European Journal of Plant Pathology (2005) 113:395–405
DOI 10.1007/s10658-005-4511-7

Influence of stigmatic morphology on flower colonization by *Erwinia amylovora* and *Pantoea agglomerans*

F. Spinelli[1,*], F. Ciampolini[2], M. Cresti[2], K. Geider[3] and G. Costa[1]
[1]*Dipartimento di Colture Arboree, University of Bologna, viale Fanin, 46-40127, Bologna, Italy;* [2]*Dipartimento di Scienze Ambientali, University of Siena, via Mattioli, 4-53100, Siena, Italy;* [3]*Max-Planck-Institut für Zellbiologie, c/o BBA Dossenheim, Schwabenheimer Str. 101, D-69221, Dossenheim, Germany; *Author for correspondence (Phone: +39-051-209-6447; Fax: +39-051-209-6401; E-mail: spinelli@agrsci.unibo.it)*

Accepted 20 October 2005

Key words: biological control, epiphytes, fire blight, flower morphology, *gfp*-labelling

Abstract

The morphology of apple and pear stigma was investigated with confocal laser scanning microscopy and scanning electron microscopy. The floral colonization process by *Erwinia amylovora* was studied with *gfp*-labelled bacteria and confocal laser scanning microscopy to allow the *in vivo* observation of the pathogen colonization on intact, viable plant tissues without any kind of staining of the specimens. The interaction on the stigma between *Erwinia amylovora* and *Pantoea agglomerans*, both labelled with genes encoding for fluorescent proteins (DsRed-GFP), was also investigated. A stylar groove, covered by papillae and dwelling from the stigma along the style, was visualized. In laboratory conditions, this groove was shown to be an important way for *E. amylovora* migration towards the nectarthodes. Due to its anatomical structure the groove can sustain bacterial multiplication and thus may play an important role on the interactions between the pathogen and the bacterial antagonist *P. agglomerans*.

Abbreviations: Confocal laser scanning microscopy – CLSM; scanning electron microscopy – SEM

Introduction

The Gram-negative bacterium *Erwinia amylovora* is the causative agent of fire blight (Burrill, 1883), which is the most devastating bacterial disease of apples and pears (Vanneste, 2000). This bacterium infects particularly the plants belonging to the subfamily of *Maloideae* such as *Cotoneater*, *Crateagus* and *Pyracantha* and economically important apple and pear varieties (Eden-Green and Billing, 1974). The primary infection occurs through flowers (Eden-Green and Billing, 1974; Wilson and Lindow, 1993; Johnson and Stockwell, 2000). The infection process can be divided into distinct steps: the colonization of stigma, the multiplication of *E. amylovora* on the stigmatic surface, the migration along the style and, finally, the invasion into the host tissue through the nectarthodes (Thomson, 1986).

On the stigma, *E. amylovora* epiphytically multiplies mainly in the large intercellular spaces among the stigmatic papillae where it can exploit the abundant nourishing exudates (Hattingh et al., 1986; Wilson et al., 1989, 1990a; Johnson and Stockwell, 2000). These exudates allow also the multiplication of other bacteria including antagonists. The stigmatic surface is therefore the site, where the biological control agents must interact with the pathogen to reduce its multiplication and prevent the floral infection (Hattingh et al., 1986; Thomson, 1986; Wilson et al., 1989; Johnson et al., 1993; Vanneste, 1995). Accordingly, the stigmatic morphology and the distribution of stigmatic papillae may play an important role on

the pathogen colonization of pistils and interactions with biological control agents. Considering application of *Pantoea agglomerans* strains as bacterial antagonists, special attention was paid to the colonization of the stigma by strain *Eh*C9-1 (Ishimaru et al., 1988). *Eh*C9-1 is a good colonizer of apple and pear stigmas: its population can rapidly reach the carrying capacity of the stigma that is close to 10^4–10^6 CFU/pistil (Stockwell et al., 1992, 1996, 1998; Wilson et al., 1992; Johnson et al., 1993; Wilson and Lindow, 1993). *Eh*C9-1 produces antibiotics inhibiting growth of *E. amylovora*. Antibiotic production plays a major role in the inhibition of pathogen multiplication, but also competition for growth sites and nutrients contributes to the overall effectiveness of biological control (Johnson and Stockwell, 2000). No direct observation of the contemporary colonization of the stigma and style by *E. amylovora* and *P. agglomerans* has been described. Our data shows that the pathogen and the antagonist colonize the same spaces not only on the stigma, but also on the whole pistil. Also the stylar groove as a direct prolongation of the stigma can be considered to provide favourable conditions for multiplication of *E. amylovora* and *P. agglomerans*.

Materials and methods

Bacterial strains

The following *E. amylovora* strains were used: Ea1/79, Ea286, and EaDCA289/01 (Falkenstein et al., 1988; Jock et al., 2002; Spinelli et al., 2002). These strains were transformed with a plasmid carrying the *gfp* or the *DsRed*-gene. As biological control agent, *Pantoea agglomerans Eh*C9-1 (Ishimaru et al., 1988), labelled with a *gfp* or *DsRed*-plasmid, was used. All the bacterial strains used in this research were obtained from the Microbial Collection of the Max-Planck-Institut für Zellbiologie (Dossenheim, Germany). Plasmid pfdC1Z' with the *gfp* gene inserted in lacŹ linker (Geider et al., 1995; Bogs et al., 1998), and plasmid pDsRed (Bloemberg et al., 2000) were used for the transformation of competent cells. These plasmids confer kanamicin and ampicillin resistance, respectively. For each bacterial strain, 1 ml of overnight culture was transferred in 1.5 ml sterile Eppendorf tubes. The bacterial cells were collected by centrifugation at $8000\times g$ for 5 min, washed several times with distilled water and, finally, resuspended in 100 μl of sterile water. The competent cells were used immediately. For electroporation, 1–2 μl of plasmid DNA (pfdC1Ź-gfp or pDsRed) was added to the bacterial suspension and mixed. The suspension was then transferred to a 2 mm electroporation cuvette (Eurogenetics, Belgium) and pulsed at 800 Ohms, 2.5 kV, 2.5 μFD with a BioRad gene pulser. After electroporation, 1 ml of SOC medium was added instantly, incubated 60 min at 28 °C with slight shaking and then spread on a selective agar plate with proper dilution. Transformed cells were observed under a fluorescence microscope to confirm expression of the inserted plasmids.

To enhance the plasmid stability without the antibiotic selective pressure, the transformed cells were plated several times on antibiotic lacking Luria-Bertani (LB) agar. In addition, to check the stability of the plasmid also in the bacteria growing in the plant tissues, apple seedling were inoculated with the labelled pathogen. Five grams of fresh leaves and stem were collected between 5 and 12 days after inoculation and placed in vials containing 5 ml of sterile phosphate-buffered saline (pH 7.4) amended with 2% (w/v) of polyvinylpolypyrrolidone. The plant tissues were triturated using a Polytron homogeniser (Brinkman Instruments, Westbury, NY) and appropriate dilutions of the homogenate were plated on MS medium (Miller and Schroth, 1972). The total number of *E. amylovora*-like colonies and the percentage fluorescent were determined after 48 h incubation at 28 °C.

Morphology of apple and pear stigma

To investigate the stigmatic morphology of the *Maloideae* plants, the stigmas from different cultivars of *Malus domestica* (Golden Delicious, Royal Gala and Primiera) and *Pyrus communis* (Tosca and William) and from *Crataegus* sp. were sampled and observed. For the SEM observation, 30 freshly open flowers without dehiscent anthers were considered per cultivar and species. In addition, for each apple and pear cultivar, 20 flowers were observed under a stereomicroscope at four different phenological stages: popcorn stage, opening of petals, dehiscence of anthers and petal fall.

Stigma colonization by fluorescent bacteria

Flowering scions of *Malus domestica* (cv. Golden Delicious and Primiera) and *Pyrus communis* (cv. William) were used for the experiments. Preliminary experiments, carried on pear flowers inoculated with all the possible combinations of the labelled bacterial strains, were performed to determine the most appropriate antagonist/pathogen combination. Two different bacterial combinations, the *rfp*-labelled EhC9-1 + g*fp*-labelled Ea1/79 and *gfp*-labelled EhC9-1 + *rfp*-labelled Ea286 were considered. Only flowers at popcorn stage were used and all the already opened flowers were detached from the branches and discarded. Before the inoculation, the flowers were artificially opened to expose the stigmas. According to Vanneste and Yu (1996), flower inoculation with the antagonist was performed by spraying the blossoms with an aqueous suspension of *Eh*C9-1 containing approximately 1×10^8 CFU ml^{-1}. The pathogen inoculation was performed 24 h later by spraying the flowers with an aqueous suspension of *E. amylovora* containing approximately 5×10^6 CFU ml^{-1}. Prior to inoculation, the bacteria were grown for 24 h in Luria broth amended with the appropriate antibiotic. The consistency of the sprayed inocula was assessed by tenfold sequential dilutions on the appropriate agar medium plates. The plants were kept in controlled conditions (22 °C, 70% RH, 12 h day–night cycle) during the experiment. Flowers inoculated only with *E. amylovora*, *P. agglomerans* or sterile water were used as controls. For each treatment, 20 flowers were monitored daily from 1 to 5 days after artificial inoculations. Additional experiments were carried out on *Crataegus* sp. and *Pyracantha* sp. flowers.

Quantitative determination of fluorescent bacteria

Flowering brunches of *Malus domestica* (cv. Golden Delicious) were used for the experiment. Also in this case, only flowers at popcorn stage were considered. The flowers were inoculated with the antagonist by dipping the distal part of the pistils in an aqueous suspension of *rfp*-labelled *Eh*C9-1 containing 1×10^7 CFU ml^{-1}. The pathogen inoculation was performed 24 h later using an aqueous suspension of gfp-labelled Ea286 containing 4×10^6 CFU ml^{-1}. The detached brunches were kept in water under controlled conditions (22 °C, 70% RH, 12 h day–night cycle) during the experiment. Flowers inoculated only with *E. amylovora*, *P. agglomerans* or sterile water were used as controls. To assess the bacterial population of different parts of the blossom, ten flowers per treatment were sampled at 3 h and 1, 2, 3, 4, 5 days after artificial inoculation. For each flower, the hypanthium and the pistils were detached aseptically. Successively, under the fluorescence stereomicroscope, for each pistil, the distal part of the stigma was dissected by the style. The stigmas, the styles and the hypanthium were washed separately in 1 ml of sterile 10 mM $MgSO_4$. Tenfold sequential dilutions were made and plated on LA medium amended with the appropriate antibiotic and cycloheximide (50 mg l^{-1}).

In addition, to verify if the stylar groove is able to sustain the bacterial growth, the stigmas of several Golden Delicious and Primiera flowers were covered by dipping them in melted paraffin. The flowers treated in this way were successively spray-inoculated with an aqueous suspension of *Eh*C9-1 (pfdC1Ź-gfp) or Ea286 (pfdC1Ź-gfp) containing approximately 5×10^7 viable cells ml^{-1}. The bacterial populations of the style and hypanthium were determined, as previously described, at 3 h and 1, 2, 3, 4, 5 days after artificial inoculation.

Scanning electron microscope (SEM) equipment

Samples collected for SEM analysis were fixed in 5% glutaraldeyde and dehydrated in 100% ethanol. After critical-point drying and gold-sputter coating, samples were analysed by Philips 501B SEM

Confocal laser scanning microscope (CSLM) equipment

Optical sections were obtained with a Confocal Laser Scanning Microscope (CLSM) (Microradiance, Bio-Rad Instruments, Henel, UK) mounted on a Nikon Optiphot microscope and equipped with an Argon laser. A 60× objective and the BHS (GHS) filterset were used for imaging. All images were collected using a stepper motor to make Z-series. The projections of the individual colour channels (RGB) were merged with the Confocal Assistant™ software (Version 4.02, © 1994–1996, Todd Clark Brelje) to facilitate visualization.

Fluorescence microscope

For two-dimensional observation, a Zeiss Axiophot optical microscope (Neofluar 100× oil immersion objective), with BHS (GHS) filterset was used. BHS: excitation light 546 nm, emission 580 nm; GHS: excitation light 450–490 nm, emission 510 nm. These two filtersets were used to visualized the fluorescence of the DsRed and GFP proteins, respectively. For the three-dimensional observation, a Leica MacroFluo™ stereomicroscope equipped with the appropriate filtersets for GFP and DsRed was used.

Results

Morphology of apple and pear stigma

Apple and pear stigmas were club-shaped, bilobate and covered by stigmatic papillae. In freshly opened flowers, the papillae presented a round-columnar shape and were covered by a thin cuticle. The SEM and stereomicroscope observations of non-inoculated pistils allowed the detection of a particular anatomical feature: a groove running all along the pistil. It originated from the stigma and continued along the style to the nectar cup both in apple (Figure 1) and in pear (Figure 2). This groove was found in all the plant material observed and also in preliminary observations on *Crataegus* sp. and *Pyracantha* sp. stigmas using a fluorescence stereomicroscope. In these observations, the bacteria were mainly localized between the papillae and inside the stylar groove indicated by bacterial fluorescence (Figure 3c–d). The groove is formed by the carpels which fold during pistil development and the attaching margins interlock and adhere through the epidermis (Takhtajan, 1991). The epidermis of the groove was constituted by stigmatic papillae (Figure 1). The groove was a direct continuation of the stigma along the style: the canalicular papillae were morphologically identical to those present on the stigma and no discontinuity among the stigmatic and canalicular epidermis was found (Figure 1). Even when the stylar groove deeply penetrated inside the style, it remained separate from the transmitting tissue by several layers of parenchymatous and vascular tissue. This observation confirmed the results obtained with TEM and light microscopy by Cresti et al. (1980).

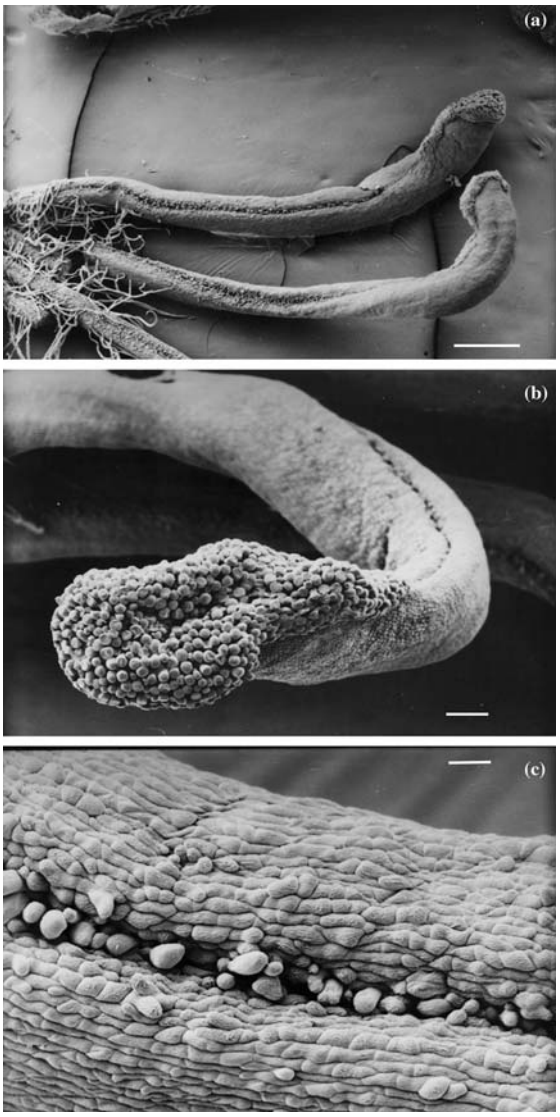

Figure 1. Morphology of the apple pistil investigated by SEM. (a) Micrograph of pistils (cv. Golden Delicious) from a newly opened flower with a stylar groove originating from the stigma and present along the pistil. The groove epidermis is completely occupied by stigmatic papillae. On the left, at the base of the style, surrounded by multicellular trichomes, the number of stigmatic papillae is higher and the groove is wider than at the distal part of the stigma. Scale bar = 500 μm. (b) Close-up of the apple stigma. The stigma is bilobe and covered by turgid, columnar papillae. The stylar groove originates directly from the stigma. Scale bar = 100 μm. (c) Part of the stigmatic papillae covering the apple stylar groove. The papillae on the internal and external surfaces of the groove are distinguishable from the epidermal cells by their columnar shape. No morphological differences between the stylar and stigmatic papillae were detected. Scale bar = 25 μm.

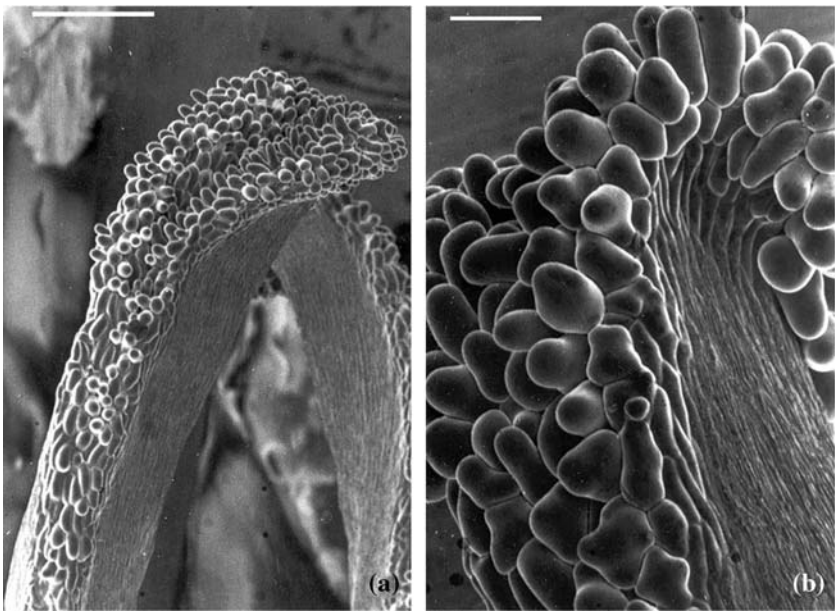

Figure 2. Morphology of the stylar groove in a newly opened pear flower investigated by SEM. (a) Micrograph of a pear pistil with the stylar groove originating from the stigma. Scale bar = 250 μm. (b) Part of the papillae covering the pear stigma. Scale bar = 50 μm.

Stigma colonization by fluorescent bacteria

The insertion of the *DsRed* and *gfp*-plasmid in *E. amylovora* and in *P. agglomerans* strains and the subsequent expression of the fluorescent proteins was found to be stable. Also after repeated transfers on non-selective LB medium, there was no detectable loss of fluorescence. Plant assays performed on Golden Delicious micropropagated plantlets showed that the transformed *E. amylovora* continued to express high levels of GFP several days after experimental inoculation. On infected shoots, production of fluorescent bacterial exudate was observed 11 days after inoculation. Furthermore, the expression of GFP or DsRed did not affect the colony morphology, growth rate and the pathogen virulence. GFP and DsRed showed a comparable fluorescence brightness, moreover, in the bacterial species considered; also their maturation time was comparable, even if DsRed needed at least 48 h to show its maximum fluorescence.

Observing the multiplication of *gfp*-labelled *E. amylovora* on the stigma and its migration towards the hypanthium, it was possible to prove that the bacterium exploited the stylar groove during its movement from the stigmatic surface to the nectarthodes (Figure 4a). In all the observations performed, *E. amylovora* moved massively inside the groove and only very few bacteria migrated outside it along the style. Similarly, the bacterial antagonist *P. agglomerans* was mainly localized on the stigma among the papillae (Figure 4b–c) and migrated along the style primarily exploiting the groove (Figure 4d–e). Also in the stylar groove, the bacterial cells were mainly localized among the numerous papillae. Pollen grains were occasionally found on the groove surface. Nevertheless, even when they showed a green fluorescence, they were easily distinguishable from bacterial cells by the shape, the dimensions and the brightness of fluorescence. When pollen grains were hydrated, they had a spherical shape with a diameter of approximately 20 μm and presented a weak fluorescence. The pathogen and the antagonist shared exactly the same niches on the whole pistil and the antagonistic interaction between *P. agglomerans* and *E. amylovora* occured not only on the stigma, but also inside the stylar groove during pathogen migration to the nectarthodes. Furthermore, in all the observations performed, the *P. agglomerans* population on the stigma and inside the groove overwhelmed the *E. amylovora* population (Figure 4). The population dynamics of *P. agglomerans* and *E. amylovora*

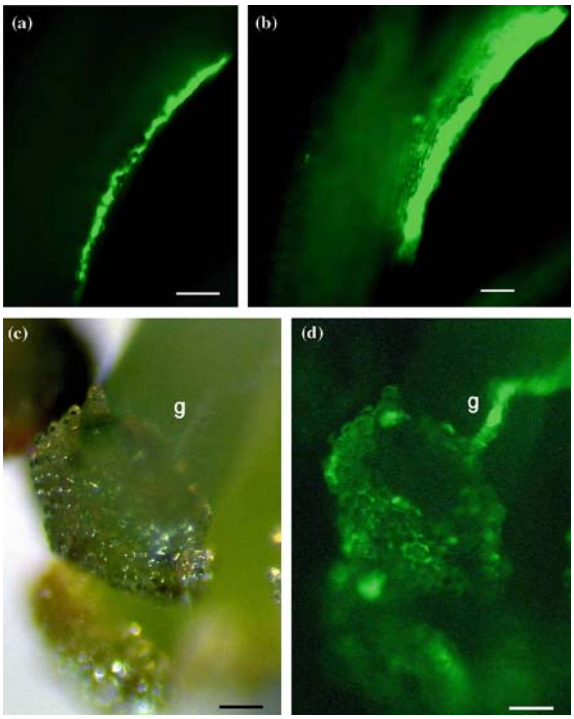

Figure 3. Colonization of pear and hawthorn flowers by *gfp*-labelled *E. amylovora* cells. Strain Eal/79(pfdC1Z'-gfp) was visualized using a fluorescence stereomicroscope. (a and b) Photographs of the style of a pear flower (cv. Tosca) at 48 h after inoculation. The green fluorescence of *E. amylovora* is localized in the stylar groove. Scale bar = 1 mm. (c) Bright field photograph with stigma, style and groove. (d) The green fluorescence of bacteria are mainly localized between the papillae and inside the groove (g). Scale bar = 250 μm.

when inoculated alone or in combination is reported in Figures 5–7.

Finally, in all the observations from 24 to 72 h after pathogen inoculation, even in heavily colonized stigmas, the papillae maintained their structural integrity and no damage due to *E. amylovora* colonization was found. After 72 h, the stigma integrity started to decrease as a consequence of both the pathogen colonization and the natural senescence processes. The stigma and the style started to degenerate also in the pistils previously co-inoculated with *P. agglomerans*. Between 72 and 120 h after inoculation, the majority of papillae collapsed both on the stigma and the groove. Nevertheless, the sectioning of the style by means of CLSM confirmed that even after 96 h, no penetration of the pathogen inside the stylar tissues was ever occurred.

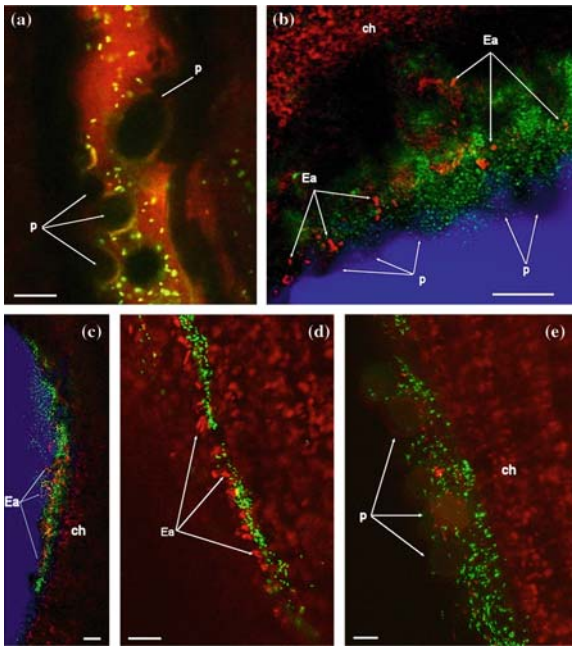

Figure 4. Colonization of apple flowers by *rfp*-labelled *E. amylovora* and *gfp*-labelled *P. agglomerans* cells. Strains Ea286(pDsRed), Ea1/79(pDsRed) and EhC91(pfdC1Z'-gfp) were visualized in the CLSM. (a) Micrograph of a Golden Delicious apple style 24 h after *E. amylovora* Ea1/79 (pDsRed) inoculation. The stylar groove is clearly distinguishable. The bacterial cells are mainly localized among the numerous stigmatic papillae (p) inside the groove. Scale bar = 100 μm. (b) Micrograph of a Primiera apple stigma heavily colonized with bacteria. The photograph was taken 48 h after pathogen inoculation and 72 h after antagonist inoculation. In this case, the abundance of *gfp*-labelled *P. agglomerans* results in a diffused green fluorescence, whereas the red fluorescence of *rfp*-labelled *E. amylovora* (Ea) is difficult to distinguish from the natural fluorescence of chloroplasts (ch). *Erwinia amylovora* cells occur in aggregates resembling patterns found for pathogen movement in the xylem. The stigmatic papillae are indicated with a p. Scale bar = 500 μm. (c and d) Micrographs of a Primiera (c) and a Golden Delicious (d) apple style. The bright red cells of *Erwinia amylovora* Ea286(pDsRed) can be distinguished from the chloroplasts (ch) by the stronger red fluorescence. The photograph was taken 48 h after pathogen inoculation and 72 h after antagonist inoculation. *E. amylovora* cells are organized in chains. Scale bars = 500 μm (c), 250 μm (d). (e) Micrograph of a Golden Delicious apple stylar groove. The bacteria are localized among the stigmatic papillae (p). The photograph was taken 48 h after pathogen inoculation and 72 h after antagonist inoculation. The red colouration of plant tissues is due to the chlorophyll fluorescence (ch). The micrograph was acquired as an RGB image, only the Red and Green canals but not the Blue transmission canal were considered. Scale bar = 250 μm.

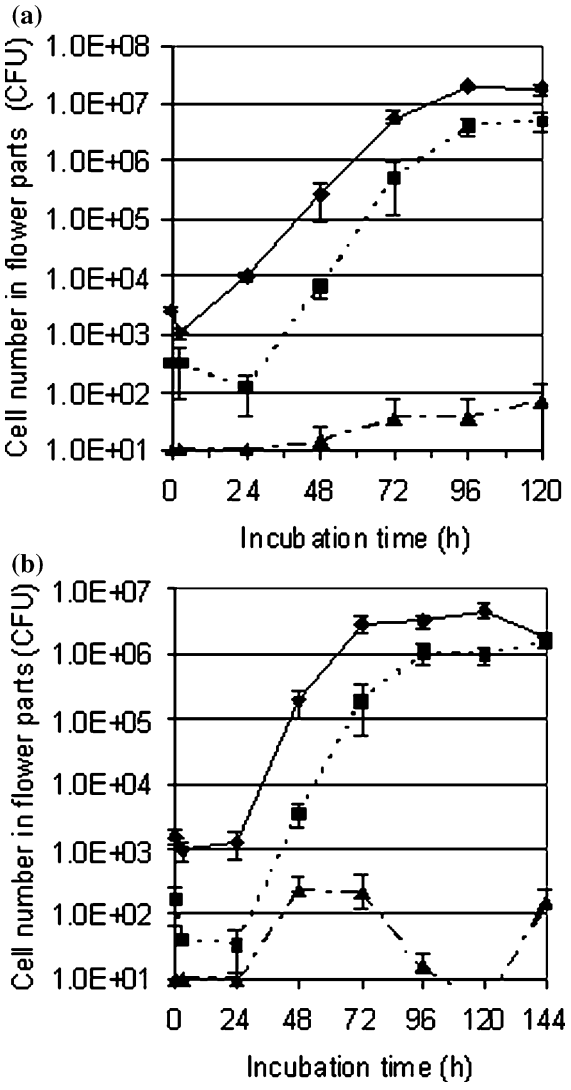

Figure 5. *Erwinia amylovora* and *P. agglomerans* populations on the different parts of the flower. The graphs reports the Ea286 (a) and EhC9-1 (b) populations established on an apple Golden Delicious stigma (–♦–), style (--■--) and hypanthium (nectar cup) (··▲··). The standard error is shown.

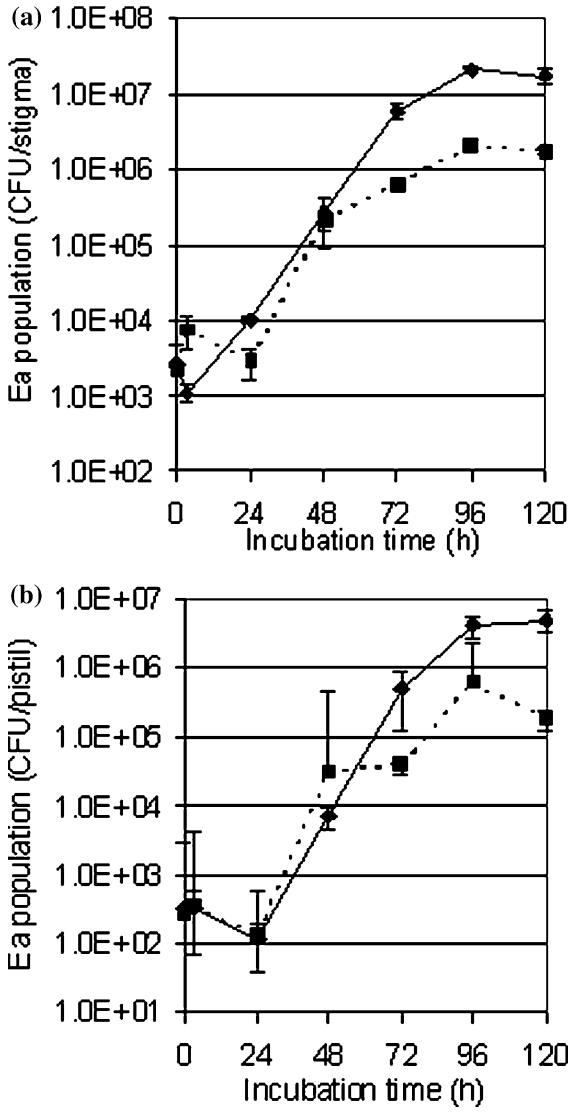

Figure 6. *Erwinia amylovora* Ea286 populations a Golden Delicious apple pistil when inoculated alone or in combination with *P. agglomerans* EhC9-1. (a) The graph shows the population of *E. amylovora* established on an apple stigma inoculated by spraying EhC9-1 (1×10^7 CFU ml^{-1}) (–♦–) and then Ea286 (3.75×10^6 CFU ml^{-1}) after 24 h (--■--). The *E. amylovora* population is significantly reduced in the presence of *P. agglomerans*. Standard error is shown. (b) A similar effect was observed for *E. amylovora* with (–♦–) and without addition of EhC9-1 (--■--) established on the style. The standard error is shown.

Quantitative determination of fluorescent bacteria

Erwinia amylovora and *P. agglomerans* populations were determined on different parts of the pistil and on the nectar cup. The bacterial populations were always higher on the stigma than on the style (Figure 5a–b). Both on the stigma and style the bacteria rapidly grew until they reached the estimated carrying capacity of pistil. On the hypanthium, the bacterial populations remained under the threshold of detectability (10 viable bacteria) for the first 24 h. They slowly grew successively and their populations barely exceeded 10^2 viable cells.

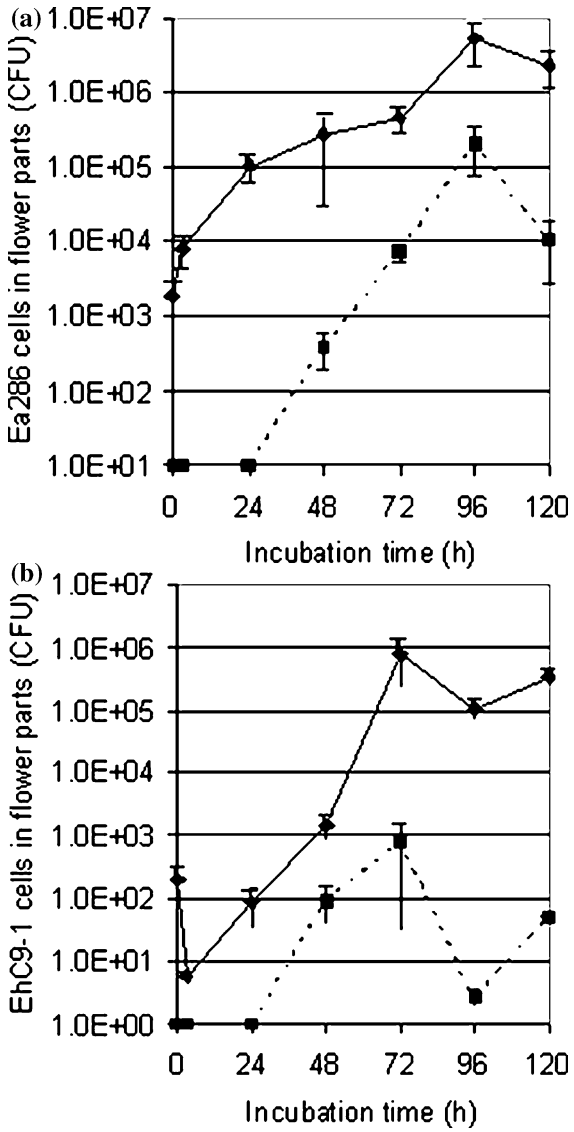

Figure 7. Erwinia amylovora Ea286 and *P. agglomerans* EhC9-1 populations established on Golden Delicious apple style and hypanthium. For this experiment the stigma were covered with melted paraffin to prevent bacterial colonization. Therefore, the bacterial populations showed in the graphs are only due to direct colonization and growth on the style without contribution of the stigma. (a) *Erwinia amylovora* colonization of the style (–♦–) and subsequent migration to the hypanthium (--■--). (b) *Pantoea agglomerans* colonization of the style (–♦–) and the nectar cup (--■--). The standard error is shown.

Pantoea agglomerans colonized both the stigma and style and it inhibited the *E. amylovora* growth in these sites. The pathogen population was significantly lower on the stigmas and styles pre-treated with the antagonist (Figures 6a–b). The *Pantoea agglomerans* population was weakly influenced by the presence of the pathogen (data not shown). In addition, when the bacteria were inoculated only onto the style, the population rapidly increased, thus showing that the style was able to sustain the bacterial growth (Figure 7a–b). Again, *E. amylovora* and *P. agglomerans* reached the hypanthium only after 24 h. The pathogen grew rapidly on the hypanthium and after 96 h, reached a maximum population of 2×10^5 cells (Figure 7a). *Pantoea agglomerans* showed poor growth on the hypanthium (Figure 7b).

Discussion

Apple and pear flowers present a solid style. Different from hollow styles, which are characterized by an internal cavity filled with mucilage (Labarca et al., 1970), in a solid style, the inner part is constituted by a bundle of glandular cells, which form the transmitting tissue (Cresti et al., 1980). The transmitting tissue is surrounded by several layers of parenchymatous tissue and it is therefore completely separate from the stylar groove described here (Cresti et al., 1980).

In the present work, the papillate tissue which formed the groove epidermis was a direct continuation of the stigmatic tissue, and the stigmatic secretions also embedded the stylar papillae. Therefore, it is likely that the stylar papillate tissue had similar functions to the real stigmatic tissue. The observation that the pollen grains, rarely found on the style, germinated on this papillate tissue corroborates this hypothesis.

Biological control of fire blight is a promising method to reduce blossom infection. Nevertheless, the efficacy of biocontrol requires further improvement. Therefore, a better knowledge of the trilateral interactions between the pathogen, the plant and antagonistic microorganisms is needed. The use of multiple autofluorescent proteins to label the pathogen and antagonistic bacteria is a powerful, non-invasive method to visualize *in planta* the spatiotemporal interactions occurring in microbial biocoenosis (Bloemberg et al., 2000; Monier and Lindow, 2003, 2004). Moreover, the combination of multiple autofluorescent proteins with CLSM is essential to elucidate the infection

process and host–pathogen interactions at a single cell level (Bloemberg et al., 1997). These techniques are especially interesting since plant tissues are not exposed to any kind of treatment or manipulation: no fixation, sectioning or staining is needed.

The observations reported here show that *E. amylovora* migrates from the stigma to the nectar cup inside the described stylar groove. It is noteworthy that the groove epidermis is completely constituted by stigmatic papillae, which provide a nutrient-rich, humid and protected environment where bacteria can find a favourable environment for their multiplication (Wilson et al., 1989). According to these observations, *E. amylovora* easily reaches the groove that is continuous with the stigma where the pathogen firstly multiplies. It can also be hypothesised that *E. amylovora* migration inside the groove might be facilitated by the stigmatic secretions abundantly produced on the stigma and conveyed into the groove. Moreover, also inside the groove, secretions are produced (Cresti et al., 1985) thus allowing a fast bacterial multiplication that might force the bacteria towards the nectar cup. The bacterial multiplication inside the stylar groove is also confirmed by population studies performed with traditional methods and by the observation of chains of fluorescent bacterial cells, which indicate the presence of bacteria, which actively multiply. The stylar groove is a peculiar site for bacterial growth and the size of *E. amylovora* and *P. agglomerans* populations on the style and stigma are comparable. The presence of a protected and humid stylar groove probably reduces the influence of climatic conditions on the pathogen migration to the nectar cup (Lindow and Brandl, 2003). Our observations confirm those of Wilson et al. (1990a, b; 1992) , that *E. amylovora* initially grows on the stigma as an epiphyte without producing any damage to the stigmatic papillae and pistil integrity. Even 120 h after inoculation, in a heavily colonized pistil, with severe damage to structural integrity, no pathogen penetration inside the style was ever observed. Therefore, *E. amylovora* invasion of the style and consequent infection of the plant tissues seems unlikely.

Previous reports (Pierstorff, 1931; Rosen, 1936; Hildebrand, 1937; Rundle and Beer, 1987), suggested that *E. amylovora* penetrates through the stigma and migrates inside the style towards the other plant tissues. In fact, the presence of *E. amylovora* within the groove, which deeply penetrates inside the pistil, might misleadingly give the impression of an improbable bacterial infection through stylar tissues.

Several authors have indicated that *P. agglomerans* excludes *E. amylovora* populations on the stigma (Wilson et al., 1990b; Vanneste and Yu, 1996) and Wilson et al. (1992) showed that *E. amylovora* and *P. agglomerans* share the same stigmatic niche when inoculated on separate flowers. However, no direct *in vivo* observation of the contemporary colonization of the stigma and style by *E. amylovora* and *P. agglomerans* has yet been made. Our research showed that the pathogen and the antagonist colonize the same spaces not only on the stigma, but also on the whole pistil. In fact, inside the stylar groove that can be considered as a direct prolongation of the stigma, both *E. amylovora* and *P. agglomerans* found favourable conditions for multiplication. Therefore, also within the groove biological control agents could interact with the pathogen and colonization by *P. agglomerans* may prevent further pathogen multiplication, thus providing efficient biological control of fire blight.

In all the observations performed, the fluorescence signal due to *gfp*-labelled *P. agglomerans* greatly surpassed the DsRed signal from *E. amylovora*. This was mainly attributable to a higher *P. agglomerans* population rather than to differences in maturation time, photobleaching, pH sensitivity and plasmid stability between DsRed and GFP (Baird et al., 2000). This was confirmed both by the assessed population density by serial dilutions and the use of rfp-labelled *P. agglomerans* in combination with *gfp-E. amylovora*. Since the chlorophyll fluorescence interferes with DsRed detection, the population of *rfp*-labelled bacteria might be underestimated.

Preliminary observations on *Crataegus* sp. and *Pyracantha* sp. flowers confirmed the presence of a similar groove in other plants belonging to the *Maloideae* subfamily and showed that also on these species it may play a role during *E. amylovora* colonization of the blossoms. In conclusion, we were able to visualize the simultaneous colonization of apple flowers by *P. agglomerans* and *E. amylovora* and clearly distinguish the two bacterial populations at a single cell level. Therefore the results presented in this paper provide

novel information on the distribution and establishment of antagonistic and pathogenic bacteria on apple and pear flowers.

Acknowledgements

We thank J.L. Vanneste and S. Lindow for comments and advice on the manuscript, W. Rademacher for all his suggestions, M. Noferini for his help in preparing the figures and E. Sabatini and E. Rondelli for performing part of the work.

References

Baird GS, Zacharias DA and Tsien R (2000) Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proceedings of the National Academy of Science of the United States of America 97: 11984–11989.

Bloemberg GV, O'Toole GA, Lugtenber BJJ and Kolter R (1997) Green fluorescent protein as a marker for *Pseudomonas* spp. Applied and Environonmental Microbiology 63: 4543–4551.

Bloemberg GV, Wijfjes AHM, Lamers GEM, Stuurman N and Lugtenberg BJJ (2000) Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: New perspectives for studying microbial communities. Molecular Plant–Microbe Interactions 13: 1170–1176.

Bogs J, Bruchmüller I, Erbar C and Geider K (1998) Colonization of host plants by the fireblight pathogen *Erwinia amylovora* marked with genes for bioluminescence and fluorescence. Phytopathology 88: 416–421.

Burrill TJ (1883) New species of *Micrococcus*. American Naturalist 17: 319.

Cresti M, Ciampolini F and Sansavini S (1980) Ultrastructural and histochemical features of the pistil of *Malus communis*: the stylar transmitting tissue. Scientia Horticulturae 12: 327–337.

Cresti M, Ciampolini F and Sansavini S (1985) The morphological characteristics of the stigma in various fruit plants. Rivista della Ortoflorofrutticoltura Italiana 69: 49–62.

Eden-Green SJ and Billing E (1974) Fireblight. Review of Plant Pathology 53: 353–365.

Falkenstein H, Bellemann P, Walter S, Zeller W and Geider K (1988) Identification of *Erwinia amylovora*, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. Applied and Environmental Microbiology 54: 2798–2802.

Geider K, Baldes R, Bellemann P, Metzger M and Schwartz T (1995) Mutual adaptation of bacteriophage fd, pfd plasmids and their host strains. Microbiological Research 15: 337–346.

Hattingh MJ, Beer SV and Lawson EW (1986) Scanning electron microscopy of apple blossom colonized by *Erwinia amylovora* and *Erwinia herbicola*. Phytopathology 76: 900–904.

Hildebrand EM (1937) The blossom-blight phase of fireblight? and methods of control, Cornell University Agriculture Experiment Station Memoirs 207, Ithaca, New York, 17–40.

Ishimaru CA, Klos EJ and Brubaker RR (1988) Multiple antibiotic production by *Erwinia herbicola*. Phytopathology 78: 746–750.

Jock S, Donat V, López MM, Bazzi C and Geider K (2002) Following spread of fire blight in western, central and southern Europe by molecular differentiation of *Erwinia amylovora* strains with PFGE analysis. Environmental Microbiology 4: 106–114.

Johnson KB and Stockwell VO (2000) Biological control of fire blight. In: Vanneste JL (ed) Fire Blight: the Disease and its Causative Agent Erwinia amylovora CAB International Wallingford, Oxon, United Kingdom.

Johnson KB, Stockwell VO, McLaughlin Loper MJ JE and Roberts RG (1993) Effects of bacterial antagonists on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossom and on fire blight control. Phytopathology 83: 995–1002.

Labarca CM, Kroh M and Loewus F (1970) The composition of stigmatic exudate from *Lilium longiflorum* labelling studies with myo-inositol, D-glucose and L-proline. Plant Physiology 46: 150–156.

Lindow SE and Brandl MT (2003) Microbiology of the phyllosphere. Applied and Environmental Microbiology 69: 1875–1883.

Miller TD and Schroth MN (1972) Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62: 1175–1182.

Monier JM and Lindow SE (2003) Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surface. Proceedings of the National Academy of Science of the United States of America 100: 15977–15982.

Monier JM and Lindow SE (2004) Frequency, size and localization of bacterial aggregates on bean leaf surface. Applied and Environmental Microbiology 70: 346–355.

Pierstorff AL (1931) Studies on the fire-blight organism, Bacillus amylovorus, Cornell University Agricultural Experiment Station Memoir 136, Ithaca, New York.

Rosen HR (1936) Mode of penetration and progressive invasion of fire-blight bacteria into apple and pear blossoms. University of Arkansas College of Agriculture, Agricultural Experiment Station Bulletin no 331.

Rundle JR and Beer S (1987) Population dynamics of *Erwinia amylovora* and biological control agent, *Erwinia herbicola*, on? apple blossom parts. Acta Horticulturae 217: 221–222.

Spinelli F, Marcazzan GL and Sabatini AG (2000) Effetto degli inibitori delle diossigenasi sulla composizione glucidica del nettare di pomacee e sulle dinamiche di biocontrollo del colpo di fuoco batterico. Atti VI Giornate Scientifiche SOI, Spoleto 23–25 aprile 2002. 1: 109–110.

Stockwell VO, Johnson KB and Loper JE (1996) Compatibility of bacterial antagonists of *Erwinia amylovora* with antibiotics used for fire blight control. Phytopathology 86: 834–840.

Stockwell VO, Loper JE and Johnson KB (1992) Establishment of bacterial antagonists on pear blossom. Phytopathology 82: 1128 (Abstr).

Stockwell VO, Sugar D, Spotts R, Johnson KB and Loper JE (1998) Establishment of bacterial antagonists of *Erwinia amylovora* on pear and apple blossoms as influenced by inoculum preparation. Phytopathology 88: 506–513.

Takhtajan A (1991) Evolutionary trends in flowering plants, Columbia University Press, New York.

Thomson SV (1986) The role of the stigma in fire blight infections. Phytopathology 76: 476–482.

Vanneste JL (1995) Erwinia amylovora. In: Singh US, Singh RP and Kohomoto K (eds) Pathogenesis and Host Specificity in Plant Disease: Histopathological, Biochemical, Genetic and Molecular Basis, Vol. 1 (pp. 21–46) Prokaryotes, Pergammon Press, Oxford and London.

Vanneste JL (2000) Fire Blight, the Disease and its Causative Agent Erwinia amylovora, CABI Publishing, Wallingford, UK, 370.

Vanneste JL and Yu J (1996) Biological control of fire blight using *Erwinia herbicola Eh*252 and *Pseudomonas fluorescens* A506 separately or in combination. Acta Horticulturae 411: 351–353.

Wilson M, Epton HAS and Sigee DC (1989) *Erwinia amylovora* infection of hawthorn blossom: II. The stigma. Journal of Phytopathology 127: 15–28.

Wilson M, Epton HAS and Sigee DC (1990a) *Erwinia amylovora* infection of hawthorn blossom: III. The nectary. Journal of Phytopathology 187: 62–74.

Wilson M, Epton HAS and Sigee DC (1990b) Biological control of fire blight of hawthorn (*Crateagus monogyna*) with *Erwinia herbicola* under protected conditions. Plant Pathology 39: 301–308.

Wilson M, Epton HAS and Sigee DC (1992) Interactions between *Erwinia herbicola* and *Erwinia amylovora* on the stigma of hawthorn blossom. Phytopathology 82: 914–918.

Wilson M and Lindow SE (1993) Interactions between the biological control agent *Pseudomonas fluorescens* strain A506 and *Erwinia amylovora* in pear blossom. Phytopathology 83: 117–123.