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## Chapter

# Choosing an Adequate Pesticide Delivery System for Managing Pathogens with Difficult Biologies: Case Studies on *Diplodia corticola*, *Venturia inaequalis* and *Erwinia amylovora*

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## Abstract

With the challenges that negatively impact tree-based agriculture, landscapes and forests, such as climate change, plant pathogen and insect range expansion, invasive species and limited new pesticides, it is important to introduce new and effective tree protection options. In the last 20 years, pathogens that invade wood i.e. vascular tissues of trees causing wilt, yellowing, premature defoliation, cankers and tree death, have been on the rise. *Diplodia corticola* causes Bot canker of oak species which can kill trees diminishing the valuable ecological services they provide and reducing profits from wood and cork production. Since this and similar pathogens have difficult biologies because they reside in wood and cause severe internal damage and tree death, their management is difficult or inefficient with classical pesticide application methods that cannot reach and distribute the active ingredient in vascular wood tissues. As practical management options for this and other vascular tissue pathogens of trees are limited, we evaluated efficacy of several trunk injected fungicides in control of *D. corticola* and compared it with the efficacy of trunk injection of similar compounds for control of *Venturia inaequalis* and *Erwinia amylovora*, as two well-studied apple tree pathogens with different or partially similar lifestyles to *D. corticola*, respectively.

**Keywords:** trunk injection of pesticides, tree disease management, *Diplodia corticola*, *Venturia inaequalis*, *Erwinia amylovora*

## 1. Introduction

Agricultural, urban, and natural tree stands have been the focus of extensive plant pathogen diagnostic and disease management research in recent decades [1–16]

which recorded an increase in the number of new fungal and bacterial pathogens and their detrimental impact on agroecosystems, ecosystems, and the human society. The economic effects of these pathogens are reflected in lost fresh fruit produce [17–19], reduced yields and quality of fruit or wood and cork products [20, 21], diminished ecological tree services, and death of whole trees, stands, and forest regions or decimation of fruit industries [19, 22].

If left unmanaged, apple scab fungus *Venturia inaequalis*, a subcuticular leaf and fruit pathogen can cause 70–100% reduction in marketable fruit yield in each year [23, 24]. A pathogenic fungus *Diplodia corticola*, the causal agent of Bot canker of oak which invades tree xylem is the most widely distributed and virulent fungal pathogen causing canker and decline of cork oak (*Quercus suber*) forests in Europe [25, 26] and of southern live oak (*Q. virginiana*), coast live oak (*Q. agrifolia*), and canyon live oak (*Q. chrysolepis*) in the United States [3, 27]. Recently, there has been a rising incidence of this pathogen in the United States on northern red oak, *Q. rubra* [5, 8], black oak, *Q. velutina* [7], white oak, *Q. alba* [28], and bur oak (*Q. macrocarpa*) [29]. Other Botryosphaeriaceae, like *Neofusicoccum australe*, *N. luteum*, *N. parvum* and *N. mediterraneum* infect woody and green plant tissues and are destructive canker pathogens of avocado [1], grapevine, olive, pistachio and ash [30–33] and are recently reported as very virulent pathogens of coast redwood leading to severe decline in urban stands of California [9]. The Blue stain fungi *Grosmannia clavigera* and *Leptographium longiclavatum* are the plant pathogenic insect symbionts vectored by the mountain pine beetle *Dendroctonus ponderosae* [13]. They infect xylem and have led to death of millions of pine trees from 1990 to 2013 and decimation of pine forests in western North America [22, 34, 35]. Under favorable weather conditions during apple bloom and shoot growth, a destructive fire blight bacterium *Erwinia amylovora* inhabits and infects apple flowers and shoots and after spreading through xylem and causing cankers on wood it can kill whole trees. The resulting losses can range from \$3.8 to 100 million due to removal of as much as 450,000 apple trees in only one or two years [17, 18, 36, 37]. Fire blight severely reduced both pear and quince production primarily in continental climate regions of the world. Asiatic citrus canker caused by *Xanthomonas axonopodis* pv. *citri* invades leaf mesophyll tissue and is estimated to cause yield losses and cost of disease management of \$342 million per year [38]. The citrus greening or Huanglongbing disease is caused by a bacterium *Candidatus Liberibacter asiaticus* which proliferates and is limited to phloem vascular tissue of citrus trees. This pathogen led to \$4.5 billion negative economic impact in just 5 years after introduction in the United States [19]. These devastating internal tree pathogens further create a barrier in international trade of fresh fruit and wood products in an attempt to prevent their introduction to new regions [39, 40].

The biology of majority of these microorganisms, excluding *V. inaequalis* and *X. axonopodis* pv. *citri*, has three key shared traits: (1) they invade, reside, and spread in xylem or phloem of woody host tissues, where a significant part of pathogenesis and internal host damage is taking place, and (2) due to specific lifestyle depicted in impacting the internal wood tissues, these pathogens successfully evade exposure to the contact, local-systemic, and green-tissue systemic pesticides applied to plant surfaces, and (3) their management is extremely difficult or inefficient with the classical pesticide delivery methods. Therefore, any pesticide active ingredient in a formulated form needs to distribute in these vascular tissues to reach pathogen propagules at an effective concentration and move systemically to all the uninfected or infected tissue parts to achieve an efficient preventive or curative control, respectively.

Tree injection, often referred to as trunk or stem injection, is a method of target precise delivery or application of pesticides, plant resistance activators and fertilizers into the xylem vascular tissue of a tree with the aim to protect trees from insect pests

and pathogens or to provide tree nutrition and/or correction of micronutrient deficiencies. It primarily harnesses the transport capacity of the tree's vascular system driven by transpiration stream of water in these tissues to translocate and distribute the active compounds into the trunk, branches, canopy and roots where protection or nutrition is needed. Tree injection as a plant protection method is viewed as environmentally safer alternative for pesticide application because it secures significant reduction of non-target exposure of water, soil, air and wildlife to pesticides and fertilizers in landscapes and urban greening areas. The active ingredients are delivered within the tree, thus providing selective exposure to plant pests, with limited negative effect of weather conditions like rain or sun radiation on the injected compound and with creating no immediate pesticide residue losses outside the tree.

Trunk injection relies significantly on tree physiology processes related to water transport, xylem and phloem tissue functions, and the weather conditions that influence these specific plant processes. To achieve delivery of an effective pesticide dose, its distribution and expected management of plant detrimental organism or nutrient deficiency, there are several key factors which should be monitored by an applicator as they influence success of trunk injection for these purposes. Besides the plant pathogen biology, ecology, and epidemiology, several factors play a key role in success of trunk injection efficacy: the time of application in relation to detrimental organism establishment and symptom occurrence [11], the season and time of the day of application [41], the chemical properties of pesticide active ingredient and its formulation [42], the injected volume or dose of a pesticide, and the type of tree injection device or technology. For example, a more effective management of plant disease or insect infestation can be achieved by the preventive injections of pesticides in comparison to the therapeutic pesticide applications after the disease or infestation has already occurred. Tree injection of active compounds is much faster and easier during spring and early summer months in comparison to the late or mid-summer, late fall and winter, because water in the soil is abundant and the green leaf canopy is facilitating intensive transpiration pull and flow of water through the xylem tissue in hardwood trees, starting from the roots and branches to the leaves [41]. The three key properties of injected active ingredient that determine its mobility or binding in xylem of the tree are organic carbon-water partitioning coefficient (ml/g or  $\mu\text{g/g}$ ) or carbon adsorption coefficient ( $K_{o/c}$ ), water solubility, and formulation type.  $K_{o/c}$  expresses the level of adhesion or adsorption of pesticide active ingredient to the carbon rich compounds in certain environments such as soil or xylem and is defined as a ratio of 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 a solution. Active ingredients that have high  $K_{o/c}$  values will strongly bind to the organic compounds present in soil, sap or xylem and reduce their systemic movement i.e. translocation, accumulation and distribution in the canopy, thus reducing the efficacy in pathogen or pest control. In contrast, the ingredients with low or moderate  $K_{o/c}$  values move and distribute fast after tree injection and distribute well in the canopy, securing good pest or pathogen control. Pesticide formulation is a form of a pesticide active ingredient ready for use or which quite often requires dilution in water prior to application. Formulation is made by adding different inactive ingredients with the aim to improve the properties of an active ingredient such as solubility, surface adhesion, distribution, effectiveness, shelf life, stability, handling or application (e.g. solvents, emulsifiers, surfactants and other adjuvants). Formulation of a pesticide or a fertilizer determines the properties and residue stability of an active ingredient and can modulate its mobility in xylem of phloem after tree injection for pest or plant management [12, 42]. Finally, trunk injection devices can loosely be divided into the ones using drill- or needle-based technology [43]. In case of the



first one, access to xylem for pesticide delivery device is enabled by drilling into the trunk or root flare wood, removing a small part of the wood, and sealing of the opened injection port with an inserted plastic plug or not (plug contains an injection valve with a one-way silicone septum) [44]. For the injection application to be faster and hence economical in urban tree care, this system uses compressed air or hydraulic pressure to force-inject the pesticide solution into the wood. The second technology uses a knife-like or a flat, screwdriver-like needle with a lenticular profile, which is inserted into the wood by a hammer thus separating the wood fibers and creating a crevice while the delivery of a pesticide solution is conducted through the needle and infused into the wood [45]. This system can use force of hydraulic or compressed air pressure to deliver the pesticide solution into the xylem or is solely relying on the Venturi effect (vacuum) created by a transpiration stream in xylem to infuse the pesticide solution into the wood [45–47]. This injection technology requires longer time for injection solution delivery, especially when transpiration is limited, and thus is often less economical in urban tree care.

Tree injection was initially developed for pesticide and fertilizer application on large size trees in proximity of urban areas where ground- and air-spray applications are impractical due to substantial pesticide losses through drift, lack of proper canopy coverage, or are prohibited due to possible human and domestic animal exposure. The second driver for development of tree injection and its more frequent use in recent decades has been the destructive nature and an increasing need for effective management options for invasive tree pathogens like *Ophiostoma* fungi that cause Dutch elm disease, *Bretziella fagacearum* fungus that causes oak wilt, and insects pests like Emerald ash borer, *Agilus planipennis* and Hemlock Woolly Adelgid, *Adelges tsugae*. Because of the unique biology of these organisms which leads to severe internal damage of wood and ultimately causes tree death, their management is extremely difficult or inefficient with classical pesticide application methods like topical spraying. Therefore, the goal of trunk injection to deliver the plant protective materials into the xylem or phloem vascular tissues of trees matches the specific pathogen or pest biologies and pesticide exposure requirements for the most effective management of these detrimental organisms.

Due to a demonstrated ability of single trunk injection to increase the efficacy of injected pesticides over multiple years, a possibility to reduce the of number of topical spray applications [10, 12, 48] and a rising incidence of woody plant pathogens and insect pests in the environment [31, 33, 49–51], this approach has recently been investigated in agriculture where topical pesticide applications for plant food production is intensive. The most investigated tree fruit crops and their pathogens and insect pests are citrus (e.g. *Candidatus Liberibacter asiaticus*) [14, 15], avocado (e.g. avocado thrips, *Scirtothrips perseae*; *Phytophthora* root rot, *Phytophthora cinnamomi*) [49, 50], apple (e.g. fire blight, *Erwinia amylovora*; apple scab, *V. inaequalis* [11, 12]; oblique banded leaf roller, *Choristoneura rosaceana* [10, 52]), and grapevine (e.g. grapevine downy mildew, *Plasmopara viticola* [53]; powdery mildew, *Uncinula necator* [54]). Domesticated apple, *Malus pumila* is an important research model in continental climate because management of *V. inaequalis* in humid regions requires intensive spray programs with as many as 15–22 spray applications of fungicides in one growing season [55]. Since this research is novel for tree disease management in contemporary agriculture, the proof of concept experiments on cultivated trees are conducted by using the trunk injection devices primarily designed for delivering pesticides and fertilizers for tree protection purposes in landscapes and urban forestry [10, 44, 52, 56]. Besides the smaller tree sizes in orchards in comparison to the urban landscapes as an obvious advantage driving the investigation of efficacy of pesticides delivered with this method [52, 57, 58], some of the key researched topics are efficacy and its lasting [10, 11], application

timing optimization for season- and two-seasons-long control [12, 50, 59], trunk wounding by injection ports i.e. points [43], pesticide residue accumulation in fruit, nectar, and leaves [12, 59, 60] and their spatial and temporal distribution in the tree [61]. Even though tree injection originated from widely present needs for pest and disease control and plant nutrition in urban forestry, it holds an important potential for use in tree fruit agriculture where in the last 30 or so years there has been an increase of public pressure on apple producers to reduce pesticide use, while maintaining a high level of fruit quality [62]. Since the tree injection of pesticides and nutrients as a delivery approach is currently gaining more popularity in urban greening, landscapes and forestry management [13, 48, 63–65], we predict intensification of research for insect pest and pathogen control in tree-based agriculture and silviculture in the near future.

While trunk injection for pesticide delivery is a relatively new technology investigated in tree-based agriculture for managing diseases like citrus greening [14–16, 66–68] or fire blight [11, 44], research in agricultural engineering will first need to design or invent an application system/s that allow scalability, i.e. achieving simultaneous trunk injection of large number of trees in a short period of time. Besides this end goal many other key questions arising from research outlined above will need to be addressed through experimental work before tree injection is used in agriculture, even in limited fashion. The first steps are providing enough evidence i.e. providing proof of concept that injected pesticides are effective in tree pathogen and insect management and that injected materials have minimal negative effect on fresh fruit consumer and beneficial orchard fauna. Because effective management options for Bot canker and decline of different *Quercus* species caused by *D. corticola* and other aforementioned plant pathogens of vascular tissues are limited or lacking, we evaluated the efficacy of trunk injected fungicides for *D. corticola* control and compared it to the efficacy of similar active ingredients for management of *V. inaequalis* and *E. amylovora* which are more intensively studied models in continental climate. Our goal was to present new disease management data that argues in favor of a hypothesis that for plant pathogens with difficult biologies, i.e. for those that impact internal wood tissues, it is necessary to select appropriate pesticide delivery system/s such as trunk injection to achieve the maximum disease control through increasing pathogen exposure and thus efficiency of applied pesticides. We present efficacy data of trunk-injected pesticides in management of these three woody plant pathogens with different or partially similar lifestyles to elucidate and promote the translation of tree injection as a target precise delivery system for plant protection in agriculture and silviculture of the future.

## **2. Trunk injection delivery of pesticides for management of three important plant pathogens in continental climate**

### **2.1 Biology of *Diplodia corticola*, *Venturia inaequalis* and *Erwinia amylovora***

#### *2.1.1 Diplodia corticola*

In the binomial nomenclature of fungi, Bot canker pathogen *D. corticola* is a commonly found asexual stage of an ascomycete *Botryosphaeria corticola*, a sexual stage of this fungus [69]. Asexual stage forms white to dark olive-green aerial mycelium with a dark green to black underside [5]. During 24 weeks after the host plant infection, the fungus forms dark brown to black, circular or flask-shaped fruiting bodies called pycnidia, that are up to 1 mm in diameter and emerge through the dead bark of oak. Pycnidia can form on all above ground tree parts and are

visible on bark as black masses of fungal tissue called stromata. Pycnidia have multiple chambers or locules in them, each 200–300 µm in diameter, in which spores called conidia are produced for around 2 years and serve as source of inoculum for new infections [69]. When pycnidia are mature they are partially erumpent through the host bark and form a circular opening on the top called ostiole serving for conidia release. Conidia are oval-shaped to cylindrical, straight, with both ends rounded, usually translucent and single-celled, but with aging rarely become brown and with multiple cells [69]. Inside, conidia are usually without an oil vesicle called guttule but can form one in the center. The sexual stage *B. corticola* forms spores called ascospores. Eight ascospores arranged in two rows form in one sac called ascus and multiple asci are formed in the dark brown to black fruiting bodies called pseudothecia [69]. Pseudothecia are up to 1 mm in diameter, circular and partially erumpent on the host bark when mature. Each pseudothecium has multiple chambers or locules 200–300 µm in diameter [69]. For release of ascospores, pseudothecia form a circular opening on the top called ostiole. Ascospores are spindle-shaped to rhomboid and translucent, one-celled, or rarely becoming light brown and with age two- or three-celled [69].

Because *D. corticola* has been described only recently [69], its life cycle describing how this pathogen reaches and colonizes oak species as a primary host is not fully known. Based on the other pathogenic and opportunistic pathogen members of the family Botryosphaeriaceae, *D. corticola* can probably infect through wounds and maybe plant natural openings. Once infection is established it spreads within the host via xylem to large distances with xylem sections exhibiting black streaking and cankers can form intermittently on the bark of trunk or branches [5, 8, 28, 29]. Some trees with xylem necrosis do not always exhibit visible external cankers [7]. With time, crown sections of infected trees show wilting and eventually host can die [5, 8]. It is assumed that conidia and ascospore production on cankers and germination are favored by moisture and high relative air humidity. Recently, in a study analyzing aerial fungal spore samples collected by the air spore traps and passive rain collectors, *D. corticola* was detected in 16 of the 32 sampled locations in Canada by a highly specific molecular detection method which targets a pathogen-specific DNA region [70]. This data indicates that *D. corticola* has aerial disseminated spores. It is reported that conidia of *D. corticola* are dispersed by wind [71], water and/or insect vectors [26]. A pest of oak wood, oak pinhole borer (*Platypus cylindrus*), acts as a vector of *D. corticola* spores as this fungus was detected in the insects gut and mycangia (structures in the insect body adapted for transport of spores of insect-symbiotic fungi) [72]. The invasive insects *Xyleborus affinis* and *Xylosandrus crassiusculus* in Florida have also been found to vector *D. corticola* as this fungus was frequently isolated from their mycangia [73]. Even though the knowledge on timing of seasonal spore release and relative inoculum abundance is limited [71], under natural infection pressure originating from a declining cork oak forest, *D. corticola* was found to infect cork oak seedlings at two infections peaks, in May and in September [71]. The role of *B. corticola* ascospores and their importance in pathogen dissemination have not been discussed in literature so far and much about epidemiology of this pathogen is unknown.

Majority of evidence indicates that *D. corticola* is a true pathogen of *Quercus* species [5, 8, 28, 29]. *D. corticola* was isolated from northern red oak trees in an urban stand where no obvious signs of environmental or other biotic stresses were visible, aside of the Bot canker occurrence [5]. It is not clear how environmental and biotic factors that can stress oak trees favor *D. corticola* infections and their severity, but it is possible they could worsen the disease. In the northeastern USA, this fungus has been isolated from infected black oak trees (*Quercus velutina*) which were at the same time infested with a damaging gall wasp, *Zapatella davisae* [7]. However, in



support of the true pathogen lifestyle, the inoculation tests with isolated *D. corticola* strains conducted on young, healthy, naturally established trees of black oak clearly demonstrated the pathogenicity of *D. corticola*. In a similar case in California, closely related Botryosphaeriaceae fungi *Neofusicoccum australe*, *N. luteum*, *N. parvum*, *N. mediterraneum* and *Botryosphaeria dothidea* have been isolated from declining coast redwood trees (*Sequoia sempervirens*) in the urban stands, which were severely drought stressed [9]. However, the inoculation tests with the isolates of these fungal species on potted healthy young trees of coast redwood clearly showed that *Neofusicoccum* species were true and very virulent pathogens, while *B. dothidea* was an opportunistic pathogen that did not cause severe infection on healthy trees [9]. The other members of Botryosphaeriaceae family, such as *B. dothidea* [23, 74, 75] or *Diplodia sapinea* [76], are well-known opportunistic pathogens that dwell on the tree asymptotically for months or even years, until the plant becomes weakened through any number of abiotic or biotic stresses (e.g. drought or insect infestation), and then infect it [9, 77–81]. The stressed plant host is the key conducive condition for these pathogens to establish infection and express the disease symptoms. Future experiments, similar to the drought contribution studies done for *B. dothidea* infection [9, 74, 77–79], should demonstrate which role the different plant stresses play in predisposing the oak species to *D. corticola* infection in continental and other climate types of the world where this pathogen is also widely present.

### 2.1.2 *Venturia inaequalis*

The sexual stage of an ascomycete fungus *V. inaequalis* (Cooke) Winter, 1875, a cause of apple scab disease, starts by sexual reproduction in fall which results in formation of round initials of fungal fruiting bodies called pseudothecia. These bodies are embedded in a stroma or dense mat of fungal mycelia inside the mesophyll tissue of dead apple leaves. Late in winter and the beginning of spring, pseudothecia mature, gain pear-like shape and form sexual spores called ascospores. There are eight ascospores arranged linearly in each of the 50–100 elongated cylindrical sacs called asci that form in each pseudothecium. Ascospores are translucent to brown, two-celled, with one cell always larger than the other giving them a typical shape of a shoe “footprint”. Mature pseudothecia form a circular opening or ostiole at the top of the pseudothecium, which protrudes through the surface of the dead apple leaves and allows ascospore release. There is only one cycle of ascospore production in spring of each year and they cause primary scab infections. The asexual stage *Spilocaea pomi* (Fr.) (syn. *Fusicladium dendriticum*) forms a translucent mycelium below the cuticle of the infected green apple tissues. With time, mycelia become dark gray to black, forming a dense mycelial mat that gives rise to asexual spores called conidia. Conidia are dark green, teardrop-shaped, i.e. pointed at one end and rounded on the other, single-celled or rarely two-celled. Conidia cause secondary infections during the apple growing season. There can be many cycles of conidia production and thus secondary infections, sometimes even more than 20 [82].

Depending on the substrate it colonizes over the year, the life cycle of apple scab fungus has the saprophytic and the parasitic phase in its development. The saprophytic phase starts with apple leaf drop in autumn. *V. inaequalis*, the sexual stage of apple scab fungus, overwinters in the dead fallen leaves and fruit debris of apple on the orchard floor as initials of fruiting bodies called pseudothecia. Fungus rarely overwinters as *S. pomi* in the form of mycelium on twig lesions or in the inner bud tissues [23, 83, 84]. After winter rest, pseudothecia mature in early spring and release ascospores that enable first or the primary infections on newly developing



green apple tissues. Hence, ascospores in the leaf litter and debris are prime inoculum sources in spring. With each wetting from rain or heavy dew [85], pseudothecia absorb water, swell and asci expand through the ostiole, allowing forcible ascospore discharge. Ejected ascospores can reach a height of about 5–30 mm above the ostiole and are further disseminated by air currents, wind and rain aerosol [86]. They germinate only in water droplets or film coating the plant surfaces and their germination ends the saprophytic phase of pathogen's life cycle. Depending on region, the period of ascospore discharge triggered by wetting events can last from 3 to 9 weeks during late March and over April, May and mid-June. Ascospores are airborne and can reach at least 45 m away from the inoculum source [87]. *Spilocaea pomi* as the asexual stage of this fungus begins with ascospore germination and infection of the newly developing green tissues of apple leaves, flowers and fruit. The infection of current season's apple growth starts the parasitic phase of the pathogen's life cycle. Fungal mycelium penetrates below the waxy cuticle and grows between the outer cell wall of host's epidermal cells and the cuticle covering them. If the primary infections with ascospores are successful, after incubation period the plant cuticle of infected organ ruptures due to pressure created by thickening mycelium and masses of asexual spores of *S. pomi* called conidia. The infections are first visible as light chlorotic spots on leaves against the light, then gradually turn into pale olive to dark gray and ultimately black, velvety apple scab lesions. Infections on flower pedicel lead to flower drop. Infected leaves senesce and drop, while young fruit become deformed and fall off. Conidia cause new or often known secondary infections during the season and are dislodged and dispersed primarily with the help of rain water and dew. Their dispersal allowing more new infections is occurring primarily within the tree because very few conidia can reach 10 m or more from the inoculum source [88].

During the time after spores of apple scab fungus land on the susceptible plant surface, while they germinate, and up to 72 h after they penetrate below the cuticle on green tissue, they are vulnerable to spray-applied contact and systemic fungicides, respectively. However, once the infection is established by formation of mycelium under the cuticle, almost all fungicides applied to green plant surfaces have no efficacy in eradicating these infections and eventually lesions with conidia, or their effect is minimal. Furthermore, continued post-infection scab management with spray applications of fungicides that aim to prevent new infections on green tissues is complicated by large populations of conidia, which if exposed to fungicides with specific modes of action might increase the potential for fungicide resistance selection in this devastating pathogen. Because of the specific lifestyle of *S. pomi* to reside in subcuticular spaces of green tissues during the parasitic phase of the life cycle, successful management of the apple scab is crucially dependent on preventive fungicide applications that are delivered to leaves and fruit before the major infection periods occur. To time fungicide spray applications, several epidemiological models based on pathogen ecology and biology have been developed and can predict discharge of *V. inaequalis* ascospores and the infection occurrence by using the weather forecast for up to 10 days (NEWA, RIMpro) [89–92]. They are used in all the major apple growing regions.

### 2.1.3 *Erwinia amylovora*

Fire blight is caused by a Gram-negative bacterium *Erwinia amylovora* (Burrill 1882) Winslow et al. [93] in the family Enterobacteriaceae. The bacterial cells are rod-shaped,  $0.3 \times 1\text{--}3 \mu\text{m}$  in size and occur as single cells or pairs and sometimes short chains. They are motile by 2–7 peritrichous flagella per cell [94]. The pathogen cells are not visible to the naked eye. On infected hosts, pathogen is visible in the

form of droplets or smears of bacterial ooze which are opaque white, amber or orange. Ooze is a sticky mixture of bacterial cells and exopolysaccharides which plays a major role in pathogen dissemination within and between the apple trees and orchards [95, 96]. In humid conditions, ooze exudes from the cracks on infected wood tissues with fire blight cankers and through stomata and lenticels on green, succulent parts of infected flowers, immature fruit, and shoots. In low humidity conditions, fire blight bacteria can survive in dry ooze for more than a year [97]. In the laboratory, *E. amylovora* forms domed, circular, mucoid colonies on microbiological media that contain sucrose nutrient agar [98, 99] which differ in color depending on specific contents of agar medium, ranging from red to orange, yellow white, and light blue opalescent [100]. This pathogen has a wide range of cultivated, landscape or forest plant hosts from Rosaceae family: apple, crabapple (*Malus*), pear, Asian pear, Callery pear (*Pyrus*), quince (*Cydonia*), raspberry (*Rubus*), as well as hawthorn (*Crataegus*), firethorn (*Pyracantha*), mountain ash (*Sorbus*), serviceberry (*Amelanchier*), *Cotoneaster*, loquat (*Eriobotrya*), flowering quince (*Chaenomeles*), etc.

*E. amylovora* survives through the winter in the bark around the canker edge and below the fire blight cankers initiated after pathogen progression from flower and shoot infections established in the previous years. Bacterial cells can also overwinter in asymptomatic host buds or as latent infections in asymptomatic wood [101]. It is possible that cells of this pathogen are in a viable but non-culturable physiological state in asymptomatic tissues [102, 103]. With warm spring weather, pathogen cells multiply and emerge in bacterial ooze exuding on the edges of overwintered cankers. On apple, this process usually occurs during late bloom and petal fall growth stages. Every single droplet of ooze may contain up to 1 billion cells of *E. amylovora* [95]. The ooze protects bacteria from unfavorable weather conditions and bacteria are disseminated from ooze to flowers, shoots and injured tissues by rain, wind, birds and presumably insects that touch or feed on ooze [95, 96, 104, 105]. For example, in experimental conditions, *E. amylovora* was found to survive and can be transmitted for up to 8 days in the digestive tract of Mediterranean fruit fly *Ceratitis capitata* and up to 28 days on its surface [106]. Insects that might vector *E. amylovora* are still investigated, but it is probable that the insect vectors will vary depending on the region of the world. Recent investigation shows that flies are attracted to feed on ooze and can acquire from 100 to 100,000 viable *E. amylovora* cells per fly individual [96]. It appears that honeybees do not visit ooze droplets on cankers in spring and might not spread the bacteria from cankers to flowers. It is assumed that in low humidity conditions, dry ooze strings or particles exuded through lenticels or stomata on the bark can break off and reach or settle on nearby susceptible flowers or shoots after carried by wind or insects. The closer the fire blight cankers are to any open flowers or shoots, the higher is the chance for *E. amylovora* cells to reach the flower surfaces by previously mentioned pathways. The period of apple susceptibility to *E. amylovora* infection lasts from the day when the first flowers open in the orchard and ends when the last terminal buds set on shoots. However, risk for severe infections becomes lower after flowering ends because the number of flowers as entry points for the pathogen significantly declines. Shoots initiate growth just before flowering ends and are susceptible until their growth stops. Terminal bud set is an apple growth stage when the current year vegetative growth stops, and a bud is formed at the top of the shoot. In continental climate, this usually occurs during July, varying somewhat among different apple cultivars. After this stage, risk from fire blight infections is negligent due to age-related resistance, unless a hail injury occurs on trees providing new entry points for fire blight infections (trauma blight).

Once *E. amylovora* bacteria reach the apple flower surfaces, they multiply rapidly on the surfaces of nutrient-rich flower stigmas if temperatures are favorably

warm [107]. After colonizing young, just opened flowers [108], bacteria require achieving necessary population size and presence of moisture for infection establishment. The bacteria only have few days to grow their numbers on young flowers to reach at least 100,000 and up to 1 million live cells before a possible infection event can be triggered by rain, dew, or hail. During this time and before the moisture becomes available to allow infection, honeybees could spread the bacteria from contaminated flowers to newly opened flowers [109, 101]. This pollinator-facilitated spreading continues the necessary pathogen population increase. Flower surfaces of many other species of Rosaceae family, except European plum *Prunus domestica*, which are not susceptible to fire blight were found to be potential sites for population increase of *E. amylovora* during their periods of bloom [110]. With a wetting event in the form of rain, dew or hail, the pathogen is washed down from stigmas to the nectar glands located in the floral cup where pathogen enters the host and causes the infection. Infection of succulent green shoots occurs either via (1) internal pathogen spread through green tissues from the infected flowers to the base of the nearby shoots, (2) direct transfer of the pathogen from cankers or contaminated plant or tool surfaces to the shoots, or (3) by pathogen dispersal from contaminated or infected flowers to the shoot tips and leaves. Insects might play a vector role in these three pathways. For limited amount of time, *E. amylovora* cells can survive on other healthy surfaces of leaves and branches. However, population growth on these surfaces does not occur. Pathogen enters and colonizes the cortical parenchyma through stomata on the leaves or green stem, or through the microinjuries i.e. punctures and tears caused by wind, wind-carried soil particles, hail, friction of plant parts, or sucking or chewing insects [111]. During the time before infections take place, the lifestyle of *E. amylovora* involves inhabiting and growing on the plant surfaces and is influenced by the temperature and moisture from the environment and by nutrients on the plant host. This is known as epiphytic phase of *E. amylovora* biology during which successful management of fire blight is achieved by preventive spray applications of antibiotics that are delivered to flowers, before or up to 24 h after the predicted rain event/s that would trigger the infection/s. Several fire blight epidemiological models have been designed based on environmental and biological requirements of *E. amylovora* and can predict infection events by using the weather forecast for up to 10 days in advance to calculate the near-future infection risks (NEWA's EIP, Maryblyt, RIMpro, Cougarblight).

During the incubation, i.e. usually sometime around 10 – 14 days before the first conspicuous blossom or shoot blight symptoms are visible, small white, amber or orange droplets of bacterial ooze can emerge and drip from the infected green tissues (flower pedicels, floral cup, sepals, immature fruit and shoots). With more wetting events and insect activity, ooze can spread to new flowers and actively growing shoots across the whole orchard. Since blossom and shoot blight symptoms are not yet visible, this dissemination of ooze allows secondary infections and can propel a fire blight outbreak into an epidemic, especially if the antibiotic spray application/s were not conducted during bloom. Once incubation is over, blossom blight is visible as dead, black or brown flower clusters with more droplets of bacterial ooze developing if weather conditions are humid. Shoot blight and immature fruit infections are visible as black or brown “flags” or “strikes” and brown to black shriveled fruitlets, respectively. Blighted shoot tips often bend in the typical shape of Shepherd's crook. Fire blight cankers on branches, trunk and rootstock are formed by pathogen's progress via xylem or the cortical parenchyma from the established infections on flowers, shoots and suckers, into the wood bark tissues.

When *E. amylovora* enters the succulent tissues of flowers or shoots, it begins the pathogenic phase of its lifestyle when it causes the disease and acquires moisture and nutrients from the host and can migrate to other close or far host tissues and



organs. During this phase, pathogen colonizes the cortical parenchyma tissue and xylem vessels and can continue with systemic migration and distribution in the plant [112–114]. Fire blight bacteria migrate internally via xylem of symptomless branch and trunk tissues and ahead of the visible blight or canker symptoms, thus reaching uninfected plant parts and apple rootstocks and causing infection far from the visible infections in the canopy [115]. On susceptible rootstocks, the resulting infections can express as cankers, often causing tree death due to trunk or rootstock girdling, or can remain latent i.e. as a symptomless infections of trunk or rootstock. Rootstock or trunk infections can also be initiated by *E. amylovora* migration from the externally infected rootstock suckers (shoot growth from the root system or rootstock stem) or water sprouts (shoot growth from the trunk or thick branches). When *E. amylovora* resides as an endophyte in an apparently healthy plant tissues of branches, rootstock or budwood, this lifestyle is referred to as an endophytic phase of its biology. Finally, in nurseries, *E. amylovora* cells which survive on bark surfaces can infect rootstock or scion when either are bruised or injured during the processes of vegetative material harvest, transport, or grafting.

## 2.2 Materials and methods

### 2.2.1 Trunk injection of pesticides for *Diplodia corticola* management

To test the effect of injected fungicides and activators of plant systemic acquired resistance (SAR) [11] for reduction of Bot canker caused by *D. corticola* we conducted experiments on potted northern red oak trees (*Q. rubra*) with fully developed canopy. We evaluated trunk-injected fungicides and application rates listed in **Table 1**, which were selected based on the EPA labels of pesticides for landscape use (**Table 1**) and the preliminary fungicide screenings *in vitro* for suppression of *D. corticola* colonies on fungicide-amended Petri of plates with potato dextrose agar medium (Aćimović et al. unpublished data). Since in year one of the experiment Phosphojet at 1.5 ml dose caused phytotoxicity on tree trunks, we reduced the dose to 0.75 ml in year two repetition of the experiment (**Table 1**).

Year one			
Treatment	Active ingredient	Dose and dilution	Total volume
Propizol	Propiconazole 14.3% (Propizol, Arborjet Inc.)	1.5 ml + 1.5 ml water	3 ml
Arbotect	Thiabendazole 20% (Arbotect 20-S, Syngenta)	0.06 ml + 2.94 ml water	3 ml
Phosphojet	Mono- and di-potassium salts of phosphorous acid <sup>x</sup> 45.8% (Phosphojet, Arborjet Inc.)	1.5 ml + 1.5 ml	3 ml
Water control	-	3 ml	3 ml
Year two			
Treatment	Active ingredient	Dose	Total volume
Propizol	Propiconazole 14.3% (Propizol, Arborjet Inc., Woburn, MA)	1.5 ml + 1.5 ml water	3 ml
Arbotect	Thiabendazole 20% (Arbotect 20-S, Syngenta)	0.06 ml + 2.94 ml water	3 ml
Phosphojet	Mono- and di-potassium salts of phosphorous acid 45.8% (Phosphojet, Arborjet Inc.)	0.75 ml + 2.25 ml water	3 ml
Water control	-	3 ml	3 ml

<sup>x</sup> Commonly called potassium phosphites

**Table 1.**

Trunk injected fungicide treatments evaluated for management of Bot canker fungus *Diplodia corticola* on northern red oak trees, *Quercus rubra*.



One injection point i.e. port per trunk of each potted tree, positioned ca. 5–7 cm above the ground level, was created by drilling 7–10 mm into the xylem tissue with a 4.3 mm diameter drill attached to a cordless drill. To inject the protective liquid solutions listed in **Table 1**, we used a Stinger needle for plugless trunk injection assembled on an individual feed line attached to the Tree IV air/hydraulic microinjection system, which operated at 60 psi air pressure (Arborjet Inc., Woburn, MA). The Stinger needles are used for injection of trees when trunk injection ports of large diameter (9.5 mm) are of concern or should be avoided and for injection of trunks with small diameters. The diameter of injection port for inserting a Stinger needle is smaller and does not require sealing with an Arborplug. In year one, the injected potted oak trees had trunk diameter at 5 cm height averaging 1.3 cm and ranging from 1 to 2.1 cm. In year two, a new set of injected trees had the diameter at 5 cm height averaging 1.5 cm and ranging from 1.1 to 2.2 cm. Trunk injection were conducted on 12 June in year one and on 16 August in year two.

Trees were inoculated with *D. corticola* on 21 June and on 25 August, i.e. 9 days after injection of fungicides. Trunk bark on the opposite side from the injection port and 10 cm above the port was cut at three sides of rectangle to create a sleeve which was peeled longitudinally. A PDA plug 5 mm in diameter from 10-day-old colony of *D. corticola* isolate from our previous work [8] was placed in the sleeve on each injected tree and wrapped with parafilm (**Table 1**). Once the first symptoms of canopy wilt were observed, trees were destructively examined by stripping the bark off above and below the inoculation point. The necrosis length (cm) and width (cm) of Bot canker in xylem of the oak trunks were measured on 10 July in year one and on 27 September in year two. Xylem necrosis area (cm<sup>2</sup>) was calculated by multiplying the length and width for each individual tree and treatment mean was calculated from six replicate trees.

Statistical analysis was done with MIXED procedure in SAS Studio software (SAS Institute Inc. 2017, Cary, NC) using the xylem necrosis areas (cm<sup>2</sup>). If the fungicide effect was found to be statistically significant ( $p < 0.1$  in year one;  $p < 0.05$  in year two), treatment comparisons were done with LSD test. We presented the fungicide management results as percent reduction of Bot canker necrosis area, also known as percent disease control, calculated as: percent reduction of necrosis area = [percent necrosis area in water control – percent necrosis area in specific treatment] × 100/percent necrosis area in water control.

### 2.2.2 Trunk injection of pesticides for *Venturia inaequalis* management

With the goal to optimize timing and number of fungicide injections for management of apple scab fungus *V. inaequalis*, we conducted two experiments. In the experiment 1, we trunk-injected fungicides listed in **Table 2** on 29-year-old ‘Mac Spur’ apple trees four times, with the first injection applied in the fall of year one and the next three injections conducted in the spring of next, year two. In the experiment 1, the injection on 11 April was conducted at 50% apple bloom (**Table 2**). In the experiment 2, we injected 29-year-old ‘Mac Spur’ apple trees with fungicides only one to two times in total, but by delivering them at different seasons i.e. in fall or spring, as per schedule listed in **Table 2**. In the experiment 2, the injection on 21 April was conducted at the silver tip growth stage of apple (**Table 2**).

On each trunk injection date with fungicides listed in **Table 2**, a separate set of four cardinaly-oriented trunk injection ports per each tree of ‘Mac Spur’ was created by drilling 25 mm into the xylem with a 9.5 mm diameter drill bit attached to a cordless drill. The first set of four injection ports was positioned ca. 25 cm above the ground level. The subsequent sets of four injection port were positioned ca. 5 cm above and between the lower four-port sets. Every port was sealed with Arborplug no. 4 (Arborjet

EXPERIMENT 1					
Treatment	Active ingredient	Dose	Dates of injections or sprays		
			Year one	Year two	
Phosphojet low F <sup>u</sup> + 3S <sup>v</sup>	Mono- and di-potassium salts of phosphorous acid <sup>w</sup> 45.8% (Phosphojet, Arborjet Inc.)	2.59 ml / 2.5 cm of DFH <sup>x</sup>	15 October	11 April, 11 May, 8 June	
Phosphojet high F + 3S		5.17 ml / 2.5 cm of DFH	15 October	11 April, 11 May, 8 June	
Alamo low F + 3S	Propiconazole 14.3% (Alamo, Arborjet Inc.)	8.3 ml / 2.5 cm of DFH	15 October	11 April, 11 May, 8 June	
Alamo high F + 3S		16.6 ml / 2.5 cm of DFH	15 October	11 April, 11 May, 8 June	
Water control	-	8.3 ml / 2.5 cm of DFH	15 October	11 April, 11 May, 8 June	
Spray standard	Mancozeb 75%, (Penncozeb 75 DF, Cerexagri Inc.) + Fenarimol 12% (Rubigan EC, Dow AgroSciences LLC)	2.7 kg / 0.405 ha + 354.9 ml / 0.405 ha		27 March; 3, 13, 18 <sup>y</sup> April; and 1 May 2012	

EXPERIMENT 2					
Treatments	Active ingredient	Dose	Dates of injections or sprays		
			Year one	Year two	
Phosphojet F	Mono- and di-potassium salts of phosphorous acid 45.8% (Phosphojet, Arborjet Inc.)	5.17 ml / 2.5 cm of DFH	11 October	-	-
Phosphojet F + S <sup>z</sup>		5.17 ml / 2.5 cm of DFH	11 October	21 April	-
Phosphojet S	Arborjet Inc.;	5.17 ml / 2.5 cm of DFH	-	21 April	-
Phosphojet S + S		5.17 ml / 2.5 cm of DFH	-	21 April	22 May
AgriFos sprays	AgriFos, Agrichem PTY.)	1,892.7 ml / 0.405 ha	1, 8, 16, 21, 31 May and 5, 11, 19, 26 June 2013		
Inspire Super F	Difenoconazole 8.4% +	7 ml/ tree	11 October	-	-
Inspire Super F + S	Cyprodinil 24.1% (Inspire Super EW, Syngenta)	3.5 ml/ tree	11 October	21 April	-
Inspire Super S		7 ml/ tree	-	21 April	-
Inspire Super S + S	Inspire Super sprays	7 ml/ tree	-	21 April	22 May
Inspire Super sprays		354.8 ml/ 0.405 ha	1, 8, 16, 21, 31 May 2013		
Water control	-	500 ml/ tree	11 October	21 April	22 May

<sup>u</sup> F - one fall injection  
<sup>v</sup> 3S - three spring injections  
<sup>w</sup> Commonly called potassium phosphites  
<sup>x</sup> DFH - trunk diameter at one-foot height (30.5 cm)  
<sup>y</sup> Date with single fenarimol application  
<sup>z</sup> S - one spring injection

**Table 2.** Fungicide treatments trunk-injected across two seasons and sprayed for management of apple scab fungus *Venturia inaequalis* on 'Mac Spur' apple trees.

Inc., Woburn, MA) positioned just below the bark level to allow port closure with cambium callus [44]. To inject the fungicides, we used the Quik-jet microinjection system (Arborjet Inc.) operating at hand-generated hydraulic pressure to deliver low volumes of liquid for injection, thus allowing faster application times, and the Tree IV air/hydraulic microinjection system (Arborjet Inc.) operating at up to 60 psi of air pressure to deliver large solution volumes of liquid for injection ( $\geq 600$  ml). In the experiment 1 (Table 2), we injected all the treatments listed for 15 October in year one with Quik-jet (Table 2). On 11 April in year two, we injected propiconazole using the Viper air/hydraulic microinjection system set at 90 psi air pressure (Arborjet Inc.). At the later dates, we injected Alamo using the Tree IV and Phosphojet using the Quik-jet. In the experiment 2, we injected Phosphojet with Quik-jet and cyprodinil + difenoconazole with Tree IV. The needle/s of each of the used injection devices was inserted through the Arborplugs allowing the total liquid volume per tree, at one injection time, to be divided and delivered equally among the four ports.

All the experiments were conducted under naturally high infection pressure during the primary season of *V. inaequalis* ascospore release in spring. In the experiment 1, we rated percent incidence of apple scab only on leaves, since fruits were lost due to spring frosts. In the experiment 2, we rated percent incidence of scab on leaves and fruit. A total of chose 20 spurs and 20 terminal shoots per tree were selected, with about five from each crown quadrant, and rated for leaf scab incidence. The fruit scab incidence was rated by selecting and rating 100 fruits per tree, with about 25 per crown quadrant, and if less was found we rated all the fruits per tree. The data were analyzed using MIXED procedure in SAS 9.3 (SAS Institute, Cary, NC). The tree was the subject of repeated measures. If the main effects or their interactions were found to be statistically significant ( $p < 0.05$ ),

Treatment	Active ingredient	Dose
<b>For control of blossom and shoot blight incidence on 'Gala' apple trees</b>		
Actigard 1	Acibenzolar-S-methyl 50% (Actigard, Syngenta)	1 x 0.34 g/tree
Actigard 2		2 x 0.34 g/tree
Phosphojet	Mono- and di-potassium salts of phosphorous acid <sup>x</sup> 45.8% (Phosphojet, Arborjet Inc.)	2 x 22.5 ml/tree
Agrimycin	Streptomycin 17% (Agrimycin, Nufarm Ltd.)	2 x 1.82 g/tree
Water control	-	2 x 520 ml/tree
<b>For control of shoot blight severity on 'Jonathan' apple trees</b>		
Arborbiotic	Oxytetracycline hydrochloride 39.6% (Arborbiotic™, MFG Scientific Inc.)	1 x 0.28 g + 2.52 ml water / each 25.4 mm of DFH <sup>y</sup>
Water control	-	2.52 ml water / each 25.4 mm of DFH

<sup>x</sup> Commonly called potassium phosphites

<sup>y</sup> DFH - trunk diameter at one-foot height (30.5 cm)

**Table 3.**

*Trunk-injected treatments of bactericides and SAR-activators for management of fire blight bacterium Erwinia amylovora on flowers and shoots of 'Gala' and 'Jonathan' apple trees.*

examination, i.e. slicing of interactions within main effects was performed, *F*-tests conducted and pairwise or specific time or treatment comparisons were done with *t*-tests ( $\alpha = 0.05$ ). We presented the fungicide management results as percent of disease reduction, also known as percent disease control, calculated as: percent reduction of disease incidence = [percent disease incidence in water control – percent disease incidence in specific treatment]  $\times$  100/percent disease incidence in water control.

### 2.2.3 Trunk injection of pesticides for *Erwinia amylovora* management

#### 2.2.3.1 Treatments for reducing blossom and shoot blight incidence

To test the effect of injected bactericides and activators of plant systemic acquired resistance (SAR) for blossom and shoot blight incidence reduction, the orchard experiments were conducted over 2 years (**Table 3**). The early spring injections in year one (26 March) were conducted with Viper air/hydraulic micro-injection system<sup>®</sup> at under 110 psi of air pressure and late spring injections (23 April) were done with Tree IV<sup>®</sup> air/hydraulic micro-injection system, at 60 psi air pressure (Arborjet Inc., Woburn, MA). In the year two, trunk injections on 1 and 22 May were applied using Tree IV<sup>®</sup> air/hydraulic micro-injection system at 60 psi of air pressure. The injection needles of these devices were inserted through the one-way valve silicone septum in the Arborplugs<sup>®</sup> which allowed delivery of protective solutions into the drilled injection ports. In each injection, the total injected volume per tree was divided equally among the four ports (**Table 3**). Four injection ports per each apple per tree, positioned ca. 10–15 cm above the ground level, were cardinally oriented and created by drilling 25 mm into the xylem tissue using a 9.5 mm diameter drill bit attached to a cordless drill. Each port was sealed with Arborplug<sup>®</sup> no. 4, by pushing the plug with a specialized screwdriver-like tapper hit with a hammer (Arborjet Inc., Woburn, MA, USA). The plug was positioned just below the bark level to allow port closure with cambium callus.

In the year one, we used 14-year-old 'Gala' apple trees which were trunk-injected using the compounds and dosages listed in **Table 3**. Injections were performed at the tight cluster growth stage in apples (26 March), or 21 days before 80% bloom, and at petal fall growth stage (23 April). In the year two, experiments were conducted on a new set of 21-year-old 'Gala' apple trees, injected with the same doses in **Table 3**. Injections were applied at early tight cluster growth stage (1 May) or 13 days before 80% bloom and at petal fall (22 May). The treatment



Actigard 1 was injected only on the first date in both years (**Table 3**). Each dose in every treatment, except the Phosphojet, was diluted and injected with 520 ml of water per tree. The doses per tree were chosen according to the four rules: (1) the dose was equivalent to the US EPA pesticide label rate for a maximum amount per 0.405 ha with 250 planted apple trees; (2) the dose was one half of the maximum US EPA label rate allowed per one season; (3) the dose was equal to a rate delivered in one spray application treatment per 0.405 ha with 250 apple trees; or (4) the dose was selected based on previous research with trunk injection of similar pesticides [116]. Trees injected with water and the non-injected non-inoculated trees served as negative controls for efficacy comparisons. In year one, each treatment was replicated on four trees arranged in a randomized complete block design, where blocking controlled the variable crown tree sizes (large, medium, medium-small, and small) [117]. In the year two, we used the same number of replicate trees per treatment but arranged in a completely randomized design (CRD).

In year one, on 16 April at 80% bloom, apple flowers of all experimental trees were inoculated with a suspension of *E. amylovora* strain in distilled water using a hand-sprayer ( $5.4 \times 10^6$  CFU/ml; CFU—colony forming units). In year two, on 14 May at 80% bloom, flowers were inoculated with *E. amylovora* ( $0.7 \times 10^6$  CFU/ml). In year one, we evaluated blossom blight incidence 22, 29 May and 5 June, and in year two on 11, 18, and 25 June. Rating of blossom blight incidence consisted of random selection of flower clusters to form a 100-cluster sample per tree and calculating the percent of diseased and healthy blossom clusters in that sample. Shoot blight incidence was evaluated on 29 May and 5 June in year one and in year two on 11, 18, and 25 June. After randomly selecting enough shoots to form a sample of 100-shoots per tree, shoot blight incidence percent was calculated for each tree from the number of blighted and healthy shoots. Mean percent of blossom and shoot blight incidences for each treatment were calculated from the disease incidences on four replicate trees. For clarity, presented means consist of four replicate trees averaged across two or three time points i.e. dates listed above when fire blight incidences on flowers or shoots were rated.

We analyzed the data with MIXED procedure in SAS 9.3 (SAS Institute, 2012). The main effect of treatments on blossom and shoot blight incidence were analyzed using *F* test ( $\alpha = 0.05$ ) and if found significant, pairwise treatment comparisons were done using *t*-tests ( $\alpha = 0.05$ ). We presented the fire blight management results as percent of disease reduction, also known as percent disease control, calculated as: percent reduction of blossom or shoot blight incidence = [percent blossom or shoot blight incidence in water control – percent blossom or shoot blight incidence in specific treatment]  $\times$  100/percent blossom or shoot blight incidence in water control.

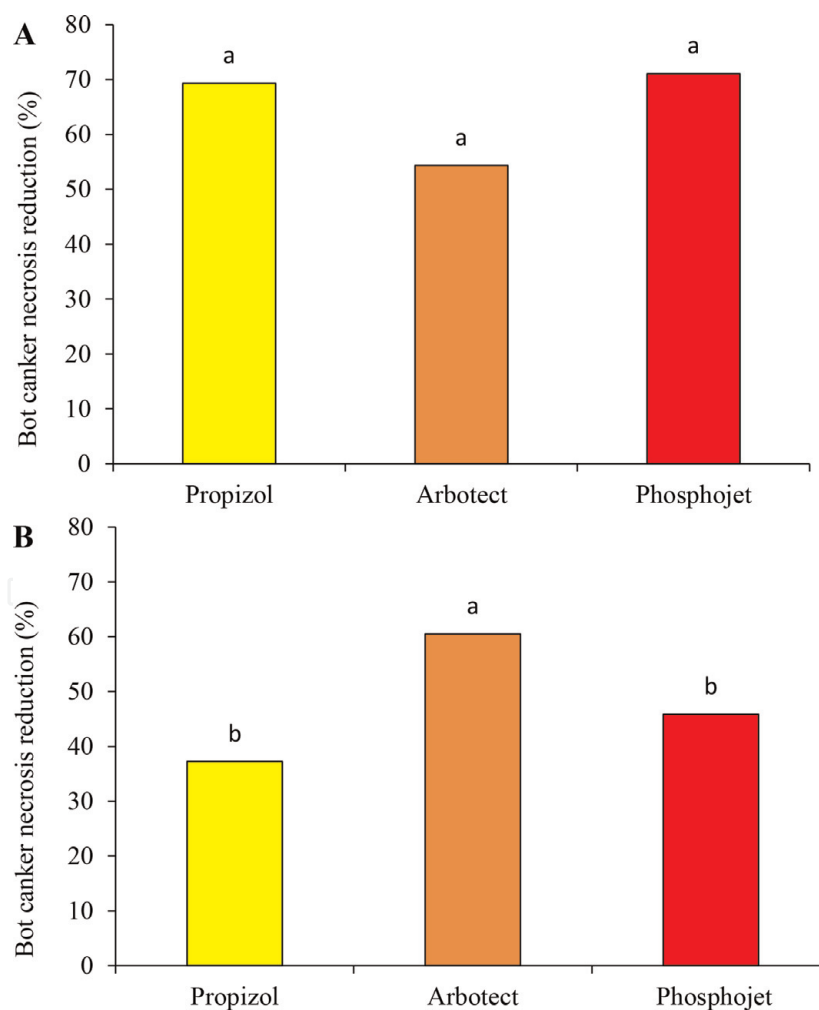
### 2.2.3.2 Treatments for reducing shoot blight severity

To test the reduction of shoot blight severity with bactericide oxytetracycline hydrochloride (Arborbionic, MFG Scientific Inc., EPA Reg. No 88482-1; Arbor-OTC<sup>®</sup> Injectable Tree Antibiotic, Arborjet Inc., Reg No. 74578-7), apple trunk injections were performed in a similar fashion described above, but by using a Quik-jet<sup>®</sup> micro-injection system instead (Arborjet Inc., Woburn, MA, USA). This device relies solely on hand-generated hydraulic pressure to inject the necessary pesticide solution volume in each port. The injection ports were created and sealed with Arborplugs (Arborjet Inc.) in the same way as described above and injected volume per tree was divided equally among the four ports. The experiments were conducted in 2 years. In year one, at petal fall growth stage (23 April) mature 12-year-old apple trees of cv. 'Jonathan' were trunk-injected with Arborbionic using dose in **Table 3** diluted at 10% in water. The total dose per tree was calculated based



on the unique trunk diameters at 30 cm height using the EPA label instructions. In year two, the same apple trees injected in year one were re-injected at petal fall (22 May) using the same dose in **Table 3** delivered via a fresh set of drilled injection ports above the previous year's set of injection ports. In both years, Arborbiotic treatment as well as water control were replicated on four trees arranged in a CRD.

A total of 10 terminal shoots per each tree were inoculated on 7 May in year one and on 30 May in year two. We used a previously reported inoculation method [114]. In brief, the upper third of leaf blade of the second or the third youngest leaf on each shoot tip was cut perpendicular to the leaf midvein with scissors dipped in *E. amylovora* suspension (year one:  $4.7 \times 10^7$  CFU/ml; year two:  $5 \times 10^8$  CFU/ml). An additional 10 shoots per each tree were wounded with scissors dipped in distilled water and used as an in-per-tree negative control. When the disease started developing on inoculated shoots, the length of shoot blight lesion (necrosis) and the total shoot length was measured for each inoculated shoot and the shoot blight severity percent was calculated by comparing the ratio of necrotic lesion length and the total shoot length (cm). Only the total shoot length was measured for negative control shoots. The shoot necrosis lesions and total shoot lengths were measured at 7-day intervals after inoculation and were ceased when terminal bud set on shoots occurred (year one: 14, 21, and 28 May and 4, 11, and 18 June; year two: 10, 17, and



**Figure 1.**

Percent reduction i.e. control of Bot canker necrosis area in trunk xylem in relation to water control on *Quercus rubra* trees in year one (A) and year two (B) achieved with trunk injections of fungicides Propizol (propiconazole), Arbotect (thiabendazole) and Phosphojet (potassium phosphites). Means followed by different letters are significantly different (A:  $p < 0.1$ , LSD test; B:  $p < 0.05$ , LSD test). In year one (A), the area of Bot canker necrosis in trunk xylem in water control was  $5.15 \text{ cm}^2$  and in year two (B)  $5.8 \text{ cm}^2$ . Each mean consists of six replicate trees.

24 June and 1, 8, and 15, 2013 July). The mean of shoot blight severity percent in per tree basis was calculated from the 10 shoot replicates. For each time point when the disease was rated, the average shoot blight severity was calculated from the four tree replicate means. For clarity, presented means consist of four replicate trees averaged across five or six time points i.e. dates listed above when fire blight severity on shoots was rated.

We analyzed the data using MIXED procedure in SAS 9.3 (SAS Institute, 2012). If the main effect of treatment on shoot blight severity was found significant ( $F$  test,  $\alpha = 0.05$ ), comparison to water control was conducted using re  $t$ -tests ( $\alpha = 0.05$ ). We presented the fire blight management results as percent of disease reduction, also known as percent disease control, calculated as: percent reduction of shoot blight severity = [percent shoot blight severity in water control – percent shoot blight severity in specific treatment]  $\times$  100/percent shoot blight severity in water control.

### 3. Results

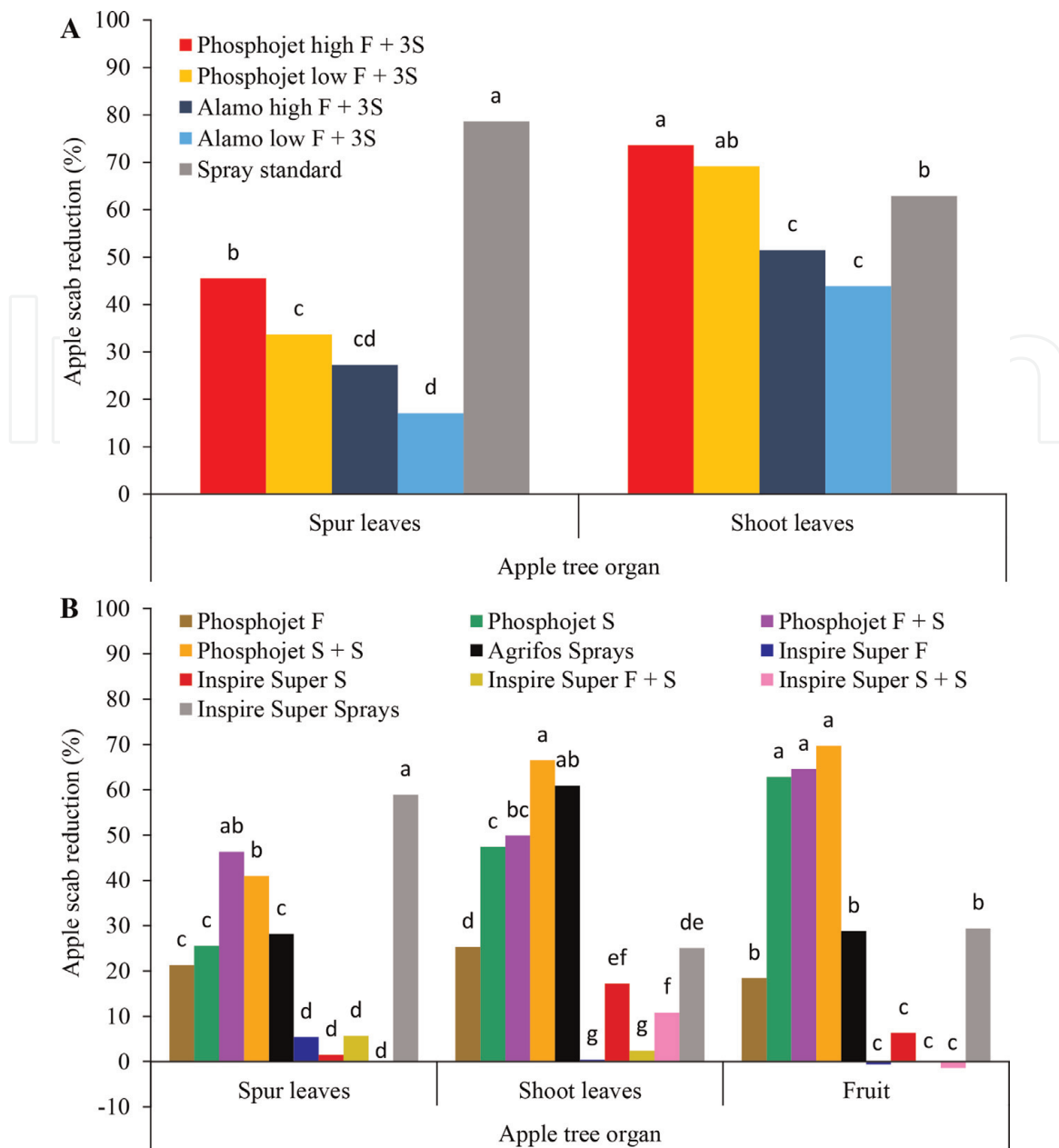
#### 3.1 Trunk injection of pesticides for *Diplodia corticola* management

All the three fungicides trunk-injected preventively provided significant reduction of Bot canker caused by *D. corticola* for 37.2–71.1% (**Figure 1**). Phosphojet at 1.5 ml per tree gave the best disease control when averaged across both years (58.5%) but caused phytotoxicity on four out of six tree replicates in year one and these trees died before the disease was rated. In year two, Phosphojet rate was reduced to 0.75 ml and this negative effect was not detected again. Averaged across both years, Arbotect provided the second-best control of 57.5%, followed by Propizol with 53.3% (**Figure 1**).

#### 3.2 Trunk injection of pesticides for *Venturia inaequalis* management

In the experiment 1, fungicides injected four times in total, once in fall and then three additional times in spring, during the primary scab infection period, provided significant reduction of apple scab incidence on spur and shoot leaves (**Figure 2A**). On spur leaves, the best scab reduction of 45.5% was achieved with injected Phosphojet high, but this control was not better in comparison to 78.6% in spray standard applied in spring during the primary scab season (**Figure 2A**). In contrast, control with injected Phosphojet high on shoots outperformed the spray standard with 73.6 vs. 62.9% in scab reduction (**Figure 2A**). Similarly, Alamo performed better on shoot leaved than on spur leaves (**Figure 2A**).

In the experiment 2, fungicides injected 1–2 times in total, across or within two seasons of fall and spring, revealed that the injected Inspire Super treatments largely did not significantly reduce disease incidence on spur and shoot leaves when compared to the water control. In contrast, all the injected Phosphojet treatments and Agrifos sprays did. Comparisons among these treatments clearly demonstrated that on all the three rated apple organs (**Figure 2B**), Phosphojet trunk injections provided statistically better apple scab reduction i.e. control in comparison to all the Inspire Super trunk injections. On spur leaves, two Phosphojet trunk injections, fall plus spring, was the best treatment among injections by providing 46.3% control which was similar to the Inspire Super sprays (**Figure 2B**). On shoot leaves, two Phosphojet trunk injections both done in spring, provided the best scab control of 66.5% similar to nine sprays of Agrifos (**Figure 2B**). On fruit, scab control was the best in Phosphojet trunk injection done once or twice in spring, and in fall plus



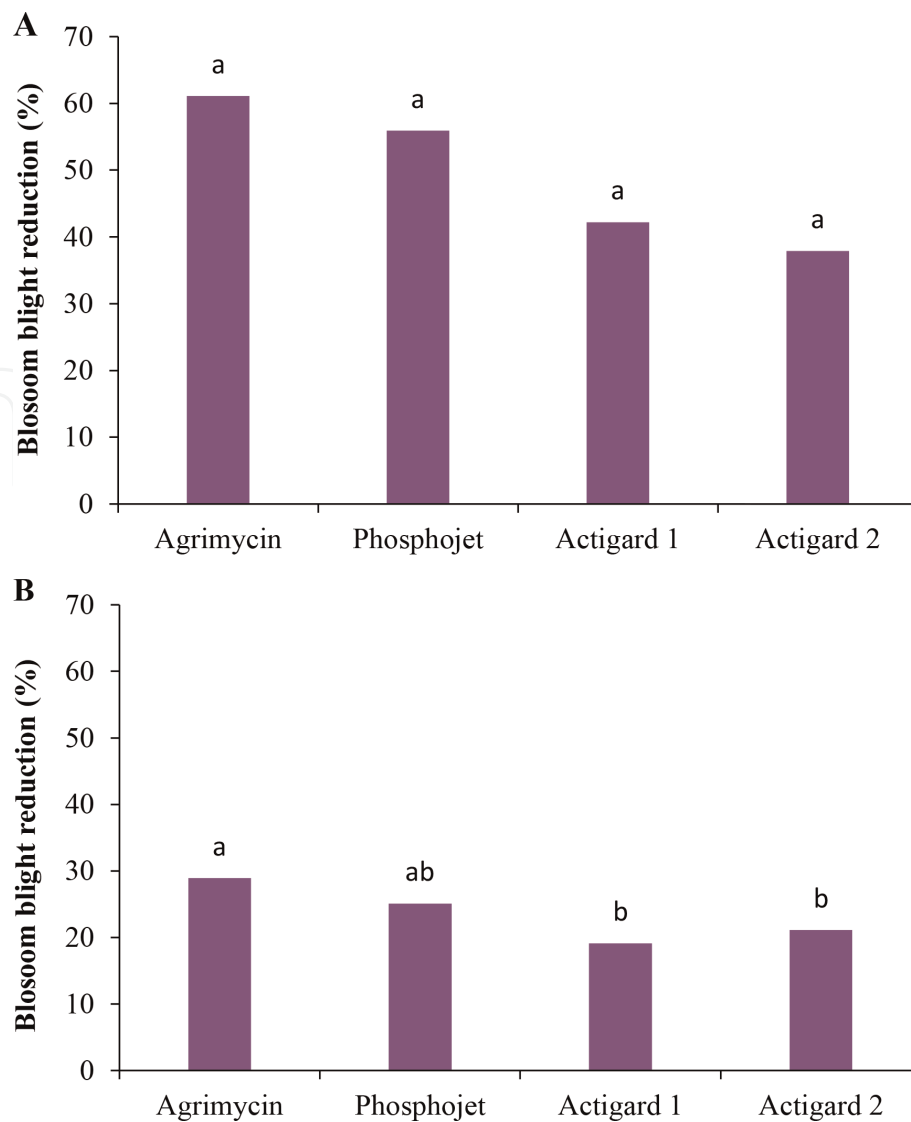
**Figure 2.** Percent reduction i.e. control of apple scab in relation to water control on ‘Mac Spur’ trees after in experiment 1 (A) and experiment 2 (B) achieved with trunk injections and sprays of potassium phosphites (Phosphojet, Arborfos) and of difenoconazole + cyprodinil (Inspire Super). Means within each graph section i.e. apple organ followed by different letters are significantly different (*t*-test,  $p < 0.05$ ). F - one fall injection; 3S - three spring injections; S - one spring injection. In experiment 1 (A), scab incidences in water control on spur and shoot leaves were 72.2 and 54%. In experiment 2 (B) scab incidences in water control on spur leaves, shoot leaves and fruit were 88.3, 94.4 and 95.5%, respectively. Each mean consists of six replicate trees.

spring: 62.8, 69.7 and 64.6%, significantly outperforming both the Agrifos and the Inspire Super sprays (Figure 2B).

### 3.3 Trunk injection of pesticides for *Erwinia amylovora* management

#### 3.3.1 Treatments for reducing blossom and shoot blight incidence

In both year one and year two, all the trunk-injected bactericides (Agrimycin) and SAR-activators (Actigard, Phosphojet) provided significant reduction of

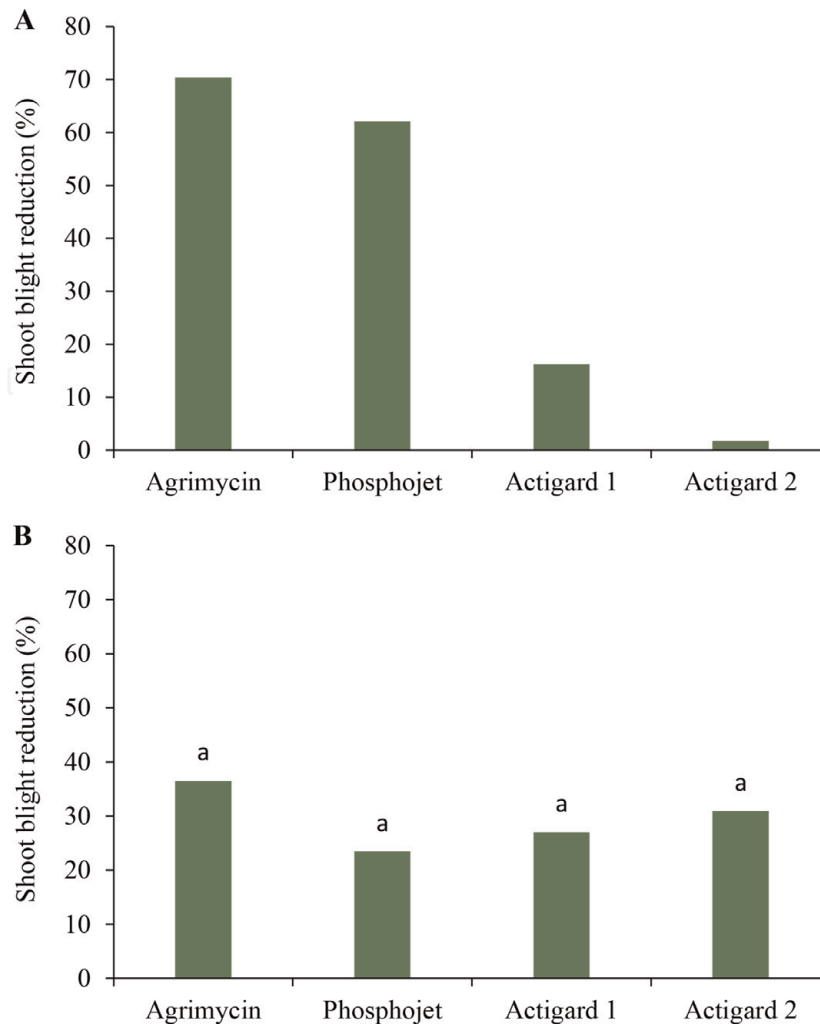


**Figure 3.** Percent reduction i.e. control of blossom blight incidence in relation to water control on ‘Gala’ apple trees in year one (A) and year two (B) achieved with one to two trunk injections of ‘Gala’ apple trees with Agrimycin (streptomycin), Phosphojet (potassium phosphites) and Actigard (acibenzolar-S-methyl). Means within each graph followed by different letters are significantly different (*t*-test,  $p < 0.05$ ). Blossom blight incidence in water control in year one was 47.2% (A) and in year two 72.9% (B). Each mean consists of four replicate trees averaged across three time points when disease was rated.

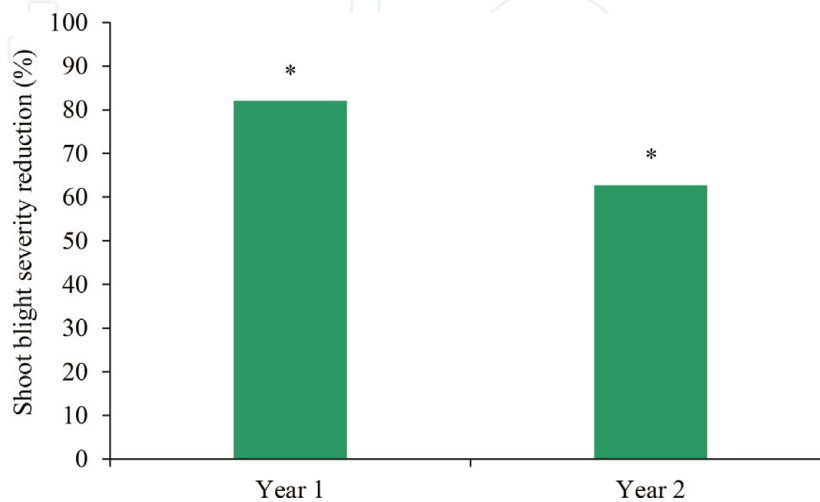
blossom blight incidence in comparison to the water control (**Figure 3**). In year one, which had low disease pressure (**Figure 3A**), there was no significant difference among all the treatments in disease reduction i.e. control (37.9–61.1%). In year two, with high infection pressure, Agrimycin was the best providing 28.9% blossom blight control (**Figure 3B**). Averaged across both years, Agrimycin and then Phosphojet were the best treatments with 45 and 40.5% achieved control, respectively (**Figure 3**).

In year one, none of the trunk-injected products provided significant reduction of shoot blight incidence in comparison to the water control, hence did not differ among each other (**Figure 4A**). In year two, under high disease pressure, all the injected products significantly reduced shoot blight incidence for 23.4–36.5% in comparison to the water control, but when compared they did not significantly differ between each other (**Figure 4B**). If averaged across both years, Agrimycin and then Phosphojet achieved the best control of 53.5 and 42.8%, respectively (**Figure 4**).





**Figure 4.** Percent reduction i.e. control of shoot blight incidence in relation to water control on 'Gala' apple trees in year one (A) and year two (B) achieved with one to two trunk injections of Agrimycin (streptomycin), Phosphojet (potassium phosphites) and Actigard (acibenzolar-S-methyl). (A) In year one, the injected treatments did not significantly reduce shoot blight incidence relative to water control. (B) Means followed by different letters are significantly different ( $t$ -test,  $p < 0.05$ ). Shoot blight incidence in water control in year one was 22.4% (A) and in year two 68.5% (B). Each mean consists of 4 replicate trees averaged across two time points in (A) and three time points in (B) when disease was rated.



**Figure 5.** Percent reduction i.e. control of shoot blight severity relative to water control achieved from a single trunk injection of 'Jonathan' apple trees with Arborbionic (oxytetracycline hydrochloride) in each year. Means with an asterisk indicate significant reduction of shoot blight severity (year one: Tukey's HSD test; year two:  $t$ -test,  $p < 0.05$ ). Each mean consists of four replicate trees averaged across five time points in year 1 and six time points in year 2 when disease was rated.

### 3.3.2 Treatments for reducing shoot blight severity

In both years Arborbionic provided significant reduction i.e. control of shoot blight severity in comparison to the water control (**Figure 5**). When averaged across both years, the control of shoot blight severity reached 72.4% (**Figure 5**).

## 4. Discussion

### 4.1 *Diplodia corticola*

We present the first data on management of *D. corticola* on northern red oak using fungicides thiabendazole, propiconazole and potassium phosphites delivered by trunk injection as an alternative pesticide application method which offers selective exposure of this and other wood pathogens to the injected compounds. Since this fungus invades and spreads via tree xylem on different oak species as hardwood trees and causes necrosis and vascular occlusion [7, 8], ultimately killing the tree, trunk injection of fungicides seems as the most suitable fungicide delivery method for this pathogen's biology and likely more effective for managing the resulting Bot canker disease. The achieved levels in control of Bot canker in xylem ranged from 37.2 to 71.1% with an overall average of 56.4% across all the fungicides we trunk injected. Phosphojet provided control of 58.5%, but the most reliable fungicides and across-years consistent were Arbotect (thiabendazole) and Propizol (propiconazole) which achieved control of 57.5 and 53.3%. A higher efficacy was not achieved probably because of the short time between fungicide injection and inoculation with *D. corticola* which could have reduced the uniformity in distribution of these fungicides in xylem, thus hampering the efficacy. On the debarked cork oak trees and under moist conditions, the canker length caused by *D. corticola* is reduced for 25.8–98.5% by preventive spray applications of thiophanate-methyl and/or copper-calcium sulphate, delivered immediately after the cork peeling [20]. On average, across different test locations, Bot canker control in this study was 64.7% with thiophanate-methyl and/or copper-calcium sulphate [20].

The organic carbon-water partitioning coefficient ( $K_{o/c}$ ) for thiabendazole is moderate to high and ranges from 1104 to 4680 ml/g, while water solubility is 50 mg/L at pH 7 and 38 mg/ml at pH 2 [118]. These parameters indicate on low to no mobility of thianbendazole in xylem as a carbon rich environment. The  $K_{o/c}$  of propiconazole is 1086–1817 ml/g which is moderate to high [119, 120] and water solubility is low, 100–150 mg/L [121]. This could have contributed to slow and reduced uniformity in distribution of injected fungicides in xylem. However, both Arbotect and Propizol are fungicides formulated for trunk injection on hardwood trees and if properly diluted and delivered preventively they can accumulate sufficiently to secure the internal control of specific plant diseases (e.g. Dutch elm disease caused by *O. ulmi* and *O. novo-ulmi*; sycamore/London plane anthracnose caused by *Apiognomonia veneta*).

In the future studies, we predict that the efficacy of preventive fungicide applications against *D. corticola* via trunk injection delivery can be increased: (1) with more time allowed between injection and infection with *D. corticola*, (2) with more injections per season, and (3) a larger dose per tree. These factors should allow continued and better distribution of these fungicides in the wood xylem and canopy and probably secure the higher fungicide efficacy in Bot canker control, especially on larger trees.

## 4.2 *Venturia inaequalis*

We evaluated the similar fungicides on apple, *M. pumila*, another hardwood tree species. When Phosphojet and Inspire Super, where the latter one contains a DMI (demethylation inhibitor) fungicide difenoconazole from the same class as Alamo or Propizol (propiconazole), were trunk-injected for management of apple scab fungus *V. inaequalis*, the best control was achieved with Phosphojet and then by Alamo.

The efficacy against this subcuticular pathogen that infects just below the waxy layer on leaves and fruit, clearly depended on the apple canopy organ and the time/s of fungicide injection/s. Namely, on spurs which hold much fewer leaves in total in comparison to the shoots, the best leaf scab incidence reduction was 45.5 and 46.3%. In contrast, scab reduction on shoot leaves with Phosphojet reached 66.5 and 73.6%. On apple fruit, scab reduction reached up to 62.8, 64.6 and 69.7%. These efficacy patterns clearly demonstrate the differential influences of the tree's yearly and organ-specific physiology, the properties of injected compound, and the injection timing on the accumulation of fungicides in the canopy. Since the major water transport in xylem, occurs in spring, at least one to two injections of phosphites in early spring gave a good disease control, depending on the canopy organ. The best scab control with injected phosphites was achieved on the shoot leaves, followed by apple fruit, and then on the spur leaves. The injected phosphites probably accumulated more in the shoot leaves than in the spur leaves and they accumulate more in fruit than in spur leaves. This can be explained by the variable rates of transpiration from these organs, which influences the speed and abundance of fungicide accumulation after trunk injection. The total leaf area on shoots is larger in comparison to spurs. The fewer leaves on spurs, which are first to develop in spring and early reach their full size, have fewer total number of stomata on them in comparison to more numerous shoot leaves. Additionally, from petal fall up until terminal bud set, shoots keep growing and developing more leaves on the tips. Hence, apple shoots hold the higher number of stomata in total, thus allowing much higher transpiration intensity, abundant accumulation of injected fungicides and thus scab control. Similarly, apple scab control was lower on fruit than on shoots which could be explained by the fact that apple fruit hold 10- to 100-fold lower frequency of stomata on their epidermis in comparison to the apple leaves [122].

The chemical properties of different active ingredients impact their distribution and accumulation in the canopy. For example, potassium phosphites have higher water solubility of 500 g/L in comparison to propiconazole and difenoconazole which have low to very low water solubilities of 100–150 mg/L and 13 mg/L, respectively [121, 123]. Potassium phosphites have low organic carbon-water partitioning coefficient ( $K_{o/c}$ ) from 228 to 587 ml/g in comparison to moderate to high of propiconazole, 1086–1817 ml/g, and of difenoconazole, 3870–11,202 ml/g, respectively [119, 120]. This difference likely allowed phosphites to move faster in xylem [124] and accumulate more in leaves and fruit than the other injected fungicides. At the same time, propiconazole and difenoconazole were probably bound to the organic phase of xylem symplast and apoplast, thus lowering their accumulation in leaves and fruit and reducing their effect on scab incidence [65]. This is often referred to as a reservoir effect and  $K_{o/c}$  as is an important property of a pesticide that can explain its limited or abundant accumulation in the canopy [65, 125]. Besides the  $K_{o/c}$  and water solubility, the inactive components of the Inspire Super pesticide formulation we injected (stickers, emulsifiers, surfactants, etc.) could reduce the abundant accumulation of difenoconazole and a better scab control. Fungicides have to be formulated for injection to secure their upward translocation in xylem and often diluted prior to trunk injection to reduce the impact of  $K_{o/c}$  effect. Once the high solubility, low  $K_{o/c}$

and injectable formulation are possible for one active ingredient, a rapid and desired control effect on plant pathogen or insect pest can be expected [42, 45, 126].

The reduction of apple scab and our prior work on analyzing the residues of injected pesticides on apple leaves and fruit [12, 61] indicates that accumulation of trunk-injected fungicides in the wood and canopy is a time-demanding process chiefly shaped by the tree physiology and tissue resistance points [127, 128]. Trunk injection is an opposite process to the immediate deposition of fungicide solution on the tree canopy by foliar spray applications. However, even though the injected dose per tree of phosphites in Phosphojet was 1.6–2 times higher than in the Agrifos sprays, the fact that just two injections secured better control of scab on fruit and spur leaves in comparison to nine Agrifos sprays demonstrated better persistence of injected Phosphojet. This shows that trunk injection is a superior delivery method for phosphites as it enhances their activity for 1–2 growing seasons [12].

### 4.3 *Erwinia amylovora*

The fire blight bacterium *E. amylovora* is a pathogen of apple trees with a unique and complicated biology involving several lifestyles: (1) *in planta* overwintering in fire blight cankers on bark or asymptotically in host buds or as latent infections in asymptomatic wood [101], (2) residing on different plant surfaces and colonizing flower surfaces before their infection, and (3) migration after infection to other close or far host tissues and organs through colonizing the cortical parenchyma and xylem vessels. Therefore, it seems that for the stages of pathogen overwintering in wood or bark and especially for migration via xylem, the use of trunk injection delivery of compounds active against *E. amylovora* might be the most suitable way to control this pathogen. Overall, our trunk injection experiments with antibiotic bactericides, Phosphojet and Actigard, both known SAR-activators [11], demonstrated good to poor fire blight incidence reduction in years with low and high infection pressures, respectively.

The best control i.e. reduction of blossom blight incidence across both trial years was achieved with two trunk injections of Agrimycin (45%) and of Phosphojet (40.5%). However, under high and low infection pressures in the two trial years, the levels of control with these materials (28.9, 61.1%, 25.1, 55.9) were far from comparable to 92–99% control often achieved and expected with preventive flower spray application of Agrimycin and Kasugamycin in commercial apple orchards [129, 130]. In the case of injected Phosphojet and Actigard, the achieved blossom blight reduction probably originated from an SAR effect triggered in the nearby spur leaves by these compounds, as the SAR effect in flowers was inconsistent [11]. SAR is a defense plant response which is activated after localized plant exposure to a pathogen or after a spray applications of a synthetic or natural compound, known as an SAR-inducer or activator [131]. Our 1–2 trunk injections of Actigard reduced blossom blight incidence for only 19–42%, indicating that this delivery method cannot not improve the SAR-effect of Actigard on flowers to combat blossom blight successfully. Namely, different sources report from 3 to 91% of blossom blight control with foliar sprays of Actigard on other apple cultivars [132–134].

Vegetative flowers parts in *Malus* species and later fruit have 10- to 100-fold lower frequency of stomata on epidermis when compared to the epidermis of leaves [122]. Flowers also have a considerably smaller green tissue volume. This leads to a conclusion that due to a very low transpiration footprint of green flower parts with lower number of stomata in comparison to the leaves and fruit, accumulation of injected compounds in these parts was weaker and slow thus reducing their efficacy. Second, it is possible that the injected antibiotics could not reach the surface of stigmas where *E. amylovora* multiplies to reduce its populations as successfully as



after the topical spray application, or that they do reach stigma surfaces but at a too low of a dose or too late for a better reduction. The reached levels of control with the injected Agrimycin probably originated from the limited accumulation i.e. presence of a suboptimal dose of this antibiotic in the green flower tissues. This only partially stopped the progress of the infection once *E. amylovora* entered the flower tissues. Therefore, the injected compounds aiming to reduce blossom blight should be formulated to translocate and accumulate faster in flower green tissues to reach a potentially higher efficacy. Otherwise, these should be injected much earlier in comparison to our injection dates, probably in fall of the previous year, to increase the time for compound accumulation and ultimately improve the disease reduction. A process of optimizing the trunk injection timing/s is a common topic research on agricultural tree crops to maximize the effect in pathogen or pest control [10, 12, 50, 56]. It appears that higher dose of injected compounds might be necessary for longer-lasting control of fire blight on both flowers and shoots.

Even though reduction of shoot blight incidence was not statistically significant in year one, which was characterized with low infection pressure, it indicated that trunk-injected Agrimycin and Phosphojet might have potential to perform better than Actigard treatments. However, in year two, under the heavy infection pressure, this was not the case as all the injected treatments were similar. Overall, it seems that the reduction of shoot blight incidence with injected Agrimycin and Phosphojet across both years of 53.5 and 42.8%, was slightly better than the reduction of blossom blight incidence with the same materials of 45 and 40.5%, respectively. Shoots obviously have much higher green tissue area and transpiration rate in comparison to the flowers. Shoots likely accumulate higher amounts of trunk injected compounds in comparison to the green flower parts, which allowed slightly better disease reduction early after injection. Still, the shoot blight incidence reduction was far from the expected control with spray applied antibiotics in commercial apple orchards. In a trial with trunk injection of Arborfos (45.8% mono- and di-potassium salts of phosphorous acid, Mauget Inc., Arcadia, CA, USA), shoot blight was reduced for 67% on inoculated 'Paulared' apple trees [116]. The same dose per tree which we delivered in two injections of Phosphojet, achieved shoot blight incidence reduction of 23.4–62.1%. Since we have split the dose delivery temporally, this weakened shoot blight incidence reduction by Phosphojet and probably by Actigard too. In shoot inoculated trials multiple Actigard sprays achieved shoot blight reduction between 2.8 and 50.7% [135, 136] while by trunk injection we achieved only 1.7–30.9% of shoot blight reduction. Hence, the two-time trunk injection does not improve shoot blight reduction by Actigard.

The reduction of shoot blight severity with Arborbionic (MFG Scientific Inc., USA) was excellent and reached up to 82%. Such an effect with oxytetracycline hydrochloride demonstrates that this active ingredient is readily soluble in water and that the formulation we used is designed for trunk injection. Our results indicated that the trunk injected Arborbionic limits i.e. stops systemic spread of *E. amylovora* in xylem of apple shoots [11]. Even though oxytetracycline hydrochloride is a bacteriostatic, when we delivered it via trunk injection in apple trees only one time per year, it demonstrated prolonged effectiveness that was higher in comparison to spray applications [44, 137, 138]. Trunk injection delivery enhanced the efficacy of oxytetracycline hydrochloride in control of shoot blight severity. Finally, in our prior work we also showed that the injected Arborbionic at a dose of 0.31 g + 2.52 ml water per each 2.5 cm of trunk diameter at 30.5 cm height, can achieve a formidable reduction of blossom and shoot blight incidence for 60.6 and 60.7%, respectively [44]. This indicates that this bactericide in this formulation and probably at a slightly higher dose is the best candidate to achieve satisfactory accumulation inside and deposition on the susceptible apple plant tissues and surfaces to secure the higher efficacy.

## 5. Conclusion

Our results on management of three different pathogens with partially similar or different biologies, where *D. corticola* and *E. amylovora* invade and spread in xylem while *V. inaequalis* does not and infects subcuticularly, indicate that trunk injection of pesticides that are formulated for xylem translocation can be more-less similar in control of these three pathogens. However, the interaction of chemical properties of the active ingredient, the injected dose per tree, as well as the transpiration footprint of plant organs, played the key roles that determined the achieved levels of efficacy.

In the biology i.e. life cycle of *D. corticola*, it seems that the dominant phase is the invasion and necrosis of xylem, leading to vascular occlusion, canopy wilting and canker development on wood before it kills oak trees. Hence the logical approach to prevent this disease is trunk injection delivery of fungicides. In our two-year experiments on potted trees, the injected potassium phosphites (Phosphojet) achieved levels of Bot canker control in xylem of up to 71.1%. Averaged across both years, potassium phosphites achieved disease reduction of 58.5%, but the more consistent results were achieved with fungicides thiabendazole (Arbotect) and propiconazole (Propizol) which reduced xylem necrosis for 57.5 and 53.3% on average. The maximums in reduction in individual years for these two fungicides were of 60.5 and 69.3%, respectively. We predict that higher efficacy with these fungicides can be achieved with optimization of preventive fungicide injection which would increase the uniformity of distribution of these fungicides in xylem, thus increasing their efficacy.

In the case of *V. inaequalis*, for which the injected fungicides would need to translocate the farthest via xylem to reach and accumulate in and on the epidermal cells of green plant surfaces in tree canopy, the most efficient apple scab reduction of 45.5–73.6% was achieved with potassium phosphites (Phosphojet). Unlike this readily mobile compound, scab control with propiconazole that is much less xylem mobile ranged from 17.1 to 51.5%, while the least xylem mobile difenoconazole underperformed with only up to 10.8% apple scab control. It is assumed that the injected potassium phosphites secured its efficacy against *V. inaequalis* through a strong plant defense response in the tissues called SAR [11, 139], as apparently it is not directly toxic to this pathogen [140]. We speculate that better efficacy with other systemic fungicides active against apple scab might be achieved if their formulations were re-designed for trunk injection i.e. to facilitate easier and faster translocation in xylem, thus securing higher accumulation in tissues exposed to infection.

Finally, there is the case of a complex biology of *E. amylovora* which combines life stages of inhabiting and multiplying on plant surfaces, migrating through internal host tissues after infecting, dwelling and overwintering asymptotically in host buds or wood, and overwintering in fire blight cankers on bark. The injected compounds active against this pathogen would need to translocate and distribute in in xylem and phloem, reach in and onto the stigma surfaces of flowers and accumulate at effective doses in these and green tissues of the apple tree canopy. Based on presented research, it seems that these multiple difficult tasks in this and our previous study [44] were best achieved with oxytetracycline hydrochloride—both on the apple flowers [44] and on shoots [11]. Overall the injected antibiotic streptomycin (Agrimycin) formulated for foliar application gave the best reduction of blossom blight ranging from 28.9 to 61.1% and of shoot blight from 36.5 to 70.4%. The shoot blight severity reduction with Arboriotic, the injectable formulation of oxytetracycline hydrochloride, reached an excellent 82%. Hence, the effect depended on the plant organ, bactericide active ingredient, injected dose and formulation. The SAR-activating potassium phosphites (Phosphojet) were the second best to antibiotics with 25.1 and 55.9% of blossom blight reduction and 23.4 and

62.1% of shoot blight reduction. Actigard underperformed with blossom blight reduction of 19 and 42% and shoot blight reduction of only 1.7 and 30.9%.

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## **Conflict of interest**

All authors declare that the research was conducted without any commercial or financial relationships that could be interpreted as a potential conflict of interest.

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