Study of *Erwinia amylovora* colonization and migration on blossoms of susceptible and tolerant apple cultivars

Mihalik E.[1], Radvánszky A.[1], Dorgai L.[2] and Bubán T.[3]

[1]*University of Szeged Department of Botany and Botanic Garden H-6701 Szeged P.O.B.657*
[2]*Bay Zoltán Institute for Biotechnology Szeged, H 6726 Szeged Derkovits fasor 2.*
[3]*Research and Extension Center for Fruit Growing, Újfehértó Hungary*

Summary: The stigmata of detached flowers of susceptible and tolerant apple cultivars were inoculated with about 10^4 *gfp* labeled *Erwinia amylovora* . There were no apparent differences in the colonization, multiplication and survival of the bacteria on the stigmatic surface of the culivars. Bacteria were washed down to the hypanthium surface 24 hours after inoculation. The visual symptoms of the infection were the discoloration and shrinkage of the floral parts. The gradual browning associated with the infection appeared first on the surface of the hypanthium followed by the discoloration of the style. The color of the filaments turned into brown only 120 hours after the inoculation. Bacterial cells were not detected in the tissues of the styles and filaments. The traits of the hypanthium surface are of prominent importance in the progression of the infection. The wrinkled surface, the convex shape of the outer epidermal cell walls with thin cuticle and the sunken stomata helped to preserve a water film for a longer period providing medium for the motility of the bacteria in the susceptible cultivar. Bacteria were restricted to small water droplets on the flat and waxy surface of the hypanthium of the tolerant cultivar and only a few were able to enter the tissues.

Large bacterium aggregations were detected in the intercellular spaces of the parenchyma of the susceptible cultivar 48 hours after the inoculation. In the next period the *Erwinia amylovora* cells gradually invaded the intercellulars of the hypanthium wall, the wall of the ovary and the pedicel. Low level of bacterium aggregation was found in the intercellulars of the tolerant cultivars. It is suggested that the progression of the infection was inhibited also by physiological factors.

Key words: apple cultivars, *gfp* labeled *Erwinia amylovora*, colonization, migration

Introduction

Fire blight caused by *Erwinia amylovora* (Burrill) Winslow et al. is one of the oldest known bacterial disease of rosaceous taxa of which apple (*Malus domestica)* is one of the economically important species. *E. amylovora* can infect blossom, fruit, stem, leaf and young shoots. The general symptoms of the infection are rapid wilting as a result of the water loss of parenchyma cells, discoloration followed by necrosis of infected plant parts.

The spread of the bacteria can be assisted by wetting events, wind or insect pollinators. *E. amylovora* cells are carried by these agents to the surface of stigmas *(Hildebrandt & MacDaniels 1935, Rosen 1936, Sutton & Jones 1975, Thomson, 1986).*

When the stigma develops into the receptive phase the stigmatic papillae become wet by the stigmatic secretion. This hydrophylic liquid is the substrate for colonization, growth and multiplication of the bacteria. Stigma serves as a reservoir until the rain or dew washes the bacteria down to the hypanthium *(Hattingh et al. 1986).*

According to the review of *Bubán et al. (2003),* the most common site of the infection is the nectariferous surface of the floral cup, the hypanthium. *E. amylovora* prefers the openings of the floral nectaries, the nectary stomata (nectarthodes) as entry site *(Vanneste & Eden-Green 2000),* but it is also able to penetrate the apple tissues through „ordinary" stomata and through injuries *(Sobiczewski & Klos, 1998, Bogs et al 1998, Bogs & Geider 1999).* This pathogen moves rapidly from the infenction site (leaf, flower, young stem) downwards the plant body as far as the root *(Momol et al. 1999).*

The presence of the bacteria in the plant should be detetced directly and indirectly. The indirect detection means the follow up of the appearance and progression of the symptoms of the infection. These symptoms were studied by many authors from the beginning of the 20th century till the recent years *(Jones 1909, Rosen 1936, van der Zwet & Keil 1979, Vanneste 2000)*

The direct detection involves two types of methods. One of them is the demonstration of the presence of specific bacterial DNA in the plant body *(Bereswill et al. 1992, 1995, Dorgai & Bubán 2002).* This way of detection, however, does not discern the viable and dead bacteria. The visualization of the bacteria on the plant surface and in the tissues with different microscopical methods should exhibit bacteria dead or alive, depending on the technique applied.

The frequently used techniques for visualizing the bacteria on the plant surface and inside the tissues are scanning and transmission electron microscopy *(Huang & Goodman 1976, Suhayda & Goodman 1981, Goodman & White 1981, Hattingh et al. 1986, Mansfeld & Hattingh 1987).* Several

years ago the bacterium was 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. Since then the epifluorescence microscopy has also been involved in the investigation of *Erwinia amylovora* infection *(Bogs et al 1998, Bogs & Geider 1999)*. The effectivity of all these methods were compared in our previous work *(Mihalik et al. 2003)*.

Studies on fire blight of apple revealed considerable differences in susceptibility among different cultivars and in different individuals of the same cultivars. The present study was undertaken to compare:

- the colonization of *E. amylovora* on the stigmatic surface,
- the development and progression of the visual symptoms of the infection in the blossom,
- the hypanthium characters and the pattern of the bacteria on the hypanthium,
- the localisation and migration of the bacteria in the hypanthium and in the pedicel of susceptible and tolerant cultivars, and to observe the individual differences in susceptibility.

Materials and methods

Strain and plasmid

Plasmid pfdC1Z'-gfp (KmR), a gift from Dr. K. Geider *(Bogs et al. 1998)*, was electroporated into *Erwinia amylovora* (isolate number 895). Transformants were selected in the presence of 20 g/ml kanamycin. One colony displaying green fluorescence upon excitation was selected and passaged several times under selective pressure until the stabilization of the fluorescent marker. Bacteria for the experiments were grown in the presence of 20 g/ml Km.

Plant material

Two cultivars of *Malus domestica* were involved in this study: cv. Jonagold Decosta (susceptible to fireblight) and cv. Freedom (tolerant). The characters of the flowers of both apple cultivars are summarized in *Table 1*.

Microscopical techniques

50–50 flower buds of balloon stage (just before anthesis) were removed from apple trees of the two cultivars grown in the experimental orchard of the Research and Extension Centre for Fruitgrowing, Újfehértó, Hungary. Flowers used in the experiments were in the same developmental state and originated from the same position in the inflorescence.

The pedicels were sunken into test tubes containing 10% sucrose solution. The buds were incubated in a light chamber at temperature of 23 °C and at 85% relative humidity. After 24 hours of incubation the buds opened and the wet stigmatic

Table 1 Comparison of several characteristics of the flowers of apple cultivars investigated

Characteristics	Jonagold Decosta	Freedom
susceptibilty	susceptible	tolerant
flower size	large	small
pedicel	long	short
scent	no	yes
hair on the basis of the petals	many	few
hair on the surface of the petals	no	yes
shape of the stigma	asymmetric	symmetric
hair on the style	long	short
	on the whole style	the basis of the style is hairless
shape of the hypanthium	cup	plate

surface became visible. *Gfp* labeled *E. amylovora* was applied on the stigmatic surface of 25 flowers of both cultivars as follows: The inoculation was carried out with a glass capillary, the diameter of which was about the same as that of the stigma. The inoculum suspension (10^9 cfu/ml) was soaked up the capillary and the stigmatic surface had been dipped in it. With this inoculation method about the same amount of bacteria (a few times 10^4) was allocated to the stigmatic surfaces. 24 hours after the inoculation the bacteria were washed down to the hypanthium with a sterile water drop of 30 µl containing 0.1% Tween 20. As a check, 25–25 flowers of the cultivars were treated the same way, except that sterile water was substituted for the bacterium suspension. The colonization of bacteria on the stigmatic surfaces were detected using epifluorescence microscope, 36, 48 and 72 hours after the inoculation. The visual symptoms of the infection were checked 48, 72 and 96 hours after the inoculation. The level of discoloration of different floral parts (stigmatic branches, style, filaments, hypanthium surface) was detected daily. The level of discoloration has been given in percentage to compare with the control.

Epifluorescence microscopy was also applied for the localisation of the bacteria in the hypanthium tissue. Sections were made with cryostat (Leica CM 1850) mounted with glycerol:water 1:1 mixture and wieved. The dispersion and aggregation of the bacteria on the surface of the hypanthium was examined with SEM. For SEM preparations the petals, the filaments and the style were removed. The remaining flower parts were longitudinally cut into two halves with razor blade. The samples were fixed, dehydrated subsequently in graded ethanol and acetone and dried in a critical point dryer. Mounted specimens were gold coated and wieved in a Hitachi S 2400 scanning electron microscope.

Results and discussion

The colonization of E. amylovora

The succes of the inoculation was observed by the bright green fluorescence that appeared on the surface of the

stigmatic papillae. The amount of the bacteria was estimated visually on the basis of the fluorescence intensity. There was no apparent difference in the intensity of the fluorescence on the stigmata of susceptible and tolerant cultivars. The temporal increase of the fluorescence on the inoculated stigmata of both cultivar should be the sign of the multiplication of the bacteria, but it should also be feasible, that the shrinkage of the stigma surfaces concentrated the bacteria in smaller area resulting a visually higher fluorescence intensity *(Figure 3 a, b)*. The equal alteration of the stigmata of the two cultivars showed, that the bacterium tolerancy or sensitivity was not associated with stigmatic characters. The chemical composition of the secretion (carbohydrates, amino acids etc.) adapts this fluid to be proper medium for multiplication of the bacteria *(Thomson 1986)*. According to our results we suppose that there was only slight or no difference in these compounds of the cultivars investigated.

Although the stigmatic papillae were already collapsed 48 hours after the flowers were detached and the tissue of stigma and style continually shrinked and dried out, 96 hours after the inoculation the bacteria were still viable because the hygroscopic stigmatic exudate maintained the humidity essential for survival. Bacteria are not firmly adhered to the cell walls, they could easily be washed away from the stigmatic surface.

The visual symptoms of the infection

Visual symptoms of the infection are the discoloration and shrinkage of floral parts invaded by the bacteria. As we applied excised flowers, the gradual change detected is the consequence of two parallel phenomenons: the natural wilting of the detached flowers and the evolution and procession of the symptoms of the infection.

Jonagold Decosta

The appearance and the temporal development of the symptoms were different in different plant parts *(Figure 1)*. The stigmatic branches gradually turned into brown from the stigmata to the direction of the style. The browning and the subsequent drying has been completed on the second experimental day in both control and treated samples of the cultivars, indicating that this change was not associated with the infection. It is remakable, that the symptom associated unambigously with the infection was the discoloration of the style (the fused lower part of the five stigmatic branches). 72 and 94 hours after the inoculation this area remained green in all control flowers , and became completely brown and shrinked in 40% of the treated plants under the experimental circumstances.

The filaments of the control sample kept also their green color during the experimental period, while 55% of the infected flower filaments turned brown and dried.

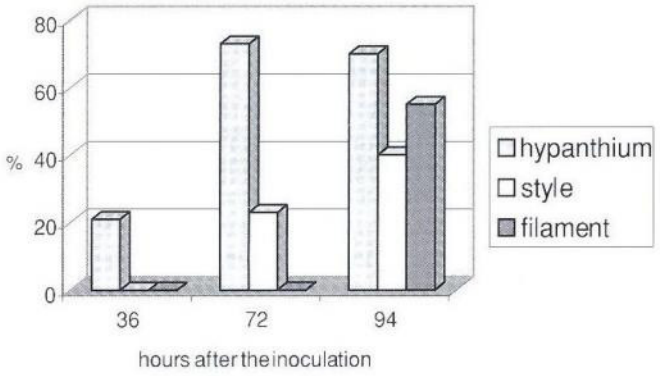

Figure 1 Percentage of discoloration of different floral parts of a susceptible apple cultivar (cv. *Jonagold Decosta*)

The hypanthium surface of both control and infected flowers showed brown coloration. In the case of the infected flowers simultaneous necrosis was also detected. During the experimental period the surface of the hypanthium grew in both the infected and the control samples. The surface of the newly extended area was green, and it is reflected in the temporal change of the ratio of green and brown hypanthium surface area.

It is remarkabe, that the individual tolerancy of the flowers were different, 45% of the treated flowers did not show definite symptoms. The reason might be the different physiological state of the flowers what we could not detect in this experiment.

Freedom

During our experimental period this cultivar did not show discoloration associated with the infection. None of the floral parts became necrotic. The stigmatic branches dried out, but it is regarded to be the natural ageing of the flowers.

Characters of the hypanthium

SEM revealed considerable differences between the susceptible and tolerant cultivars concerning the shape of the hypanthium and the pattern of their surface.

Jonagold Decosta

The deep funnel-like hypanthium of the susceptible cultivar with hairy style provided better environment for bacterial cells than the plate-like shape of the tolerant one *(Figure 2 a,d)*. Bacteria washed down from the stigma surface distributed in the hypanthium. The wrinkled surface, the convex shape of the outer epidermal cell walls with thin cuticle and the sunken stomata *(Figure 2 b)* preserved the water layer for a longer period. This water layer -in the case of nectar secretion the diluted nectar- was a medium for the motility of bacterial cells. This motility was oriented by the chemical gradient of the nectar components towards the nectary tissue. In our experiments bacterial aggregates were present on and around the nectary stomata and bacteria

penetrating the stomata were also detected.. The water loss of the cells of the hypanthium was the most characteristic effect of the infection. The guard cells of the stomata showed higher resistency for water loss than epidermal cells, therefore stomata protruded from the epidermis. Their cuticle detached the cell walls and formed different patterns *(Figure 2 c)*.

Freedom

On the flat hypanthium surface of the tolerant cultivar the evaporation of the water was fast, and there was not sufficient time for bacterial cells to enter the tissues. Frequently occurred with this cultivar that bacteria on the hypanthium surface were almost completely restricted to quickly–dried droplets. The surface of the hypanthium was much smoother *(Figure 2e)* than that of the susceptible cultivar and the shrinkage, as a result of infection, was negligible *(Figure 2 f)*.

Dispersion and migration of the bacteria in the hypanthium tissues

Jonagold Decosta

In the longitudinal and transversal sections of the hypanthium tissue masses of fluorescent bacteria were seen on the surface and in the intercellular spaces. The distribution of the bacteria was not uniform, as it was observed previously *(Radvánszky et al. 2002)*.Aggregations were also detected on and around the nectary stomata, but stomata without bacterium clustering could also be found *(Figure 3 c)*. 48 hours after the inoculation bacteria were detected in the form of large aggregations in the intercellular spaces of the parenchyma adjacent to the glandular tissue *(Figure 3 d)*. The next step in the bacterium proliferation was the fast bidirectional migration of the *Erwinia amylovora* cells which occured 24 hours after the appearance of the subhypodermal aggregations. Bacteria spread laterally to the direction of the

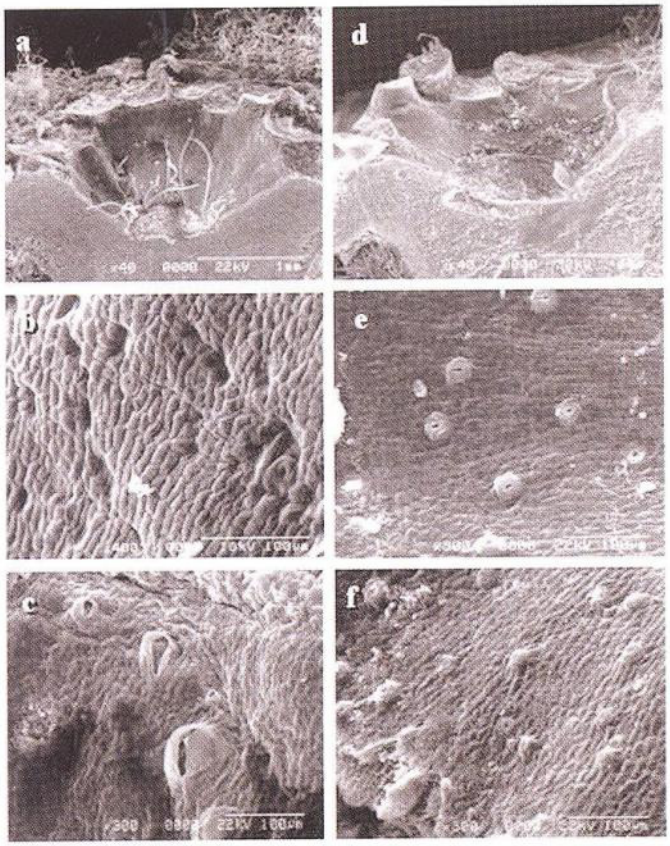

Figure 2 The shape and surface of the hypanthium
a: longitudinal section of the hypanthium of the cv. *Jonagold Decosta* (susceptible)
b: hypanthium surface of the cv. *Jonagold Decosta*
c: hypanthium surface of the cv. *Jonagold Decosta* 120 hours after the inoculation
d: longitudinal section of the hypanthium of the cv. *Freedom* (tolerant)
e: hypanthium surface of the cv. *Freedom*
f: hypanthium surface of the cv. *Freedom* 120 hours after the inoculation

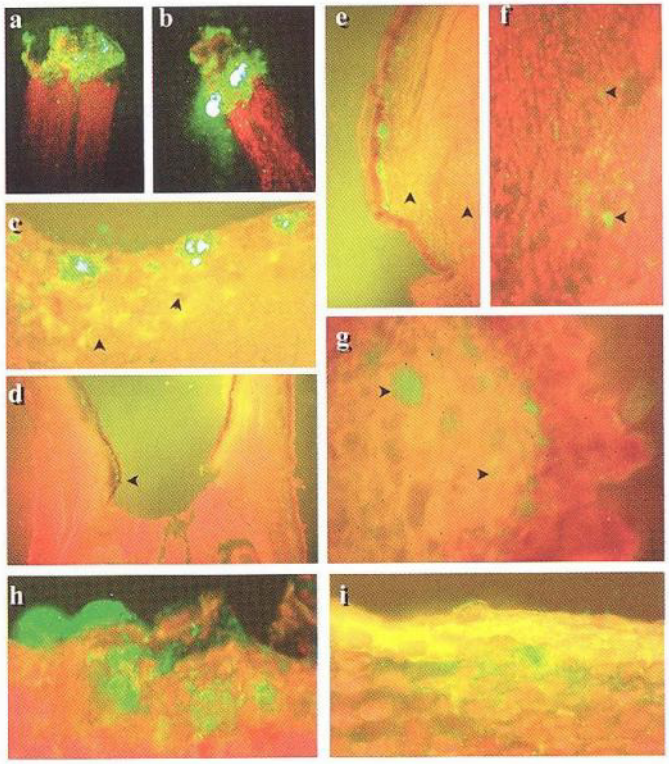

Figure 3 Colonization and migration of *E. amylovora*
a: colony of bacteria on the stigma just after the inoculation
b: colony of bacteria on the stigma 120 hours after the inoculation
c: aggregates of bacteria on the nectary stomata of the hypanthium of the cv. *Jonagold Decosta* (arrowheads: stomata without bacteria)
d: longitudinal section of the hypanthium (arrowhead: bacterium aggregates)
e: spread of bacteria in the intercellulars of the hypanthium wall (arrowheads: bacterium aggregates)
f: spread of bacteria in the intercellulars of the ovary wall (arrowheads: bacterium aggregates)
g: cross section of the pedicel (arrowheads: bacteria aggregates)
h: bacteria enclosed in water drops on the hypanthium of the cv. *Freedom*
i: single bacteria and weak aggregation in the intercellular spaces of the cv. *Freedom*

outer epidermis of the hypanthium *(Figure 3 e)* and downwards toward the pedicel, invading the parenchyma in the wall of the ovary *(Figure 3 f)*. No migration was found toward the filaments and style. When *Erwinia amylovora* cells attained to the pedicel (120 hours after the inoculation), the water loss of the tissues was almost complete, the shrinked parenchyma cells adhered only with arms. The large round intercellulars among them were occupied by bacteria *(Figure 3 g)*. Although the hypanthium wall and the ovary wall contains vascular bundles, bacteria were not detected in the xylem yet. It is probable, that *Erwinia* prefers the intercellular spaces for short distance migration.

Freedom

As *Figure 3h* demonstrates, bacteria were enclosed in water drops on the hypanthium surface of the tolerant cultivar. As a result of the restricted motility probable few bacteria entered the intercellular spaces. The low level of aggregation *(Figure 3i)* should also have physiological background.

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