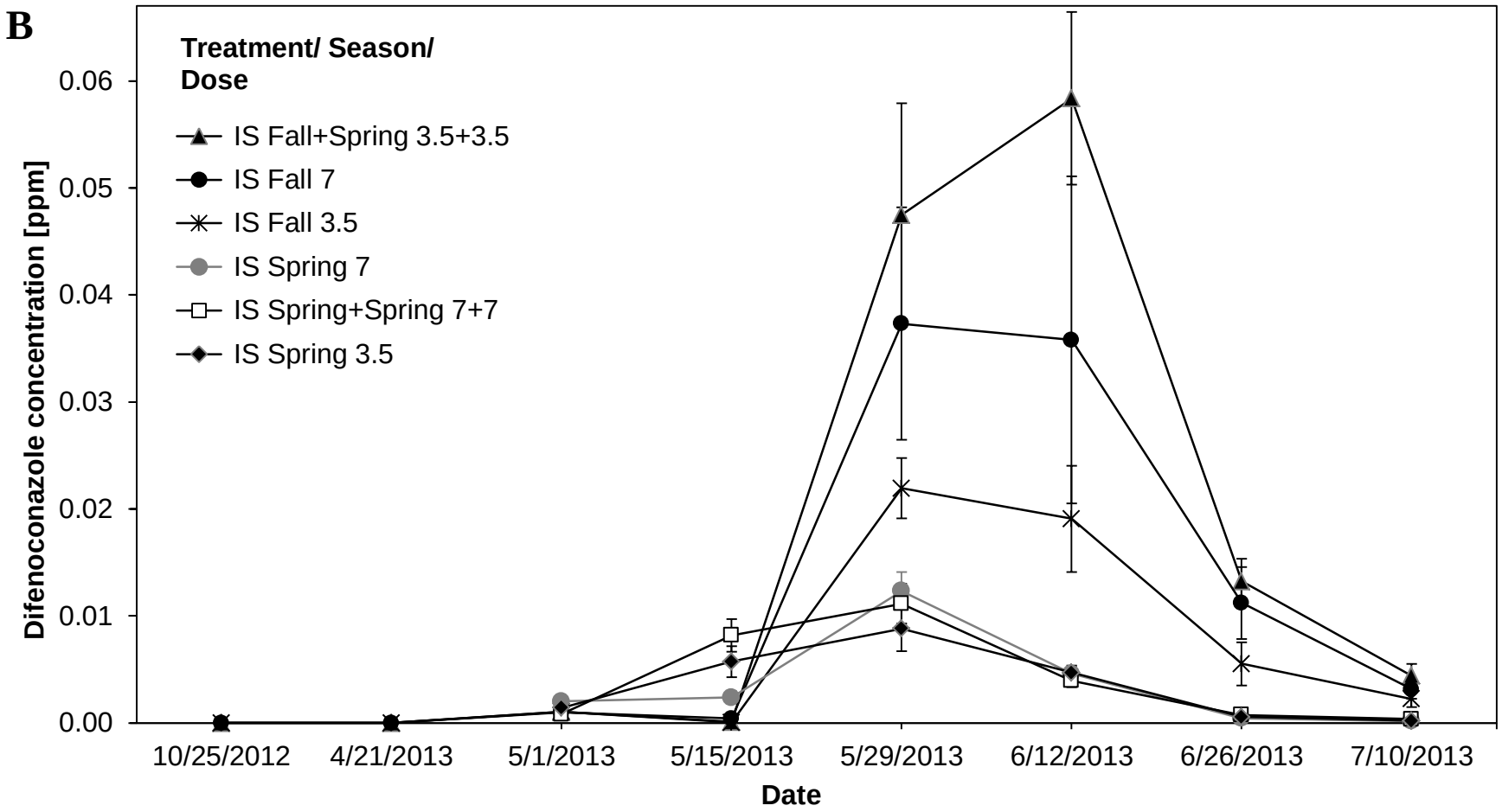

Table 30. Fungicide residues in apple fruit during 2013 after trunk injection of 'Mac Spur' trees with difenoconazole + cyprodinil ([1]IS) at different dates in 2012 and 2013 (*t*-tests, α=0.05). Spring/Fall - seasons of injection in 2012 and 2013, respectively, followed by doses of Inspire super in ml. [2]Treatment means within one date or column followed by different upper-case letters are significantly different. [3]Means within one treatment across the four dates followed by different lower-case letters are significantly different.

Treatment/ Season/ Dose per tree [ml]	Mean cyprodinil concentration [ppm] in 2013			
	Fruits			
	31 May	18 June	10 July	30 August
IS[1] Spray cyprodinil	0.16053 A[2] a[3]	0.04608 Ab	0.00583 Ac	0.00029 Ad
IS Fall 7	0.06896 Ba	0.00198 Bb	0.00087 Bb	0.00002 Bc
IS Fall+Spring 3.5+3.5	0.07109 Ba	0.00164 Bb	0.00079 Bb	0.00003 Bc
IS Fall 3.5	0.04549 Ba	0.00215 Bb	0.00046 Bc	0.00001 Bc
IS Spring 7	0.00803 Ca	0.00183 Bab	0.00096 Bbc	0.00000 Bc
IS Spring+Spring 7+7	0.01508 Ca	0.00258 Bb	0.00053 Bc	0.00001 Bc
IS Spring 3.5	0.01241 Ca	0.00309 Bb	0.00063 Bc	0.00002 Bc

Treatment/ Season/ Dose per tree [ml]	Mean difenoconazole concentration [ppm] in 2013			
	Fruits			
	31 May	18 June	10 July	30 August
IS[1] Spray difenoconazole	0.17642 A[2] a[3]	0.02605 Ab	0.00178 Ac	0.00001[4]
IS Fall 7	0.01878 Ba	0.00106 Bb	0.00034 Bc	0.00000
IS Fall+Spring 3.5+3.5	0.01954 Ba	0.00052 Bb	0.00017 BCc	0.00000
IS Fall 3.5	0.01318 Ba	0.00056 Bb	0.00006 CDc	0.00000
IS Spring 7	0.00169 BCa	0.00064 Ba	0.00086 ABa	0.00000
IS Spring+Spring 7+7	0.00312 Ca	0.00203 Ba	0.00006 Db	0.00000
IS Spring 3.5	0.00271 Ca	0.00171 Ba	0.00015 BCb	0.00000

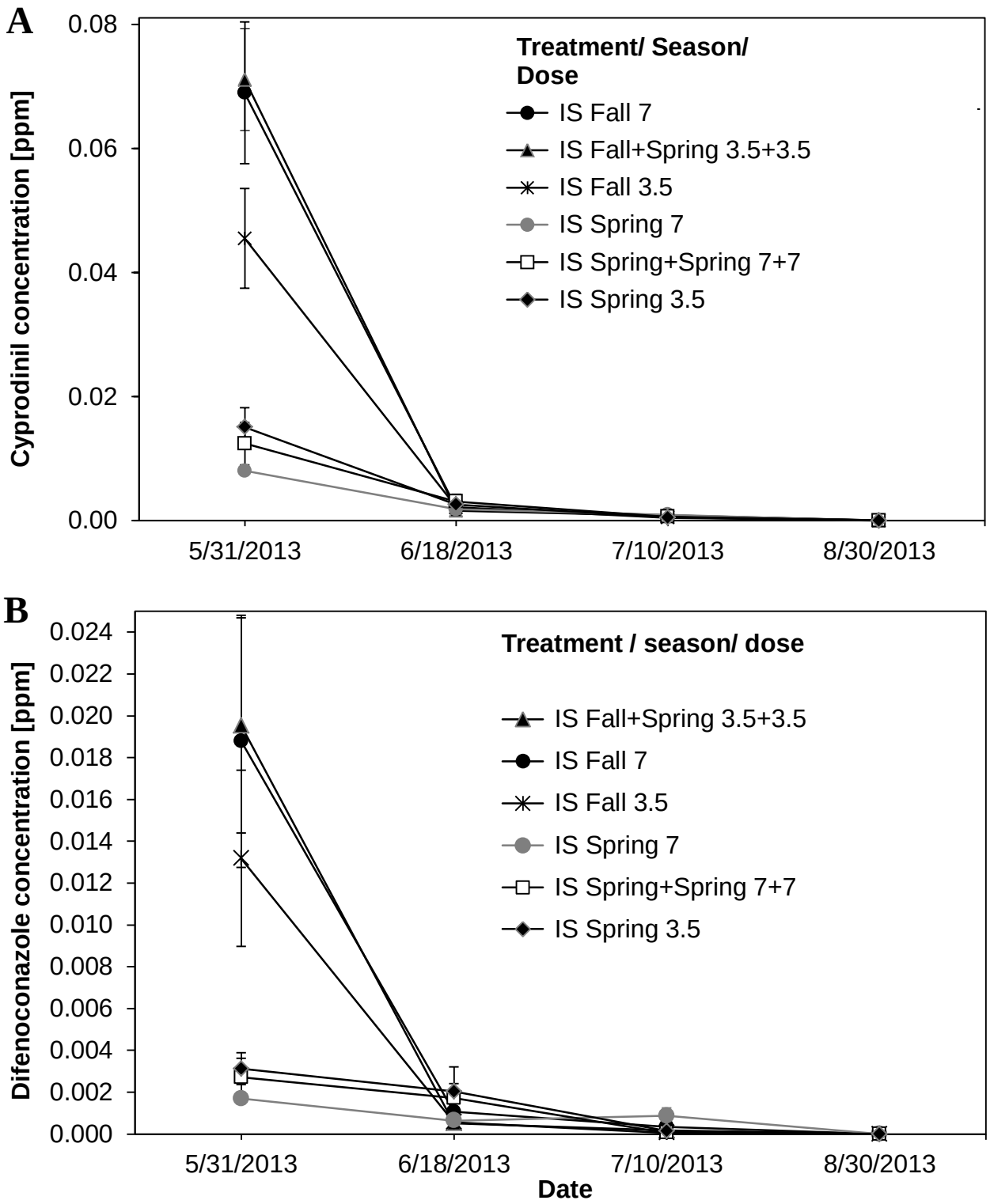

Figure 38. Fungicide residues of cyprodinil (A) and difenoconazole (B) in apple fruit during 2013 after trunk injection of 'Mac Spur' trees with difenoconazole + cyprodinil ([1]IS) at different dates in 2012 and 2013. IS - Inspire super, Fall / Spring - seasons of injection in 2012 and 2013, respectively, followed by doses of Inspire super in ml. Error bars represent standard error of the mean (SEM).

Table 31. Fungicide residues in the apple canopy during 2013 after trunk injection of 'Mac Spur' trees with potassium salts of phosphorous acid ([1]PJ) at different dates in 2012 and 2013 (*t*-tests, α=0.05). Spring/Fall - seasons of injection in 2012 and 2013, respectively, followed by doses of Phosphohet in ml/25.4 mm of trunk DFH. F - fall, S - spring. WIC - water injected untreated control. [2]Treatment means within one date or column followed by different upper-case letters are significantly different. [3]Means within one treatment across the six or eight dates followed by different lower-case letters are significantly different. [4]The treatment was not applied before these dates.

Treatment/ Season/ Dose per tree [ml]	Mean phosphorous acid concentration [ppm]							
	Buds and leaves							
	25 October 2012	21 April 2013	1 May 2013	15 May 2013	29 May 2013	12 June 2013	28 June 2013	10 July 2013
PJ[1] F+S 5.17+5.17	0.00000 A[2]d[3]	0.00014 Ad	0.25153 Bb	0.00000 Ad	0.00718 Acd	3.37272 Aa	0.29556 Ab	0.02112 Ac
PJ Fall 5.17	0.00000 Ad	0.00000 Bd	1.81648 Aa	0.00891 Abc	0.04698 Ab	2.37399 ABa	0.04922 Bb	0.00000 Bcd
WIC	0.00000 Ab	0.00000 Bb	0.00000 Db	0.00844 Ab	0.00000 Ab	0.12665 Ca	0.00000 Cb	0.00000 Bb
PJ Spring 5.17	-[4]	-	0.03413 Ccde	0.15029 Acde	0.01116 Ae	1.92137 ABa	0.37940 Ab	0.02340 Ad
PJ S+S 5.17+5.17	-	-	0.00668 Dc	0.00449 Ac	0.24699 Abc	1.75412 ABa	0.05275 Bb	0.00936 ABc
Agrifos Spray	-	-	0.00000 Dd	0.00000 Acd	0.21221 Abc	1.68112 Ba	0.00000 Ccd	0.06081 Ab

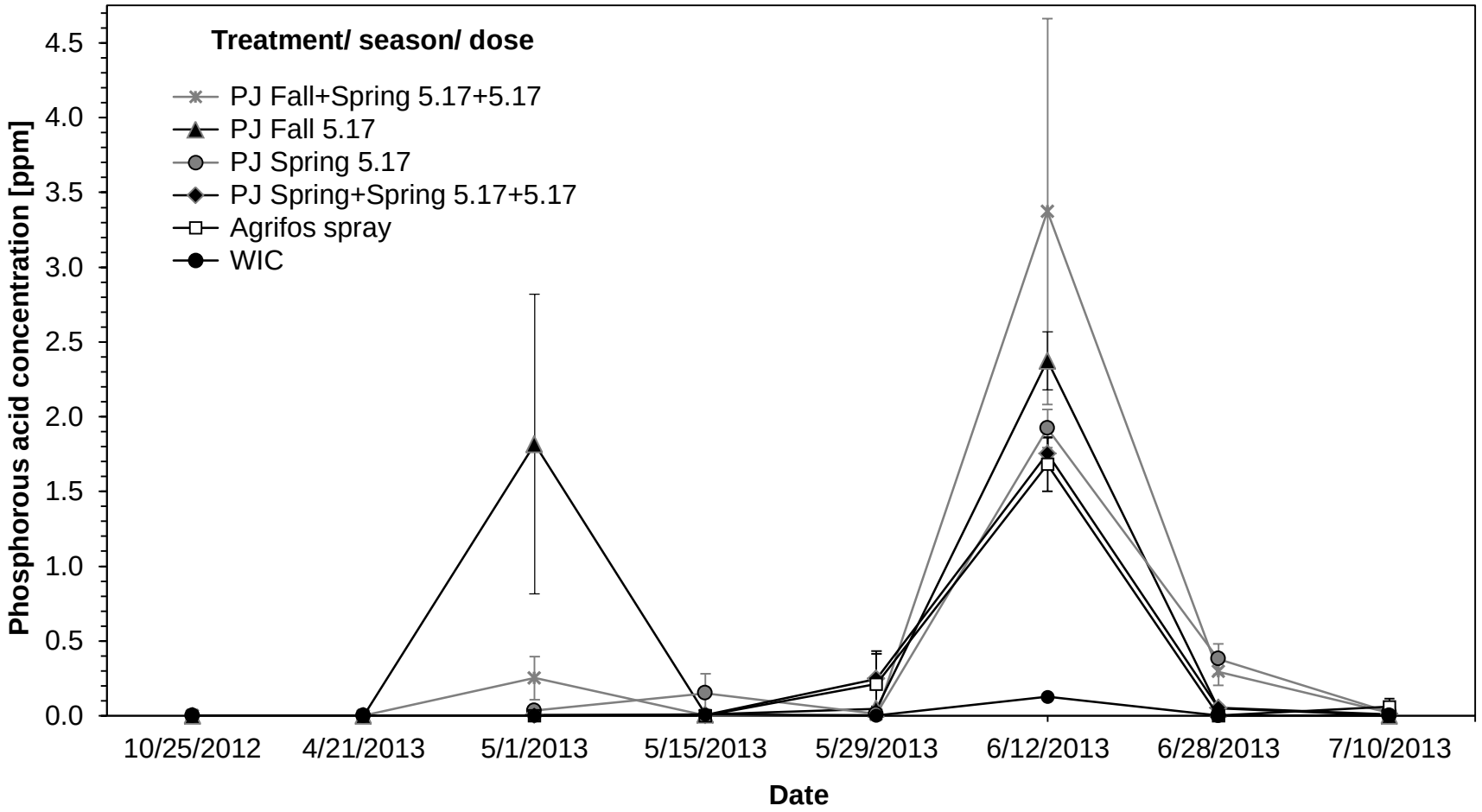

Figure 39. Fungicide residues in the apple buds and leaves during 2013 after trunk injection of 'Mac Spur' trees with potassium salts of phosphorous acid (PJ) at different dates in 2012 and 2013. Fall / Spring - seasons of injection in 2012 and 2013, respectively, followed by doses of Phosphohet in ml/25.4 mm of trunk DFH. WIC - water injected untreated control. Error bars represent standard error of the mean (SEM).

Table 32. Fungicide residues in apple fruit during 2013 after trunk injection of 'Mac Spur' trees with potassium salts of phosphorous acid ([1]PJ) at different dates in 2012 and 2013 (*t*-tests, α=0.05). Fall / Spring - seasons of injection in 2012 and 2013, respectively, followed by doses of Phosphohet in ml/25.4 mm of trunk DFH. WIC - water injected untreated control. [2]Treatment means within one date or column followed by different upper-case letters are significantly different. [3]Means within one treatment across the six or eight dates followed by different lower-case letters are significantly different.

Treatment/ Season/ Dose per tree [ml]	Mean phosphorous acid concentration [ppm]			
	Fruits			
	31 May 2013	18 June 2013	10 July 2013	30 August 2013
Agrifos Spray	1.18417 A[2] b[3]	0.26019 ABa	0.41229 Ba	2.79116 Ab
PJ[1] Spring+Spring 5.17+5.17	0.51131 ABab	0.00189 Cb	0.49882 ABa	2.48893 Aab
PJ Spring 5.17	0.49152 Bb	0.22111 ABCb	2.29607 Aa	1.05672 Ab
PJ Fall 5.17	0.32579 Bbc	0.42307 Aab	0.92019 ABa	0.00702 Ac
PJ Fall+Spring 5.17+5.17	0.01249 Bbc	0.34239 Aab	2.04503 ABa	0.00000 Ac
WIC	0.00000 Ba	0.03792 BCa	0.00000 Ca	0.00850 Aa

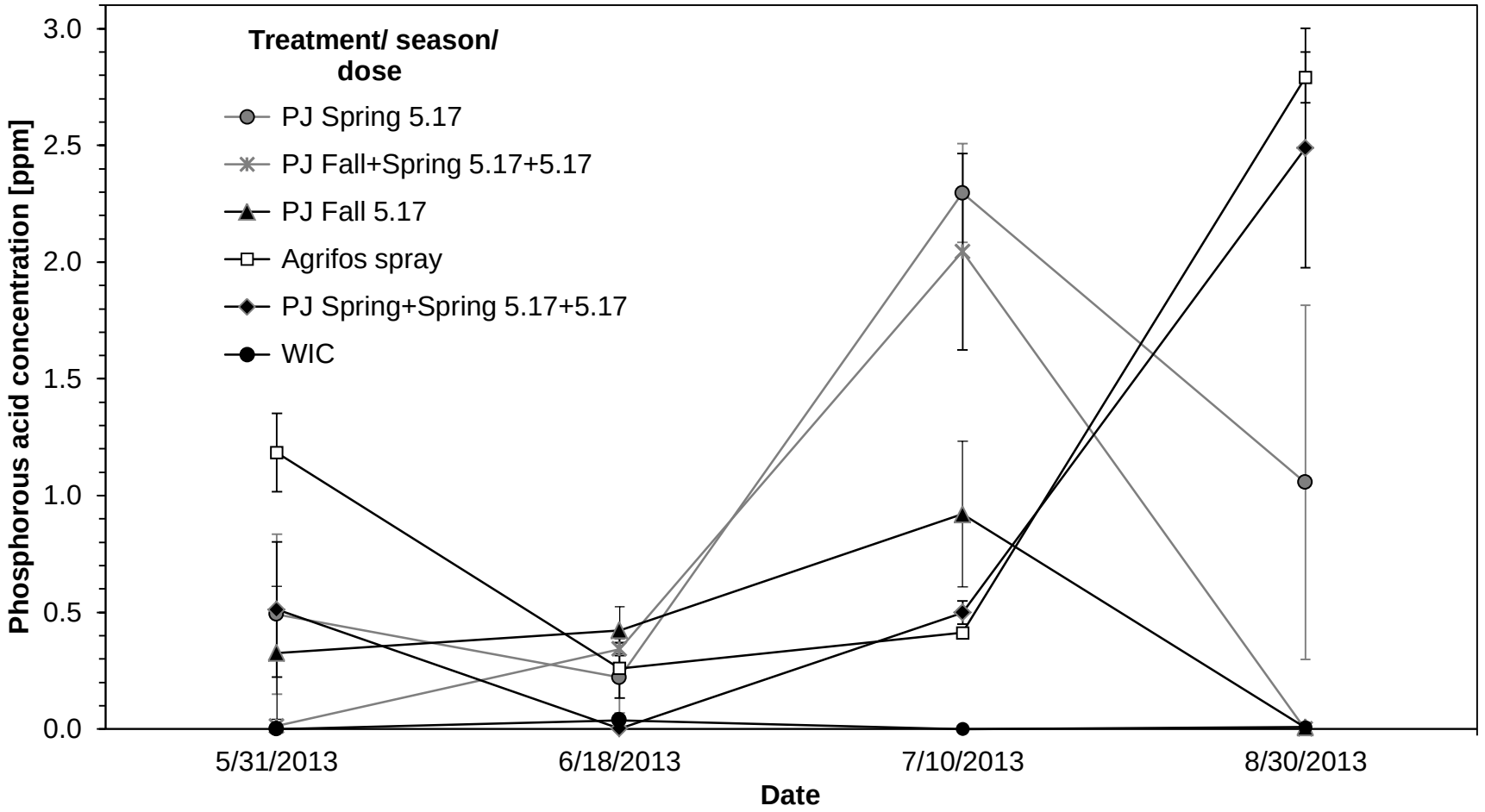

Figure 40. Fungicide residues in apple fruit during 2013 after trunk injection of 'Mac Spur' trees with potassium salts of phosphorous acid (PJ) at different dates in 2012 and 2013. Fall / Spring - seasons of injection in 2012 and 2013, respectively, followed by doses of Phosphohet in ml/25.4 mm of trunk DFH. WIC - water injected untreated control. Error bars represent standard error of the mean (SEM).

APPENDIX 3. Media, buffers and primers

Microbiology media in *Erwinia amylovora* culturing

Liquid LB medium:

In less than 1 L of distilled H_2O add:

10 g tryptone
5 g yeast extract
10 g NaCl

Adjust pH to 7
Add remaining distilled H_2O volume up to 1L
Sterilize by autoclaving

Buffers for *Erwinia amylovora* suspension preparation as inoculum

0.5X PBS:

In less than 1 L of distilled H_2O add:

4 g of NaCl
0.1 g of KCl
0.72 g of Na_2HPO_4
0.12 g of KH_2PO_4

Adjust pH to 7.4
Add remaining distilled H_2O volume up to 1L
Sterilize by autoclaving

Primers for putative apple PR-protein genes*

aj708-aj709 for PR-1
aj778-aj779 for PR-2
aj780-aj781 for PR-8
aj748-aj749 for actin

*According to: Maxson-Stein et al., 2002. Effect of treating apple trees with acibenzolar-S-methyl on fire blight and expression of pathogenesis-related protein genes. *Plant Disease*, 86(7), pp. 785-790.

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