Floral traits affecting fire blight infection and management

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Abstract *Erwinia amylovora*, the causative agent of fire blight, colonizes primarily the flowers of the sub-family Maloideae. Commercially important fruit tree species such as apple (*Malus domestica*) and pear (*Pyrus communis*) are also affected by the disease. Epiphytic bacterial populations develop on the stigma, from where the pathogen colonizes the hypanthium, aided by moisture. Under favorable conditions, nectar provides a rich medium for growth, which allows bacterial invasion of tissues through the stomata of the nectary. The paper reviews various floral traits that may play a role in the onset and progression of the infection. Flower age, stigma morphology and longevity, the size of epiphytic bacterial population, morphology of the hypanthium, anatomy of the nectary, dynamics of nectar secretion, as well as the volume, concentration and composition of the nectar are discussed in detail, comparing traits of susceptible versus tolerant apple and pear cultivars. Management programs, aiming at the suppression of *E. amylovora* on floral parts by antibiotics,

chemical compounds, natural substances or biological control agents, are also discussed.

Keywords Bacterium · Biological control agent · *Erwinia amylovora* · Hypanthium–*Malus* · Nectar · Nectary · *Pyrus* · Stigma

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora* (Burr.) Winslow et al., is one of the most devastating diseases of pome fruit trees (van der Zwet et al. 1988; Johnson and Stockwell 1998). Commercially important fruit tree species such as apple (*Malus domestica*) and pear (*Pyrus communis*) are also affected by the disease. All the aerial parts of the hosts can be infected by the pathogen. The most common and characteristic symptoms include wilt and death of flower clusters, withering and death of shoots and twigs, leaf blight, fruit blight as well as limb and trunk blight (CABI and EPPO 1997).

For blossom infection, *E. amylovora* needs to increase its population size through an epiphytic phase that may occur on the stigma and the hypanthium. The bacterium first colonizes the stigma, multiplies on its surface, followed by migration along the style to the flower cup (hypanthium), facilitated by rain or heavy dew. The pathogen gains entry to the floral tissues through the nectary stomata (nectarthodes) located on the hypanthial surface (Hattings et al. 1986; Thomson 1986; Wilson et al. 1989, 1990; Johnson and Stockwell 1998; Pusey 1999a; Bubán et al. 2003a).

Prevention of blossom infection is one of the primary goals in fire blight management, because the bacterium population growing on floral surfaces provides much of the

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inoculum for secondary phases of the disease, including the infection of shoots, fruits and rootstocks (Schroth et al. 1974; van der Zwet and Beer 1991).

The paper reviews various floral traits that may play a role in the appearance and progression of the infection. Flower age, stigma morphology and longevity, the size of epiphytic bacterial population, morphology of the hypanthium, anatomy of the nectary, dynamics of nectar secretion, as well as volume, concentration and composition of the nectar are discussed in detail, comparing traits of susceptible versus tolerant apple and pear cultivars. Management programs, aiming at the suppression of *E. amylovora* on floral parts by antibiotics, chemical compounds, natural substances or biological control agents, are also discussed.

Hosts of fire blight

Altogether 129 plant species of 37 genera in the *Rosaceae* family are known as susceptible hosts of *E. amylovora* (Bubán 2007). The principal and most susceptible hosts are in the sub-family Maloideae of the family Rosaceae, including both cultivated and native wild plants, such as serviceberry or saskatoon (*Amelanchier* spp.), Japanese quince and Chinese flowering quince (*Chaenomeles* spp.), cotoneasters (*Cotoneaster* spp.), hawthorns (*Crataegus* spp.), quinces (*Cydonia* spp.), apples (*Malus* spp.), fire-thorns (*Pyracantha* spp.), pears (*Pyrus* spp.), rowans or mountain ashes (*Sorbus* spp.) and *Stranvaesia* spp. (CABI and EPPO 1997).

Johnson et al. (2006) evaluated epiphytic growth of *E. amylovora* on flowers of plant species common to landscapes where pears and apples are grown. The investigated plants included genera regarded as important nectar and pollen sources for pollinating insects: *Acer*, *Amelanchier*, *Brassica*, *Cytisus*, *Populus*, *Prunus*, *Rubus*, *Salix*, *Taraxacum*, *Trifolium* and *Symphoricarpos*. All species from the Rosaceae family except European plum (*Prunus domestica*) supported epiphytic populations of the pathogen, whereas nonrosaceous plants were generally poor supporters of epiphytic growth. Similarly, rosaceous ornamental shrubs like *Cotoneaster*, *Crataegus*, *Malus*, *Pyracantha* and *Sorbus* taxa in the vicinity of orchards can also serve as important inoculum sources, but still, despite warnings and regulations, no sufficient attention is paid to them (Thomson et al. 1975; Al-Dahmashi and Khlaif 2004; Bubán 2007). Even though populations of *E. amylovora* present on flowers of alternate hosts may not cause disease, the flowers of rosaceous non-disease-host species could serve as potential sites of inoculum increase during their periods of bloom, because vectors of *E. amylovora*, principally bees, visit many kinds of flowers in landscape areas

between pear and apple orchards (Johnson et al. 2006; Bubán 2007).

In the Netherlands, legislation allows the Plant Protection Service to take measures against the spread of fire blight, including uprooting of diseased plants, prohibition of planting very susceptible host plants and flowering prohibition for hawthorn. As a result, the incidence of fire blight infections has decreased by more than 90% (van Teylingen 2002).

In Bulgaria, where the spread of fire blight has nearly eliminated commercial production of pear and quince during the last 20 years, quince is the host most frequently attacked by *E. amylovora*, and infected trees provide a permanent source of inoculum. The importance of quince as a fire blight host encouraged a breeding program, which resulted in the selection of new quince genotypes that combine resistance to fire blight and high fruit quality. Production of other pome fruit crops is also expected to benefit from reduced inoculum levels (Bobev et al. 2009).

Environmental factors

The development of fire blight is affected by a number of environmental factors such as air temperature and relative humidity (RH). Rain, followed by warm, cloudy weather, especially during blossoming and shoot growth, is very favorable to epidemic outbreaks of fire blight (van der Zwet and Keil 1979; van der Zwet 1986).

Growth of *E. amylovora* and antagonistic bacteria on stigmas was shown to be dependent on temperature. Pusey and Curry (2004) demonstrated that within the range from 4 to 24°C, the diurnal pattern of temperature variation did not affect growth of *E. amylovora* on stigmas; growth was always dependent on mean temperature. The highest temperatures allowing growth of *E. amylovora* strain Ea153 and *Pseudomonas fluorescens* A506 on crab apple stigmas were 39 and 35°C, respectively, and the upper limits for *Pantoea agglomerans* (syn. *Erwinia herbicola*) strain E325 and *P. vagans* strain C9-1 (formerly *P. agglomerans*) were above 40°C. Optimal temperatures for strains Ea153 and A506 were 28 and 24°C, respectively (Pusey and Curry 2004).

High temperatures can lead to sizable epiphytic populations of the pathogen before significant flower senescence occurs (Pusey and Smith 2008). This was demonstrated with experiments where newly opened detached flowers were incubated at a constant 24°C (which exceeds typical temperature averages in the orchard), and *E. amylovora* populations increased from 10³ colony-forming units (CFU) to 10⁶ or 10⁷ CFU per flower within 24 h, and yet the flowers appeared only slightly changed (Pusey 1997, 2000; Pusey and Curry 2004; Pusey and Smith 2008).

Taylor et al. (2003) showed that the ability of the pathogen to multiply to levels that cause infection was dependent on temperature, rainfall and flower age, in accordance with the results of Thomson (1986), Pusey (2000) and Shwartz et al. (2003), who found that *E. amylovora* populations on apple and pear flowers increased irrespective of humidity levels, and the main climatic factors governing flower infection were temperature and rainfall.

The availability of water is critical in the sequence of events leading to infection (Pusey 2000). However, Rosen (1933) and later Thomas and Ark (1934a, b) reported that rain was not necessary for fire blight infection. Thomson et al. (1975) confirmed that precipitation was a requisite neither for colonization, nor for disease development. At the same time they highlighted the importance of dew formation, frequently observed on trees during periods of high humidity and cool nights. Researchers generally agree that wetting events enhance both pathogen dissemination and infection (Thomson et al. 1975; Thomson 1986). Using detached flowers of crab apple, Pusey (1999b, 2000) demonstrated that wetting was required for infection to occur, however, the duration of wetting was not important.

During initial settlement of the bacterium on flower surfaces, water in the vapor form (especially in the case of high humidity levels) is an important factor, but not the only determinant of moisture availability. In the field, soil moisture affects host water potential, which relates to the secretion of fluids by stigmatic and hypanthial tissues. Comparing the two potential sites of epiphytic growth, *E. amylovora* could multiply on the stigma at a lower RH (55–100%) than in the hypanthium (RH >80%) (Pusey 2000). These observations are consistent with those of Thomson (1986), who reported the increase of bacterial populations on inoculated pear stigmas both at high (70–100%) and low (<20–30%) RH, but populations on inoculated pear hypanthia declined when RH was low. Thomson (1986) concluded that colonization of stigmas by *E. amylovora* under dry conditions did not lead to blossom blight unless rain facilitated movement of the pathogen to the hypanthium where infection generally occurs. He suggested that the role of rain in bacterial movement is more important than its role in diluting nectar, as was suggested earlier by Ivanoff and Keitt (1941) and Keitt and Ivanoff (1941).

Flower age

Both laboratory and field studies with apple (Taylor et al. 2003; Thomson and Gouk 2003; Pusey and Curry 2004) and pear (Lindow and Suslow 2003) indicate that a relationship exists between flower/stigma age and the rate of

epiphytic bacterial growth, as well as incidence of infection. Longevity of floral parts (Soltész et al. 1996) and duration of their receptivity to bacterial colonization (Lindow and Suslow 2003; Thomson and Gouk 2003; Pusey and Curry 2004) is temperature dependent (Pusey and Smith 2008).

The time period during which crab apple stigmas had the capacity to support bacterial populations decreased as temperature increased, and it was generally shorter for pollinated than non-pollinated flowers. In ‘Gala’ apple flowers even 12-day-old stigmas could support bacterial growth, and pollination had a negative effect on the capacity of stigmas to support bacterial growth (Pusey and Curry 2004), similarly to crab apples. When ‘Gala’ flowers were open for <24 h, papillae appeared bulbous and turgid. As stigmas aged, increasing portions of the papillae collapsed, interstices widened, and the underlying cuticular layers became more visible. Rate of papilla degeneration was greater at high temperatures than at low temperatures: under high temperatures most papillae were collapsed 8 days after flower opening; whereas papillae were not totally collapsed until 12 or 14 days at low temperatures (Pusey and Curry 2004).

Both laboratory and field experiments of Pusey and Curry (2004) indicated that flower stigmas can support bacterial growth at ages older than reported by Thomson and Gouk (2003), who found that *E. amylovora* populations did not increase on ‘Gala’ apple flowers when stigmas were older than 3 or 4 days at the time of inoculation under arid or humid conditions, respectively. The same pattern of bacterial growth was reported for *Ps. fluorescens* strain PfA506 and *P. agglomerans* strain Eh318. Stigmas inoculated at day 3 or 4 supported bacterial growth up to day 6 or 8, but stigmas inoculated after day 4 were not at all conducive to bacterial growth (Thomson and Gouk 2003). Differences in the study of Thomson and Gouk (2003) versus that of Pusey and Curry (2004) might be explained by the fact that the latter experiment was conducted under laboratory conditions in excised flowers, where no naturally occurring epiphytic organisms were involved. For *Ps. fluorescens* strain A506, Lindow and Suslow (2003) reported that populations in pear orchards were initially about 10^4 CFU per flower and increased to approximately 10^6 CFU per flower in flowers that were inoculated within about 5 days of opening. Eventual populations decreased with further increases in flower age at inoculation to as few as about 10^3 CFU per flower, when inoculated flowers were more than 10 days old.

In laboratory experiments of Pusey and Smith (2008), when hypanthia of apple flowers were directly inoculated with *E. amylovora*, severity and incidence of infection decreased with flower age, and the rate of declining susceptibility increased with temperature. Inoculation of

newly opened flowers resulted in maximal severity ratings and an infection incidence of 100%. As flowers aged, they became less susceptible to invasion of hypanthial tissues.

Size of the bacterial population

A single cell of *E. amylovora* can potentially infect pomaceous flowers through the hypanthium (Hildebrand 1937a; Cabrefiga and Montesinos 2005); however, the minimum infective dose depends on environmental conditions (Pusey 2000), pathogen aggressiveness (Cabrefiga and Montesinos 2005) and also on cultivar susceptibility (van der Zwet and Keil 1979).

The quantity of epiphytic bacteria transferred to the flower hypanthium during wetting is limited by populations on the stigma (Pusey and Smith 2008), which reach maximal levels between 10^6 and 10^7 CFU per flower (Johnson and Stockwell 1998; Pusey and Curry 2004). The upper limit of bacteria transferred from apple stigmas to hypanthia seems to be 10^4 CFU per hypanthium, during a few hours of wetness (Pusey and Smith 2008). The field experiment of Pusey and Smith (2008) showed that the inoculum level of 10^2 CFU per hypanthium caused a very low disease incidence or no detectable disease symptoms.

Taylor et al. (2003) observed disease symptoms only when population levels of *E. amylovora* exceeded 10^6 CFU per flower by 4 days after inoculation. They concluded that the greater the number of bacterial cells that arrive on the stigma and the younger the flower, the greater is the probability of rapid colonization, leading to infection and expression of symptoms. Taylor et al. (2003) demonstrated that even 100% infection of apple flowers could result from inoculation with 10^5 – 10^8 CFU ml⁻¹, even when infection risk periods were low. This result is consistent with the research of Shwartz et al. (2003), which suggested that a large amount of inoculum might compensate for sub-optimum conditions for infection.

Hasler and Mamming (2002) demonstrated that high pathogen populations develop not only on the stigma and stylus, but also on the anthers and hypanthium, implying that a high RH in the hypanthium (diluting nectar to concentrations that favor *E. amylovora* multiplication) can probably be sufficient for infection to occur, and bacteria must not be washed down from the stigma or anthers.

Following a wetting period, the size of *E. amylovora* populations in the hypanthium was closer to populations on the stigma in pear flowers (Thomson 1986) than in crab apple flowers (Pusey and Smith 2008). This is probably due to the more open hypanthia of pear compared with apple and crab apple, being able to receive and accumulate more bacterial cells moving via water from stigmas (Pusey and Smith 2008).

In order to reliably predict the infection risk, estimating the epiphytic population size is essential, together with local environmental data. Various methods have been worked out for a fast and reliable estimate. Thomson (1992) suggested stigma imprints as a simple, rapid and inexpensive way of monitoring epiphytic populations, enabling the sampling of large numbers of flowers directly in the orchard (Thomson et al. 1999). When applying this method, stigmas are touched to the surface of CCT medium, which was specifically designed for detecting *E. amylovora* (Ishimaru and Klos 1984). The stigma imprint technique was shown to be more sensitive than flower washing for detecting the presence of *E. amylovora* on the stigmas of inoculated flowers (Thomson et al. 1999). Dorgai and Bubán (2002) developed a PCR-based method, in which the population size was estimated by measuring and comparing the strength of the sample PCR signals to those of serial dilutions of known amounts of bacteria. Kritzman et al. (2001) developed a diagnostic, selective medium, which allows identifying *E. amylovora*. Sample flowers are touched onto the agar medium and stored at room temperature. 26–30 h later, *E. amylovora* bacteria appear in the form of typical red colonies, which are distinctively different in shape and color from other bacteria. To improve disease risk assessment, Temple and Johnson (2009, 2011) developed a rapid pathogen detection protocol that utilizes the method of loop-mediated isothermal amplification (LAMP) to target and amplify DNA of *E. amylovora*. The method quickly (1–2 h) and reliably detects a single, epiphytically colonized flower in a sample of 100 clusters (ca. 600 flowers).

Stigma and style

Rosaceae species are characterized by “wet-stigmas” (Heslop-Harrison and Shivanna 1977) that are covered with club-shaped papillae, with an additional cuticular layer. Pomaceous stigmas and their exudates sustain a wide diversity of bacteria, yeasts and other fungi, with microbial population densities far exceeding those on other aerial plant surfaces (Pusey et al. 2008a). *E. amylovora* was found to survive better on the stigma than on the hypanthium and other flower parts. The pathogen survived at least 14 days on 80% of the pistil-inoculated flowers, whereas bacteria were reisolated from only 20% of the flowers inoculated on the hypanthium (Thomson 1986). *E. amylovora* appears to be well adapted epiphytically to the stigmatic surfaces of most rosaceous species, even non-disease-host plants (Johnson et al. 2006).

The duration of stigma suitability to maintain bacteria is of crucial importance. Stigma longevity lasts longer in homogamous flowers compared to protogynous ones, both

in apple (Orosz-Kovács et al. 2001) and pear (Farkas 2001; Farkas and Orosz-Kovács 2009). Spinelli et al. (2005a, b) demonstrated that 24–72 h after pathogen inoculation, stigmatic papillae maintained their structural integrity even in heavily colonized stigmas, and no damage due to *E. amylovora* colonization was found. After 72 h stigma integrity started to decrease as a consequence of both the pathogen colonization and the natural senescence processes. Between 72 and 120 h after inoculation, the majority of papillae collapsed both on the stigma and the stylar groove. These results reinforced the previous findings of Gouk and Thomson (1999) and Thomson and Gouk (2003), who claimed that stigmatic papillae of 4-to-6-day-old apple flowers were completely collapsed and covered in mucilage.

In apple and pear, exudates are present on stigmas from the time flowers first open, or even earlier, particularly in protogynic flowers with the stigma protruding out of the closed red bud, as in some apple and pear cultivars (Orosz-Kovács et al. 2004; Farkas and Orosz-Kovács 2009). Stigmatic exudates originate from cells beneath the papillate epidermis, and subsequently emanate also from the papillae (Heslop-Harrison 1976; Stant 1981; Sanzol et al. 2003). *E. amylovora* multiplies mainly in the large inter-papillar spaces among the stigmatic papillae, where stigmatic exudates provide a protected, nutrient-rich, hydrated environment (Hattingh et al. 1986; Thomson 1986; Wilson et al. 1989, 1990; Johnson and Stockwell 2000; Spinelli et al. 2007).

Epifluorescence microscopy is one of the frequently used techniques for visualizing the bacteria on the plant surface and inside the tissues, using *E. amylovora* bacteria labeled with the gene coding for the green fluorescent protein (*gfp*). This chromophore requires no exogenous substrates for fluorescence and the labeled cells can be studied without fixation when irradiated with UV light (Bogs et al. 1998; Bogs and Geider 1999; Mihalik et al. 2003). Using the epifluorescent technique, sensitive and tolerant apple cultivars were compared by Mihalik et al. (2004), following inoculation with labeled pathogens. The colonizing bacteria covered the surface of the stigmatic papillae and could also be detected in the stigmatic fluid among and over the papillae. Bacteria did not firmly adhere to the cell walls, and they could easily be washed away from the stigmatic surface. Mihalik et al. (2004) did not observe any apparent difference between susceptible and tolerant cultivars in the intensity of fluorescence on the stigma, indicating that the tolerance or sensitivity to the bacterium was not associated with stigmatic features.

The composition of stigma exudates in pomaceous flowers has recently been analyzed by Pusey et al. (2008a). Stigma exudates in apple were made up by 4.5%

free sugars, 49.6% polysaccharides (composed of arabinose and galactose) and 45.9% proteins. The two largest components are likely to be bound together as glycoproteins in stigma exudates. At each stage of anther dehiscence, free sugars in stigma exudates of pear, apple and crab apple flowers were predominantly glucose and fructose in near equal proportions, with only trace amounts of sucrose, sorbitol and inositol. Among the three plant species studied, sugar quantities were highest for apple, followed by crab apple and pear, likely due to differences in stigma size and surface area. As expected, quantities of free sugars in stigma exudates were lower than those in floral nectars. Relative to sugar levels, the quantities of free amino acids in stigma exudates were extremely low (<1 pg per flower), with higher values for apple than pear. For all flower types, the predominant free amino acids were proline and asparagine, followed by glutamic acid, glutamine and serine. The amount of predominant free sugars and amino acids, which may serve as carbon and nitrogen sources for microbial activity, increased during anthesis (Pusey et al. 2008a).

Rosaceae flowers feature a solid style, the inner part of which is constituted by a bundle of glandular cells which form the transmitting tissue (Cresti et al. 1980). Originating from the stigma, a groove is running along the style to the nectar cup, apparent in apple and pear, as well as hawthorn and firethorn flowers. In some apple and pear cultivars, similarly to the stigmatic surface, the epidermis of the groove is constituted by stigmatic papillae (Spinelli et al. 2005a). The stylar groove epidermis is separated from the transmitting tissue by several layers of parenchymatous and vascular tissue (Cresti et al. 1980; Spinelli et al. 2005a). Both pathogenic (*E. amylovora*) and antagonistic bacteria (*P. agglomerans*) exploit the stylar groove during their movement from the stigmatic surface to the nectarhodes. Similarly to the stigmatic surface, bacterial cells are mainly localized among the numerous papillae in the stylar groove (Spinelli et al. 2005a). Higher bacterial populations were detected on the stigma than on the style. The rapid growth of bacteria was observed on both sites until they reached the estimated carrying capacity of pistil. For the first 24 h after inoculation, bacteria remained confined to the pistil (Spinelli et al. 2005a).

Rosen (1936) supposed that bacteria penetrate the stigmatic tissues mostly through intercellular spaces or between two adjoining cells by dissolution of the walls. Similarly, Pierstorff (1931), Hildebrand (1937b), Rundle and Beer (1987) suggested that *E. amylovora* penetrates through the stigma and migrates inside the style towards other plant tissues. However, Mihalik et al. (2004) and Spinelli et al. (2005a) demonstrated that bacteria never penetrated the living cells of either the stigma or the style, even 96 h after inoculation.

Hypanthium and nectary

The most common site of *E. amylovora* infection is the hypanthium, where nectar is secreted. The pathogen gains entry to the inner floral tissues via the nectar secreting stomata, called nectarhodes (Hildebrand and MacDaniels 1935; Rosen 1936; Bubán et al. 2003a). The nectary plays an important role also in the transmission of another *Erwinia* species, *E. tracheiphila*, which is the causative agent of bacterial wilt disease in cucurbits (Sasu et al. 2010a).

The nectary is mostly receptacular in the *Rosaceae* family, consisting of three histologically distinct parts: the cuticle-covered epidermis, the glandular tissue and the nectary parenchyma (Kartashova 1965). Characteristic differences can be observed in the morphology of the nectar gland between apple and pear: the calyx cup (hypanthium) in the apple flower is narrow and elongated, contrasted with the broad, open and shallow calyx cup of pear flowers (Rosen 1936), and consequently the nectaries of apple are less exposed than those of pear (Rosen 1936; Bubán et al. 2003a; Farkas et al. 2007). Under low RH, bacterial survival is probably better in apple or crab apple hypanthia than in pear hypanthia, which are relatively more open and more subject to desiccation (Pusey 2000).

The receptacular nectary of apple is located between the stamens and the ovary (Farkas et al. 2007). In apple, the cuticular pattern of the nectary (hypanthium) is highly variable and can be specific to cultivars (Orosz-Kovács et al. 1990; Scheid-Nagy Tóth 1991). Mihalik et al. (2003) reported that a susceptible apple cultivar was characterized by thin nectary cuticle, as opposed to the thick, waxy cuticle of a tolerant cultivar. The hypanthial features of sensitive ('Jonagold Decosta', 'Sampion') and tolerant ('Freedom') apple cultivars were evaluated by Mihalik et al. (2004), Orosz-Kovács et al. (2004) and Farkas et al. (2007). They demonstrated that the wrinkled hypanthium surface and the sunken stomata of the sensitive apple cultivars helped to preserve a suitable medium for bacteria to enter the floral tissues through the nectary stomata and build up bacterium aggregates around the stomatal pore. In contrast, the hypanthial surface of the tolerant cultivar was much smoother and stomatal guard cells were either at the level of the epidermis or rising above it. This arrangement of guard cells, accompanied by a smooth nectary surface, facilitates evaporation of nectar, and fails to provide optimal circumstances for the pathogen. In sensitive cultivars, the presence of a large number of sunken stomata on a highly ornamented, wrinkled nectary surface enhances the storage of nectar and increases the possibility of bacteria entering the inner parts of the flower (Farkas et al. 2007).

The experiments of Mihalik et al. (2004) with a sensitive apple cultivar demonstrated that bacterial aggregates could be detected in various tissues of the nectary also after

severe water loss caused by *E. amylovora* infection, accompanied by the shrinkage of the hypanthium tissues and detachment of the cuticle from the surface. Large bacterium colonies were observed in the subcuticular spaces above the stomata where the cuticle detached from the cell walls. Bacterium aggregates appeared gradually also in the intercellular spaces of the glandular tissue and parenchyma after the bacteria have entered the nectary tissues. Bacterial proliferation continued with the fast bidirectional migration of the pathogen, spreading laterally towards the outer epidermis of the hypanthium and downwards toward the pedicel, invading the parenchyma in the wall of the ovary. Although the hypanthium and the ovary wall contained vascular bundles, bacteria were not detected in the xylem (Mihalik et al. 2004; Mihalik and Bubán 2005). The authors suggested the pathogen preferred intercellular spaces for short distance migration. In contrast, Spinelli et al. (2007) found that after having reached the nectar cup, *E. amylovora* moved preferentially inside xylem vessels, although in heavily infected tissues the cortical parenchyma was also colonized. Sasu et al. (2010a) also documented the movement of *E. tracheiphila* through the nectary into the xylem of the pedicel.

Special combinations of flower morphological characteristics rather than a single feature were found to contribute to susceptibility of apple cultivars to fire blight, by preventing evaporation and thus providing a better environment for multiplication, migration and entry of the pathogen (Mihalik et al. 2003; Radvánszky et al. 2004). Such floral traits include a deep, narrow, funnel-like and convex hypanthium, nectar stomata below the level of epidermis, several stomata distributed evenly and rich hairiness of the style.

The floral nectary of pear is receptacular-ovarial, lining the adaxial surface of the plate-like receptacle and the apical part of the ovary (Farkas 2005; Farkas et al. 2004, 2006, 2007). The surface of the nectary is covered by a smooth cuticle, lacking ornamentation. Guard cells of nectary stomata can be found either at the level of epidermal cells or sunken a few cell rows below the epidermis. Below the stomata, among the cells of the glandular tissue, nectar chambers, i.e. nectar-storing intercellular cavities of varying size can be found (Rosen 1936; Farkas 2001, 2005).

The nectary was found to be of larger size in fire blight tolerant pear cultivars compared to susceptible ones (Farkas et al. 2004, 2007). Similarly to apple (Mihalik et al. 2004; Orosz-Kovács et al. 2004; Farkas et al. 2007), susceptible cultivars of pear are frequently characterized by sunken stomata, where guard cells are located 1–3 cell rows below the nectary epidermis. The size of nectar chambers is usually smaller in susceptible cultivars (Farkas et al. 2004, 2006). No relationship was found, however,

between the thickness of the nectary or that of the glandular tissue and the degree of susceptibility (Farkas et al. 2006). In contrast to apples (Mihalik et al. 2003, 2004), no relationship was found between the thickness of the nectary cuticle and the susceptibility of pear cultivars (Farkas et al. 2006).

Longitudinal sections cut from the median part of pear flowers, as well as from the nectarial region and the exterior region of the receptacle were tested for their antibiotic activity on agar plates seeded with *E. amylovora* suspension (Hildebrand and Schroth 1963). The zone of inhibition was found to be much narrower around the nectarial region than around the exterior region of the receptacle. Since polyphenol metabolism has been suggested to play a role in plant resistance, the arbutin levels in nectarial and exterior tissues were compared, to determine whether antibiotic activity was related to arbutin concentrations. Since no significant differences were found in arbutin concentration, Hildebrand and Schroth (1963) suggested that antibiotic activity was rather the function of the distribution and activity of the β -glucosidase enzyme, which hydrolyses arbutin into hydroquinone and glucose. Phenolics have received much attention also in later reports, and several phenyl-propanoids and poly-phenolic substances have been proved to play a significant role in the resistance-related metabolism of apples and pears (Goodman et al. 1986; Rademacher 2000; Römmelt et al. 2003a, b). Since differences in the phenolic profile of flower tissues may be related to the varying susceptibility of apple and pear cultivars, we have determined the amount of chlorogenic acid, a phenyl-propionic acid derivative, in various floral organs of pear cultivars that were susceptible to fire blight in varying degrees. The highest chlorogenic acid levels were found in the floral organs of the most tolerant cultivar ('Beurré Bosc'), containing roughly twice as much chlorogenic acid in each flower part as the most susceptible cultivar ('Hardenpont'). Comparing the various floral organs, highest levels of chlorogenic acid were detected in the pistils, whereas lowest concentrations were found in the anthers (Farkas et al. 2004).

Chalcones and dihydrochalcones are also abundant in pome fruits (Tomás-Barberán and Clifford 2000), with high levels of phloridzin and arbutin in apple and pear, respectively. The antibiotic activity of their respective aglucones, phloretin and hydroquinone, against *E. amylovora* has long been demonstrated (Römmelt et al. 2003b; Pontais et al. 2008). Burse et al. (2004) identified a multidrug efflux pump in *E. amylovora*, which confers resistance to apple phenolics like phloretin, naringenin, quercetin and catechin, contributing to bacterial virulence, which seems to confirm the role of phenolic compounds during the infection process (Pontais et al. 2008). Phloretin was detected in all the hypanthium samples of various

apple and pear cultivars, its levels in apple samples being approximately three times higher than in pear samples (Horváth et al. 2004a). However, *E. amylovora* was found to exhibit the ability to stabilize phloretin at sublethal levels even in a resistant apple cultivar, rejecting the hypothesis of this dihydrochalcone being involved in the resistance of the ornamental Evereste genotype (Pontais et al. 2008).

The mean content of nicotinic acid (NicAc), required for the multiplication of *E. amylovora*, was found to be approximately two orders of magnitude higher in pear hypanthium than in apple hypanthium. The characteristic differences between apple and pear in the levels of NicAc were supposed to contribute to the differential susceptibility of these two host species to fire blight (Paternoster et al. 2009a). The amount of NicAc on pear hypanthia was positively correlated with the altitude of the growing site and was inversely correlated with the sum of the maximum temperatures in the 30 days before flowering (Paternoster et al. 2011).

Nectar secretion and composition

In pomaceous flowers the nectar, consisting mainly of sucrose, glucose and fructose, is secreted through nectary stomata. The secretion product plays a crucial role in insect attraction, and hence pollination, but it also supports the epiphytic growth of bacteria, by providing a valuable source of energy (Wilson et al. 1989; Herrera et al. 2008). The floral nectar of apple and pear has been shown to be a chemoattractant for *E. amylovora* (Raymundo and Ries 1980) and *P. agglomerans* (Klopmeier and Ries 1987). Conditions for pathogen growth vary greatly, depending on the time of nectar secretion, concentration and sugar composition of nectar, environmental influences, as well as floral age and morphology (Ivanoff and Keitt 1941; Paulin 1987; Campbell et al. 1991; Johnson and Stockwell 1998). The sugar concentration of the nectar may influence the epiphytic population of the pathogen and biological control agents. Variations in the ability of different bacterial species and even strains to grow in nectar could translate into different levels of infection or control (Spinelli et al. 2005b).

Apple flowers are more likely to offer nectar on a regular basis (Orosz-Kovács et al. 2006) compared to pear flowers, where nectar is often undetectable on the plate-like, exposed nectary (Farkas and Orosz-Kovács 2000, 2003, 2004; Farkas et al. 2007). Sugar concentration of apple nectar is usually higher than that of pear nectar (Ivanoff and Keitt 1941; Campbell et al. 1990; Farkas et al. 2002a, b, 2004; Farkas and Orosz-Kovács 2004; Orosz-Kovács et al. 2004; Spinelli et al. 2005b).

Farkas et al. (2004) compared the volume and concentration of nectar produced in 24 h in a tolerant ('Beurré Bosc') and a susceptible pear cultivar ('Conference'). The flowers of the susceptible cultivar produced five times higher nectar volumes than those of the tolerant cultivar, accompanied by higher sugar concentrations in the tolerant cultivar compared to the sensitive one. We concluded that higher nectar volumes with lower sugar concentrations, being typical for the sensitive cultivar, ensure more favorable conditions for the pathogen than smaller volumes of more concentrated nectar in the tolerant cultivar.

Sugar concentration of nectar fluctuates widely as influenced by RH (Beutler 1930; Thomas and Ark 1934b; Ivanoff and Keitt 1941; Pusey 1999a; Farkas et al. 2002a). Rain or dew can dilute floral nectar, especially in flowers with exposed nectaries, and the decrease in osmotic pressure increases the suitability of the nectary for microbial colonization (Thomas and Ark 1934b; Ivanoff and Keitt 1941; Pusey 1999a). Infection occurs when nectar is sufficiently diluted, allowing *E. amylovora* to multiply and invade the floral tissues (Thomas and Ark 1934b; Ivanoff and Keitt 1941; Keitt and Ivanoff 1941). Dry conditions will lead to increased nectar concentration and osmotic pressure, causing a decrease or cessation of bacterial growth and eventual decline in the number of viable cells (Ivanoff and Keitt 1941; Pusey 1999a; Pusey and Smith 2008).

Diurnal fluctuation in nectar concentration is of great importance in fire blight infection. Thomas and Ark (1934a, b) reported that in pear flowers nectar sugar concentration changed from 8–10% in the morning to 20–22% at noon. Similarly, we found that sugar concentrations in pear might be as low as 2–6% in the early morning, followed by a gradual increase during the day, when concentration of nectar can reach 20–25%, or in some cultivars even 35% (Farkas 2001; Farkas and Orosz-Kovács 2004). Thomas and Ark proposed that the increase in volume and the reduction in concentration of nectar during humid weather bore an important relation to the incidence of fire blight. Similarly, Ivanoff and Keitt (1941) found that in apple the lower, early morning concentrations of nectar were favorable for infection, whereas higher concentrations later in the day were not.

As RH increases around flowers, nectar volume and nectar water potential increase, while nectar sugar concentration decreases, accompanied by an increase of the population size of *E. amylovora* in the hypanthium (Pusey 1999b, 2000). Population size of the pathogen is strongly related to nectar water potential; however, according to Pusey (2000), disease incidence and severity are more closely related to ovary water potential than to bacterial population size.

Several researchers confirmed that above a certain sugar concentration nectar may inhibit bacterial growth (Ivanoff and Keitt 1941; Billing 1976; Thomson et al. 1982; Smith 1990, 1996; Steiner 1990; Pusey 1999a). Based on artificial nectar experiments, Ivanoff and Keitt (1941) determined the optimal sugar concentration for growth of *E. amylovora* as 2–4% w/v. Growth rapidly decreased with increased sugar concentration, none occurring at 30%. The bacteria survived for 48, 24 and <24 h in drops containing 20, 30 and 40–50% of sugars, respectively. The experiments of Pusey (1999a) with artificial nectar indicated that optimal sugar concentration for growth of several bacterial strains, including *E. amylovora*, *P. agglomerans* and *Ps. fluorescens*, was in the range from 5 to 20%. Two biocontrol agents, *Ps. fluorescens* strain A506 and *P. vagans* strain C9-1 were shown to be less adapted to high concentrations of sugars than was the pathogen *E. amylovora* strain Ea153. Another experiment with artificial nectars, whose sugar concentrations corresponded to apple or pear flowers treated or not treated with ProCa, confirmed that *E. amylovora* (strain EaDCA 289/01) was less sensitive to sugar concentration than *P. agglomerans* P10c and EhDCA 296/01, and *Ps. fluorescens* A506, the latter strain being the most affected (Spinelli et al. 2005b). The relatively high tolerance of the pathogen to high sugar concentrations was revealed by earlier studies, as well (Thomas and Ark 1934b; Hildebrand and Phillips 1936; Ivanoff and Keitt 1941).

The ratio of nectar sugars (sucrose, fructose and glucose) in pomaceous nectar varies among species and even cultivars (Wykes 1952; Percival 1961; Campbell et al. 1990; Orosz-Kovács et al. 1997; Farkas et al. 2002a, b). Apple nectar is characterized by approximately equal proportions of the disaccharide sucrose and the monosaccharides glucose and fructose (Percival 1961; Campbell et al. 1990; Davary-Nejad et al. 1993; Orosz-Kovács et al. 1997; Nagy Tóth et al. 2000, 2003), whereas pear nectar is dominated by two hexoses, glucose and fructose (Thomas and Ark 1934b; Wykes 1952; Farkas et al. 2002a, b; Farkas and Orosz-Kovács 2004). Raymundo and Ries (1980) found that neither sucrose, nor glucose or fructose elicited a chemotactic response from *E. amylovora*.

The maximum total sugar concentration tolerated by the pathogen depends on the type and proportion of sugars present in the nectar (Thomas and Ark 1934a, b), determined as 30, 30 and 58% for dextrose, levulose and sucrose, respectively (Hildebrand and Phillips 1936). According to the artificial nectar experiments of Pusey (1999a), the population size of *E. amylovora* generally decreased as the disaccharide portion (sucrose) relative to the monosaccharide portion (fructose and glucose) was decreased.

Besides the most frequently occurring nectar sugars (sucrose, fructose and glucose), *E. amylovora* was reported to utilize other carbohydrates quickly and completely, including ribose, galactose, mannose, mannitol, sorbitol and trehalose (Hevesi et al. 2004), some of which have been detected as constituents of nectar (Baker and Baker 1981, 1983a, b). Although sorbitol is known as the major soluble carbohydrate in woody rosaceous plants, and is a frequent constituent of Mediterranean nectars (Petanidou 2005), virtually no sorbitol was found in the floral nectar of Rosaceae species (Bielecki and Redgwell 1980).

Erwinia amylovora exhibits positive chemotaxis to the organic acid fraction of apple nectar and to one of its amino acids, aspartate (Raymundo and Ries 1980). Bayot and Ries (1984) suggested that the organic acids attracting *E. amylovora* in nectar were either fumaric acid or malic acid or a mixture of both. Concentrations of either acid varied depending upon the nectar source. *P. agglomerans* was also shown to be attracted by various organic acids, as well as sugars and amino acids, asparagine and serine being the most attractive for this antagonist (Klopmeier and Ries 1984).

In addition to their main constituents (sugars), the nectars of several plant species contain secondary compounds like phenolic substances and proteins that are known to have antimicrobial properties (Baker and Baker 1983a; Adler 2000; Carter and Thornburg 2000, 2004; Nicolson and Thornburg 2007; Sasu et al. 2010b). Antimicrobial compounds in the nectar have been hypothesized to function in the attraction and reward of pollinators, by hindering microbial degradation of nectar sugars (Adler 2000); or to protect the floral reproductive tract from contamination by microbes brought to the flower by non-sterile pollinators or by airborne means (Carter and Thornburg 2004). Sasu et al. (2010b) demonstrated recently that nectar inhibited the growth of *E. tracheiphila*, the causative agent of wilt disease. It was also shown that a significantly larger proportion of the *Cucurbita* plants inoculated via flowers without nectar contracted wilt disease than plants with nectar.

Carter and Thornburg (2000) demonstrated that nectar proteins are involved in the production of hydrogen peroxide that accumulates to levels that are toxic to microorganisms. The production of H_2O_2 by the nectar redox cycle has been proposed to be a two-step process in which superoxide is produced from molecular oxygen and subsequently the superoxide is disproportionated into H_2O_2 and molecular oxygen (Carter et al. 1999, 2007; Carter and Thornburg 2000, 2004). The microscopical investigations of Carter et al. (2007) on ornamental tobacco have revealed that superoxide production is limited to the vicinity of the site of nectar secretion, protecting the bulk of the nectary from possible toxic effects of superoxide, and facilitating

accumulation of superoxide in soluble, secreted nectar for use in the nectar redox cycle. In addition, Thornburg et al. (2003) have identified a number of plant defense proteins that are encoded by cDNAs expressed in developing and mature floral nectaries. These include a number of genes directly involved in antimicrobial activities. They have also demonstrated that a number of specific defense genes were more strongly expressed in the floral nectary than in the foliage. Based on these evidences the nectary gland seems to play a major role in plant defense to protect the gynoecium from microbial attack.

Although hydrogen peroxide produced from the nectar redox cycle was shown to be a major factor contributing to inhibition of most microbial growth in floral nectar (Carter and Thornburg 2004); *E. amylovora* was reported to grow well in nectar, being resistant to the H_2O_2 concentrations found in nectar (Carter et al. 2007). The pathogen's tolerance to oxidative stress may contribute to the unique etiology of *E. amylovora*.

The role of honeybees in disease development and management

Insects have been considered to be the most important means of dissemination for *E. amylovora* (van der Zwet and Keil 1979). Honeybees (*Apis mellifera* L.), as the most important pollinators of apples and pears, have received much attention with regard to disease transmission (Pierstorff and Lamb 1934; Hildebrand 1935; Keitt and Ivanoff 1941; van Laere et al. 1981). Honeybees were found to be able to carry bacteria from the beehive to flowers, and particularly in transmitting *E. amylovora* from flower to flower (Pierstorff and Lamb 1934; Hildebrand and Phillips 1936; Keitt and Ivanoff 1941; van der Zwet and Keil 1979).

At the same time, honeybees have been implicated as the primary vectors of bacterial antagonists, as well (van der Zwet and Keil 1979; Thomson et al. 1992; Johnson et al. 1993a, b; Vanneste 1996; Johnson and Stockwell 1998; Pusey 2002). In the experiment of Thomson et al. (1992) when the antagonistic bacteria *Ps. fluorescens* and *P. agglomerans* were placed on pollen, honeybees were shown to be efficient vectors of these bacteria. *P. agglomerans* was detected on 92% of apple flowers 2 days after the start of the study; and 72% of pear flowers within 7.6 m from the hive were colonized with *Ps. fluorescens*.

Similarly, foraging honeybees were shown to be disseminators of *Bacillus subtilis* (Biopro[®]), and this biocontrol agent was detected in honey collected from hives where bees were exposed to Biopro[®] and from hives that were placed in sprayed orchards (Broggini et al. 2005). In comparison to the mason bee (*Osmia cornuta*), honeybees proved to be less efficient as carriers of the biocontrol agent

B. subtilis BD170 from sprayed flowers to new ones (Maccagnani et al. 2009), which is in agreement with the higher pollinating potential known for the mason bee when foraging on rosaceous plants (Monzon et al. 2004).

Honeybees were successfully used to disperse dried preparations of viable *E. amylovora* and *P. agglomerans* or *Ps. fluorescens* bacteria from hives to flowers, and bacteria vectored in this manner could become established on stigmatic surfaces (Johnson et al. 1993a, b). The average bee successfully inoculated a subsequently detectable population of bacteria to one flower per 20 blossom visits (Johnson et al. 1993a).

Alexandrova et al. (2002a) demonstrated that honeybees, while visiting pear flowers sprayed with *E. amylovora* suspension in the preceding 24 h, became contaminated with the pathogen and were able to disperse the bacteria to healthy plants. When the bees were analyzed 48 h after contamination, no live bacteria were detected and no transmission to healthy plants was observed. In another experiment of the same authors, honeybees were able to disperse *E. amylovora* from artificially contaminated beehives to pear flowers for 24 h, but not for 48 h after contamination of the hives.

Pierstorff and Lamb (1934) found the longevity of *E. amylovora* to range from 5 to 11 days in pure honey. The pathogen was obtained from the heads of honeybees taken from a hive 2 days after infestation. However, *E. amylovora* could not be detected on the combs, frames or in the honey in a beehive 24 h after it was infested with a suspension of the bacterium. Hildebrand and Phillips (1936) reported that *E. amylovora* was not capable of overwintering in the beehive or in association with the honeybee, therefore, these locations could not constitute a source of primary inoculum next spring. When the pathogen was introduced through the food of the bees, fire blight bacteria were not reisolated after 3 days from honey, comb, frame and bees. Maximum longevity in pollen and frame scrapings was 13 and 12 days, respectively. Fire blight bacteria were not found associated with the immature stages of the life cycle of the honeybee. Similarly to Pierstorff and Lamb (1934), Hildebrand and Phillips (1936) concluded that the survival of the pathogen in the beehive was highly improbable at the temperatures and sugar concentrations normally encountered in the apiary, since the bacteria survived for relatively short intervals (from 1 day to several weeks) at sugar concentrations of 30% and above.

More recently, Alexandrova et al. (2002b) investigated the longevity of *E. amylovora* in beehives and beehive associated products. Depending on the conservation temperature, the longevity period ranged from less than 2 to 42 days, from 2 to 21 days and from 1 to more than 30 weeks in honey, wax and pollen, respectively. Regardless of the conservation temperature, in propolis the

bacteria died shortly after contamination. *Erwinia* survived for 42–48 h and 30–36 h on bee corpses and in bee intestines, respectively.

Management with antibiotics

Management of fire blight in the United States has focused on suppressing *E. amylovora* on floral parts using antibiotics, and disease control has been especially dependent on the use of streptomycin (Pusey 2002). However, pathogen resistance to this antibiotic has developed in many production areas (Moller et al. 1981; Loper et al. 1991; McManus and Jones 1994; Stockwell et al. 1996a), therefore, the less effective antibiotic oxytetracycline has been used (McManus and Jones 1994; Pusey 1997). In 2009, antibiotic resistance was documented for the first time in Mexico: in a screening study all the bacterial isolates from apple flowers were found to be highly resistant to the recommended doses of both oxytetracycline and streptomycin (Robles-Hernández et al. 2009).

The emergence and spread of streptomycin-resistant strains of *E. amylovora* has necessitated the discovery and evaluation of new compounds effective in fire blight control. Kasugamycin (Kasumin[®]) targets the bacterial ribosome and is particularly active against *E. amylovora*. In field trials over three seasons, blossom blight and shoot blight control with kasugamycin was statistically equivalent to streptomycin. Development of spontaneous resistance in *E. amylovora* to 250 or 500 ppm Kasumin was not observed, however, exposure to increasing concentrations resulted in tolerance in some strains (McGhee et al. 2009). Oxolinic acid, a quinolone antibiotic, was also found to be highly effective in fire blight management, at a concentration of 300 µg a.i./l (Shtienberg et al. 2002).

The use of antibiotics in plant production is highly controversial due to the potential risk of promoting the development of antibiotic resistance in human pathogens (McManus et al. 2002). In addition, the use of streptomycin or other antibiotics against fire blight is banned in the European Union (Bubán et al. 2003b).

Prevention of fire blight with chemical substances

Inducers of systemic acquired resistance represent a considerable alternative to antibiotics for the control of fire blight. The most well-known chemical agents include prohexadione-Ca (Regalis[®] in Europe, Apogee[®] in North America), acibenzolar-S-methyl (Bion[®] 50WG, Actigard[®] 50WG) and fosetyl-aluminium (Aliette[®] 80 WP), but plant conditioners (e.g. the foliar fertiliser Biomit Plussz) can be effective as well (Bubán 2007).

Bioregulators containing acylcyclohexanediones, like prohexadione-Ca (ProCa) and trinexapac-ethyl (TrixE) have been developed as vegetative growth regulators, but have also been applied with success in fire blight prevention, being effective both against shoot blight and blossom blight (Costa et al. 2001, 2006; Aldwinckle et al. 2002; Bazzi et al. 2003; Bubán et al. 2003a, b, 2004; Sobiczewski and Bubán 2004; Spinelli et al. 2007). Acylcyclohexanediones trigger plant resistance in pome fruit trees by inducing an alternative pathway for the biosynthesis of phenylpropanoids, which leads to the formation of novel flavonoids, like luteoforol (Halbwirth et al. 2003; Römmelt et al. 1999, 2003a; Rademacher et al. 2006; Spinelli et al. 2007). Luteoforol displays phytoalexin-like properties, and has been found to be highly active against several fungi and bacteria, including different strains of *E. amylovora* (Spinelli et al. 2005c, 2007; Costa et al. 2006). Spinelli et al. (2005c) proposed that luteoforol was released upon pathogen attack from its cellular compartment and inhibited further disease development by destroying pathogen cells as well as by inducing a hypersensitive-like reaction in the host plant tissue.

Spinelli et al. (2005b) found that apples and pears treated with ProCa produced higher nectar volumes per flower, and the nectar from treated flowers contained significantly less sugar than the nectar from the control plants. In contrast, Orosz-Kovács et al. (2006) reported a decrease in nectar volumes following ProCa treatment, both in susceptible and tolerant apple cultivars. Interestingly, in the sensitive cultivar ('Sampion') sugar concentration of nectar was also negatively affected by ProCa, however, in the tolerant cultivar ('Freedom') the decrease in nectar volume was accompanied by an increase in sugar concentration. As we have seen before, higher sugar concentrations can be inhibitory for the growth of *E. amylovora*, but also for antagonistic bacteria. Lower sugar concentrations are more favorable for the colonization of antagonistic bacteria, which are more sensitive to high sugar concentrations than the pathogen (Pusey 1999a; Spinelli et al. 2005b).

Pre-treatment of pome fruit trees with ProCa gave a clear reduction of blossom blight incidence both in the field and under controlled conditions (Bubán et al. 2003b; Costa et al. 2006). In the field, the percentage of diseased flowers in treated apple and pear trees was lower by more than 50 and 45%, respectively, when compared with the control (water-treated plants). Under controlled conditions, over 50% reduction of incidence was observed on flowering pear branches, following ProCa treatment; whereas ProCa was completely ineffective on detached flowers. These data are consistent with the fact that the protective effect of ProCa is mainly due to inducing plant defense responses (Costa et al. 2006).

Both ProCa and TrixE significantly reduced pathogen migration by up to two thirds compared to water-treated

control plants inside shoot and flower tissues, as visualized with confocal laser scanning microscopy combined with *gfp*-labelled *E. amylovora*. The efficacy of acylcyclohexanediones was comparable to that of acibenzolar-S-methyl (applied as BION[®]). In the field ProCa and TrixE significantly reduced blossom blight incidence on two apple cultivars, and ProCa also proved effective in reducing blossom infection on a pear cultivar. Acylcyclohexanediones not only lowered the incidence of blossom blight, but they also limited further spread of the infection into the tissues beyond the flower cluster. The increased abscission of infected flowers in treated plants most likely also prevented infection of additional plant parts by *E. amylovora* (Spinelli et al. 2007).

A shortcoming of utilizing acylcyclohexanediones in blossom blight prevention is that it is difficult to apply ProCa prophylactically (on days 10–14 before bloom), since at the required period of treatment (i.e. green to red bud stage) there is little surface for uptake of the compound and weather conditions are often unfavorable (Bubán et al. 2003b, 2004). In countries allowing the use of antibiotics, the highest effectiveness in fire blight management may be achieved by a combined use of ProCa and streptomycin or other antibiotics; whereas in the European Union combination with other, non-antibiotic, anti-fire blight preparations may be required (Bubán et al. 2003b, 2004; Spinelli et al. 2005b).

Combinations of ProCa and two strains (P10c and *Eh*-1) of the biological control agents *P. agglomerans* showed that the effectiveness of ProCa against blossom blight in apple could be substantially raised. In this combination, both strains reduced the number of flower infections successively developing into shoot blight by 80%. The higher protective effect was supposed to result from the two different modes of action: the biological control agents prevent flower infection, but if infection occurs, the plant defense activated by ProCa stops the infection from progressing. Combination of ProCa with *P. agglomerans* could represent a viable alternative to the disputed use of antibiotics, since the control level of 80% is in the range of what is typically achieved with antibiotics (Costa et al. 2006).

Application of bioregulators with acibenzolar-S-methyl (Bion[®] or Actigard[®]) is suggested 2–4 days before the predicted time of infection (Bubán et al. 2003b). Following treatment with Bion[®], Orosz-Kovács et al. (2006) reported a significant increase both in the volume and concentration of nectar in a fire blight sensitive apple cultivar.

The prophylactic use of bioregulators containing acylcyclohexanediones or acibenzolar-S-methyl before bloom, combined with application of streptomycin at bloom—if prediction models indicate its necessity—is considered as the strategy of the twenty-first century against *E. amylovora* (Norelli et al. 2003).

Biological control

Biological control of fire blight by various microbes may provide an alternative to antibiotics (Beer et al. 1984; Lindow 1984; Johnson et al. 1993b; Epton et al. 1994; Vanneste 1996; Pusey 1997; Johnson and Stockwell 2000). Alternatively, biological control agents can complement the use of antibiotics (Lindow et al. 1996; Stockwell et al. 1996b).

The stigmatic surface, which is rich in nourishing exudates, allows the multiplication of both *E. amylovora* and antagonistic bacteria, which act as biological control agents by reducing the multiplication of the pathogen (Hattingh et al. 1986; Thomson 1986; Wilson et al. 1989; Johnson et al. 1993b). Biocontrol agents can be vectored by honeybees from flower to flower and tree to tree, and are able to colonize plant surfaces away from treated sites. This is one of their advantages over antibiotics, which protect only the contact area (Pusey 2002).

The most widely used biological control agents include various strains of *P. agglomerans* (Bloomtime BiologicalTM, Blossom Bless[®], PomaVita[®]), *P. vagans* (BlightBan[®] C9-1), *Ps. fluorescens* (BlightBan[®] A506) and *B. subtilis* (Biopro[®], Serenade[®], Micosat F[®]) (Vanneste et al. 2002; Broggin et al. 2005; Elmer et al. 2005; Fravel 2005; EPA 2006a, b). Yeasts or yeastlike species being evaluated in commercial preparations include *Aureobasidium pullulans*, *Candida sake* and *Metschnikowia pulcherrima* (Duffy et al. 2006; Jelkmann and Lindner 2008; Pusey et al. 2009).

The antagonists *Ps. fluorescens* and *P. agglomerans* colonize flower stigmas and multiply over a 48- or 72-h period to a size of 10^4 – 10^6 CFU/pistil, which is the carrying capacity of apple and pear stigmas (Stockwell et al. 1992, 1996b, 1998; Wilson et al. 1992; Johnson et al. 1993b; Wilson and Lindow 1993; Pusey 2002). Pusey (2002) reported that stigmatic populations of antagonists continued to be near maximum size beyond wetting the flowers. By contrast, hypanthial populations were barely detectable before wetting, but water caused a significant increase in the populations of *P. vagans* strain C9-1 and *P. agglomerans* E325. Hypanthial populations of *Ps. fluorescens* strain A506 remained relatively small even after wetting (Pusey 2002). The difference in the capacity of strains C9-1 and A506 to survive and multiply in the hypanthium was demonstrated earlier (Pusey 1997). Strain A506 is less adapted than strains of *P. agglomerans* to the high-sugar, low-water-potential conditions that exist in the hypanthium (Pusey 2002).

Hattingh et al. (1986) reported that *E. amylovora* 273 and *P. agglomerans* 252, which effectively inhibit the development of fire blight, occupied similar niches on stigmatic surfaces, the cells of both bacterial strains occurring in regions between papillae and in underlying

tissue. In accordance with previous findings, Spinelli et al. (2005a) demonstrated that *E. amylovora* and *P. agglomerans* share similar niches, colonizing the same spaces not only on the stigma, but also on the whole pistil. The stylar groove, which can be considered as the direct prolongation of the stigma, also provides favorable conditions for multiplication of both the pathogen and the antagonist. Antagonistic interaction occurs not only on the stigma, but also inside the stylar groove during pathogen migration to the nectarthodes. In all the observations of Spinelli et al. (2005a), *P. agglomerans* populations on the stigma and inside the groove overwhelmed the *E. amylovora* population. The pathogen population was significantly lower on the stigmas and styles pre-treated with the antagonist. Both *E. amylovora* and *P. agglomerans* reached the hypanthium only after 24 h. The pathogen population grew rapidly and reached its maximum after 96 h, whereas *P. agglomerans* showed poor growth on the hypanthium.

In field trials, *Ps. fluorescens* strain A506 and *P. vagans* strain C9-1 established epiphytic populations on pear flowers and were effective antagonists of fire blight. Trees treated with bacterial antagonists always had a significantly lower percentage of flowers with detectable *E. amylovora* populations. PfA506, because of its natural resistance to streptomycin, also spread to a high proportion of flowers treated with this antibiotic, indicating potential for mixed applications of PfA506 and streptomycin in an integrated biological and chemical control program. In fruit-growing regions where streptomycin-resistant strains are abundant, the antagonists could be more effective than streptomycin for fire blight control (Johnson et al. 1993b). The field trials of Lindow and Suslow (2003) in pear orchards suggest that optimum fire blight control can be obtained by applying antagonist strains only once or twice during the brief main bloom period followed by regular applications at 7- to 10-day intervals to achieve inoculation of delayed flowers.

The efficacy of *P. agglomerans* strain E325 and the importance of the natural dispersal of bacteria were assessed in comparative field trials under two sets of conditions. One of them allowed for the dispersal of bacteria via honeybee activity (non-enclosed trees), while the other minimized natural dispersal through the use of translucent polyethylene enclosures around single trees (enclosed trees). On enclosed apple trees, the pathogen populations on flowers pretreated with *P. agglomerans* strain E325 were always lower than those of treatments with *P. vagans* strain C9-1. After wetting, pathogen populations in the hypanthium of flowers treated with E325 were always statistically lower than those of nontreated control flowers. On non-enclosed trees, treatments with *P. agglomerans* strain E325 resulted in statistically smaller populations of *E. amylovora* as compared to treatments with *P. vagans* strain C9-1 and *Ps. fluorescens* A506 (Pusey 2002).

Biological control agents can prevent infection not only by colonizing the pistil and preemptively utilizing nutritional resources required for growth of the pathogen; but also by influencing bacterial growth on the nectary surface. Wilson and Lindow (1993) found that although *Ps. fluorescens* A506 did not colonize the nectaries as effectively as the pistil, the final population size of the pathogen was significantly reduced when flowers were inoculated with A506 72 h in advance of *E. amylovora* and flowers were incubated at high RH. Wilson and Lindow (1993) proposed that besides pre-emptive utilization of nutritional resources, other factors such as induced cessation of nectar secretion or induced accumulation of host toxins (such as oxidized phenolics) may also contribute to the suppression of pathogen populations.

Mihalik et al. (2007) found that the pretreatment of the hypanthium surface with *P. agglomerans* HIP 32, isolated from the leaves of apple trees in Hungary, blocked the penetration of the bacteria into the nectary stomata. No visual symptoms of *E. amylovora* infection could be detected, and bacterium colonies were not observed in nectary tissues, which are otherwise preferred by the pathogen. These results support the observations of Bubán et al. (2007) and Hevesi et al. (2007), suggesting that *P. agglomerans* HIP 32 is a promising biological control agent.

Secondary colonization of flowers by *P. vagans* C9-1 and *Ps. fluorescens* A506 can play a role in disease suppression. In an experiment of Nucló et al. (1998), when centre rows of pear trees were sprayed with the mixture of the two strains, as bloom progressed, populations of both strains were detected also on non-treated flowers located up to 10 m from the treated rows. *E. amylovora* populations were highest in the outermost rows, and decreased linearly with proximity to treated rows.

Johnson et al. (2000) sprayed the central three rows of blocks of pear and apple trees with a suspension of streptomycin-resistant *P. vagans* strain C9-1S. Immediately after spraying, C9-1S was detected principally on flowers sampled from inoculated trees. As bloom progressed, trees up to 18 m from inoculated trees had high proportions of flowers colonized by C9-1S. The authors found that establishment and growth of C9-1S on pome fruit flowers was not strongly affected by streptomycin or by competition from indigenous bacterial epiphytes and, as with *E. amylovora*, temperature is an important environmental variable affecting successful spread of this biological control agent from flower to flower.

Broggini et al. (2005) developed a specific molecular marker for monitoring the spread of *B. subtilis* strain BD170, the antagonist found in the biological control product Biopro®. Direct spraying resulted in efficient primary colonization of pistils in flowers that were open at the time of treatment. Secondary colonization of flowers that

were at the bud or balloon stage at the time of treatment was observed, as a result of subsequent bacterial dissemination, whose efficacy, however, depended on the timing of treatments relative to bloom stage in the orchard. In a field trial with *B. subtilis*, Maccagnani et al. (2009) found that the percentages of flower samples colonized by the bio-control agent were higher in the internal treated areas compared to the external untreated ones. However, high colonization levels were detected both in the untreated areas and in the flowers that opened several days after the treatment(s), demonstrating that pollinators can play an important role as secondary carriers. These findings are in agreement with the previous reports of Nucló et al. (1998) and Johnson et al. (2000).

Johnson et al. (2009) compared the ability of virulent and avirulent strains of *E. amylovora* to compete with each other on stigmas of pear, apple and blackberry, and to compete with a co-inoculated mixture of effective bacterial antagonists. When strains were inoculated individually, virulent *E. amylovora* strain Ea153N attained the highest population size on stigmas. In competition experiments, growth of the avirulent strain was suppressed by the antagonist mixture to a greater extent than that of the virulent strain. Virulent *E. amylovora* is supposed to modify the epiphytic habitat presented by the stigma through a pathogenesis-related process, which increases host resources available to itself and, coincidentally, to non-pathogenic competitors. In four out of nine orchard trials, avirulent Ea153 *hrpL* significantly suppressed the incidence of fire blight; but the degree of biological control achievable with an avirulent strain seems to be limited by its inability to utilize the stigmatic habitat to the same degree as a virulent strain.

The consistent difference in the capacity of various antagonists to suppress *E. amylovora* may be related to competition for nutrients and exclusion (Hattingh et al. 1986; Wilson and Lindow 1993), i.e. strains may differ in their capacity to compete for specific sites or nutrients (Pusey 2002). Besides competition, antibiotic production by biological control agents also plays a major role in the inhibition of growth of *E. amylovora*. Strains of *P. agglomerans*, such as *Eh252*, *Eh318* are known to produce antibiotics that are kept responsible for suppressing growth of the pathogen (Vanneste et al. 1992; Wodzinski et al. 1994; Wright and Beer 1996; Stockwell et al. 2002). *P. vagans* strain C9-1 produces two antibiotics, herbicolins O and I that are inhibitory to *E. amylovora* (Ishimaru et al. 1988; Johnson and Stockwell 2000). Antibiotic activity was also reported for strain E325, which—unlike many antibiotics in this group—was not affected by amino acids or enzymes. Also, under laboratory conditions, strain E325 exhibited a capacity to reduce the pH on stigmas to levels that could interfere with growth of *E. amylovora*. The

strong antibacterial activity of strain E325 was most pronounced under acidic or neutral pH (pH 5–7) (Pusey et al. 2008b). The in vitro screening of bacterial cultures that contained the naturally occurring herbicide Germination Arrest Factor (GAF) was performed by Halgren et al. (2009), who found that certain strains of *Pseudomonas* bacteria secreted GAF, which was identified as the agent responsible for inhibiting the growth of *E. amylovora*.

In field studies designed to evaluate biological control of fire blight, antagonists typically reduce infection by only 40–60%, and epiphytic establishment and growth of *E. amylovora* on flowers is suppressed only partially, although antagonists are sprayed in higher concentrations (10^8 CFU ml⁻¹) onto flowers, followed by lower dose (10^5 CFU ml⁻¹) pathogen inoculation (Stockwell et al. 2008; Johnson et al. 2009). Therefore, Johnson (2010) suggested that a refuge for the pathogen exists in the pathosystem comprising the flower, the pathogen and the antagonists. A temporal refuge is created by the dynamics of flowering on pear and apple trees: although antagonists can establish and grow rapidly in flowers open at the time of treatment, and then spread to flowers that open a short time later, some stigmas are likely to be left insufficiently colonized by antagonists and available to the pathogen at the time of inoculation. Another component of the pathogen refuge is its ability to associate more intimately on stigmas than the non-pathogenic microbial agents (Johnson et al. 2009).

Mercier and Lindow (1996) developed a method for the rapid selection of antagonists capable of suppressing growth of *E. amylovora* on pear flowers, in which the population size of the pathogen on flowers pretreated with potential antagonists was estimated by a flower-freezing assay. Among 257 bacterial and yeast strains that were selected from the predominant microflora of pear flowers, 45 (4 yeasts and 41 bacteria) proved to be antagonistic to *E. amylovora*, several being more efficient than the commercially used *Ps. fluorescens* strain A506. A number of bacterial strains selected by this method were tested for their control of flower infection by *E. amylovora* on trees in the greenhouse and the field (Mercier and Lindow 2001). There was a significant relationship between the ability of antagonists to suppress growth of *E. amylovora* in the flower-freezing assay and their ability to control fire blight on trees. None of the strains produced antibiotics inhibitory to *E. amylovora*. All strains survived well in pear flowers on trees and reached population sizes of approximately 10^5 – 10^6 cells/flower 3 days after their application.

Recently, Pusey et al. (2009) sampled apple flowers for indigenous epiphytic populations of culturable microorganisms during different stages of bloom, and tested the microbial isolates for their capacity to suppress *E. amylovora* on stigmas of detached crab apple flowers. The best bacterial antagonists were *P. agglomerans* and *Pseudomonas* spp.

The yeast taxa with highest efficacy were *Cryptococcus magnus*, an unidentified *Cryptococcus* sp. and *Pichia guilliermondii*. Yeasts or yeast-like organisms have the advantage of a greater adaptability to high osmotic pressures, particularly on hypanthial surfaces during periods of low moisture. Further evaluation of these taxa on flowers could lead to the discovery of additional biocontrol agents for fire blight (Pusey et al. 2009).

Bacteriophages can also exert an inhibitory effect on *E. amylovora*. In a recent study, Sholberg et al. (2009) isolated 19 bacteriophages active against *E. amylovora* from the soil of apple and pear orchards. The phages were classified in three groups according to host range, the first group being able to effectively lyse 87% of the 18 *E. amylovora* hosts tested. Their ability to control fire blight was tested by pear flower assays using *P. agglomerans* as a carrier for the bacteriophages.

Paternoster et al. (2009a, 2011) demonstrated that nicotinic acid (NicAc) and/or nicotinamide (NicNH₂) are essential for *E. amylovora* growth, but the amounts of these chemicals on pear and apple flowers do not limit the establishment of the pathogen when competing bacteria are lacking. Degradation of NicAc and consequent inhibition of the pathogen by microbial biocontrol agents have been recently proposed as a new approach for disease control (Paternoster et al. 2009a, b). *E. amylovora* can grow using NicNH₂ and 6-hydroxynicotinic acid as alternative growth factors to NicAc, but not using 2-hydroxynicotinic acid (Paternoster et al. 2009b).

Fire blight control with natural substances

Control of fire blight by natural substances has also received considerable attention in the last two decades. Mosch et al. (1989) reported that 24 out of 139 plant extracts tested in agar diffusion test showed antibacterial activity against *E. amylovora*. Field experiments confirmed the antibacterial activity of leaf extracts from *Rhus typhina*, *Berberis vulgaris* and *Mahonia aquifolium*. The plant extracts, applied as a protective spray, showed disease control of up to 70% (Mosch and Zeller 1989). Significant activity against fire blight was demonstrated on *Cotoneaster watereri* and *Cydonia oblonga* with the extracts from *Hedera helix* and *Viscum album* (Mosch et al. 1993). In field experiments with an apple variety, the extract from *H. helix* showed an efficacy similar to that of streptomycin (Mosch et al. 1996). When applying the *H. helix* extract on the highly susceptible apple rootstock M26, Baysal et al. (2002) observed a reduction of disease severity of up to 31%, and pathogen multiplication was lowered by up to 43%, accompanied by significant changes in the phenol metabolism of the host plant.

The antibacterial effect of essential oils is well-known, and some oils have been found to inhibit *E. amylovora* (Scortichini and Rossi 1989; Vanneste 1996). Essential oils which inhibit *E. amylovora* were also found to inhibit potential antagonistic bacteria such as *P. agglomerans* Eh252; however *E. amylovora* always appeared more susceptible than the other strains examined (Vanneste and Boyd 2002). At the same time, some of the tested oils were phytotoxic at the concentration necessary to kill the fire blight pathogen, consequently their use is probably limited to disinfestation of pruning tools. Vanneste and Boyd (2002) also performed the screening of terpenes, which constitute the essential oils, and found that hydrocarbon monoterpenes (α -pinene, β -pinene, limonene, etc.) did not show any activity against any of the bacteria tested, while the alcohol monoterpenes were all active, at least against the Enterobacteriaceae.

Horváth et al. (2002, 2004b, c) tested the inhibitory effect of various thyme (*Thymus*) essential oils on the growth of *E. amylovora*, isolated from apple (*Malus*), medlar (*Mespilus*) and pear (*Pyrus*). Thyme essential oil, as well as its main phenolic compounds thymol and carvacrol, and even some of the minor components (linalool, limonene, eucalyptol, citral, geraniol etc.) were proven to have antibacterial activity, which, however, was smaller than that of antibiotics (gentamycin, tobramycin, neomycin, chloramphenicol, tetracyclin etc.). In a preliminary field trial, thymol sprayed on apple flowers reduced the population of *E. amylovora* in infected flowers (Horváth et al. 2004c). Similarly to streptomycin and gentamycin, thyme essential oil was capable of modifying the outer membrane protein profile of *E. amylovora* (Horváth et al. 2009), which plays an important role in the interaction between the bacterium and the host plant (Denyer and Maillard 2002).

Basim et al. (2006) tested the antibacterial activity of beehive-related products like pollen and propolis against plant pathogenic bacteria, including *E. amylovora*. Both the pollen and the propolis extracts inhibited pathogen growth, and the inhibitory effect of propolis was found to be higher than that of pollen.

Final remarks

Although fire blight has been intensively investigated for more than a century, several questions still remain to be answered. In order to thoroughly understand the underlying mechanisms of tolerance or sensitivity on the host side and development of resistance on the pathogen side, further research should focus on the morphological, physiological and molecular aspects of the host–pathogen interactions.

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