

Floral biological studies on pear cultivars in relation to fire blight susceptibility

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Summary: Floral biological characteristics that may influence cultivar susceptibility to fire blight were studied in 10 pear cultivars in two Hungarian orchards from 1999 to 2003. The receptaculo-ovarial, automorphic nectary is usually bigger in tolerant cultivars than in susceptible ones. Nectary stomata are meso- or xeromorphic. Susceptible cultivars tend to have more xeromorphic stomata, where guard cells are located 1-3 cell rows below the epidermis. The size of nectar chambers is usually smaller in susceptible cultivars. Floral nectar, consisting mainly of glucose and fructose, is more abundant and less concentrated if the cultivar is susceptible to fire blight. The amount of chlorogenic acid was higher in the flowers of tolerant cultivars than in susceptible ones.

Key words: *Erwinia amylovora*, fire blight, histology, nectar, nectary, pear, *Pyrus communis* L., stomata, susceptibility to *Erwinia*, TLC

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora* (Burr.) Winslow et al., is one of the most threatening diseases of pome fruit trees (van der Zwet et al., 1988, Johnson & Stockwell, 1998). Therefore it is essential to study the possible ways of infection, including the entry of the pathogen into various flower tissues. Several floral biological traits may influence the development of the disease, such as floral age and morphology, the surface of the stigma and the hypanthium, as well as nectar characteristics (Paulin, 1987, Johnson & Stockwell, 1998).

During the epiphytic phase the bacterium first colonises the stigma, which is followed by the external washing of cells from the stigma to the hypanthium. The pathogen gains entry to the plant through nectary stomata located on the hypanthial surface (Hattingh et al., 1986, Thomson, 1986, Wilson et al., 1989, 1990, Johnson & Stockwell, 1998).

The stigma of pear flowers, similarly to other *Rosaceae* species belongs to the so-called "wet stigmas" (Heslop-Harrison & Shivanna, 1977), where the outermost layer consists of club-shaped papillae and the stigmatic fluid may provide a protected, nutrient-rich, hydrated environment for the pathogen to multiply (Hattingh et al., 1986, Wilson et al., 1989).

The nectary is mostly receptacular in the *Rosaceae* family, consisting of three histologically distinct parts: the epidermis, the glandular tissue and the nectary parenchyma (Kartashova, 1965). The intrafloral nectary of pear is receptaculo-ovarial, lining the adaxial surface of the plate-like receptacle and the apical part of the ovary. A narrow zone of the nectariferous tissue is stretching along the style, allowing nectar accumulation in the gap between the style and the nectary. 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 (mesomorphic type) or sunken a few cell rows below the epidermis (xeromorphic type). Below the stomata, among the cells of the glandular tissue, nectar chambers, i.e. nectar-storing intercellular cavities of varying size can be found (Farkas, 2004).

Nectar, getting to the surface through nectary stomata, acts as an attractant for pollinator insects, but also supports the epiphytic growth of bacteria. Conditions for the pathogen vary greatly, according to the time of nectar secretion, its sugar composition and concentration (Ivanoff & Keitt, 1941, Campbell et al., 1991, Johnson & Stockwell, 1998). The optimal sugar concentration for growth of *E. amylovora* ranges from 5 to 20% (Pusey, 1999).

According to Vansell (1946), Simidchiev (1970), Free (1970, 1993), Benedek et al. (2000) and Farkas et al. (2002) pear cultivars usually produce a small amount of diluted nectar, although certain cultivars may yield a larger amount and/or more concentrated secretory product. Nectar concentration is usually below 10%, but in some cultivars it may reach 20%. The main sugar components of pear nectar are glucose and fructose, while sucrose can be detected only in small amounts in the secretory product of few pear microtaxa (Wykes 1952, Farkas et al. 2002).

Recently, the importance of natural plant substances, often called secondary metabolic products, has increased. Many of them play a significant role in the resistance-related metabolism of apples and pears (Goodman et al., 1986, Rademacher, 2000, Roemmelt et al., 2003). Differences e.g. in the spectrum of phenolic compounds in flower tissues are supposed to be related to the varying susceptibility of apple and pear cultivars.

The development of fire blight is affected also by environmental factors such as air temperature and relative humidity. Moderately high temperatures are favourable for bacteria, the optimum for rapid development of the infection ranges from 21 to 27 °C. Rainy weather followed by warm,

cloudy weather, especially during blossoming and ultimate shoot growth, is very favourable to epiphytic outbreaks of fire blight (*van der Zwet & Keil, 1979, van der Zwet, 1986*).

The present study aims at discussing some floral biological characteristics of pear cultivars grown in Hungary, focusing on their possible relationship with *E. amylovora* infection.

Material and methods

Studied cultivars

Floral biological studies were carried out on six pear (*Pyrus communis* L.) cultivars in the cultivar collection of the Research and Extension Centre for Fruit Growing, Újfehértó, and four cultivars in a commercial orchard in Györgytarló, in the years 1999–2003. The local cultivars from the gene bank in Újfehértó may serve as future sources for breeding for fire blight resistance, whereas the cultivars from Györgytarló are among the most important ones grown in Hungary.

The studied cultivars and their *E. amylovora* susceptibility, based on incidence of fire blight symptoms:

<u>Újfehértó:</u>	<u>Györgytarló:</u>
<i>Non-susceptible:</i>	<i>Least susceptible:</i>
Bőtermő Nyári Kálmán	Beurré Bosc
Nagyasszony	
Nyári, Dunaföldvár	
	<i>Susceptible:</i>
<i>Susceptible:</i>	Olivier de Serres
Bajai 6	Conference
Fillér	
Zánka magonc	<i>Highly susceptible:</i>
	Hardenpont

Field studies – Daily nectar production

The nectar produced during 24 h was measured in the isolated flowers of 'Beurré Bosc' and 'Conference' in 2003. Flowers were isolated for 24 h by a tulle net. The volume of nectar was measured by calibrated microcapillaries, whereas refraction was measured by a hand-held refractometer (OG-101/A).

Determination of nectar sugar composition – TLC

In 2003 the nectar sugar composition of two cultivars ('Beurré Bosc' and 'Conference') was determined by thin layer chromatography (TLC). Nectar samples (5 or 10 µl) were measured into Eppendorf tubes, then were kept in an exsiccator. Standards (1 µl each) and 1 to 5 µl of the samples (after being dissolved in ethanol : distilled water 7:3) were applied to plates by microcaps.

Plate: Silica gel 60 F₂₅₄ (Merck)

Standards: sucrose, glucose, fructose (1 mg/ml)

Mobile phase: ethyl acetate : ethanol : 60% acetic acid : water coldly saturated with boric acid 50:20:10:10

Plates were run twice in developing chambers without saturation. Spots were visualised by dipping plates into a thymol-sulphuric acid reagent for 3 sec, then dried at room temperature, and finally at 105 °C for 5 min. Densitometric evaluation was performed by a CAMAG TLC Scanner at 510 nm, using the software CATS 3.14 for quantitative measurements.

Determination of phenolic compounds in flowers

The main phenolic compounds in the flowers of four pear cultivars ('Beurré Bosc', 'Conference', 'Hardenpont', 'Olivier de Serres') were determined in 2002. Extraction was done with methanol: 2 g of ground flower parts (calyx, corolla, anthers and pistil separately) was put on a water-bath (65 °C) with 10 ml methanol for 15 min. After cooling, samples were filtered. Solutions were applied to the plates by microcaps (1 µl of standards and 5 or 10 µl of the samples).

Plate: Silica gel 60 F₂₅₄ (Merck)

Standards: chlorogenic acid, caffeic acid, rutin, hyperoside

Mobile phase: ethyl acetate : formic acid : glacial acetic acid : distilled water 100:11:11:27

Plates were run once in the developing chambers, following saturation with the solvent for 30 min. Spots were visualised by briefly dipping plates into Naturstoff reagent. Plates were dried at room temperature, then at 105 °C for 5 min. Densitometry was carried out with a CAMAG TLC Scanner at 366 nm.

Histological studies

Flower samples were fixated in absolute ethanol : glycerine : distilled water 1:1:1. Flowers were embedded either in paraplast or synthetic resin.

Before embedding in paraplast, samples were dehydrated in an ascending acetone series and xylol. Longitudinal sections (7–10 µm thick) were cut with a sledge or rotary microtome.

Before embedding in the glycol methacrylate based resin, samples were dehydrated in 70% and 96% ethanol, then put into the infiltrating fluid (Technovit 7100). Longitudinal sections (5–8 µm thick) were cut with a rotary microtome.

Staining was done with toluidine blue in both cases.

In the medial longitudinal sections of the flowers the following parameters were measured with the software Image Tool 1.27:

- area of nectary
- thickness of nectary and glandular tissue
- area of nectar chamber

Mean values were calculated from data measured on 10 flowers from each cultivar, then standard deviation was determined.

Results and discussion

Histology of the nectary

In accordance with previous studies (Farkas, 2004), the floral nectary of all studied pear cultivars was receptaculo-ovarial. The gland was automorphic, protruding out of receptacular tissues at the apical part in each cultivar (Fig. 1), and also at the basal part in some cultivars (e.g. 'Beurré Bosc', 'Conference', 'Hardenpont' (Fig. 2), 'Olivier de Serres'). A part of the nectariferous tissue is located along the style. The nectar secreted here, together with the secretory product coming down from the exposed surface of the nectary, accumulates in the gap between the style and the nectary, especially if nectar is abundant and dilute. Accumulated nectar can remain in the flower for a longer time, evaporating less readily than from the exposed gland surface. This nectar reservoir increases the chance of insect pollination, but also that of bacterial growth.

Based on data from 5 years, the size of the nectary was bigger in non-/less susceptible cultivars than in highly susceptible ones (Fig. 3). No relationship was found, however, between the thickness of nectary, the thickness of glandular tissue and the degree of susceptibility (data not shown).

According to the position of guard cells of nectary stomata, cultivars belong either to the mesomorphic type (guard cells in the same level as epidermal cells) or the xeromorphic type (guard cells below the level of epidermis). Hygromorphic stoma position was not found in any of the studied cultivars. Susceptible cultivars tend to have more xeromorphic nectary stomata, where guard cells may be sunken one, two or even more cell rows below the epidermis (Fig. 4). In less susceptible cultivars the mesomorphic or slightly xeromorphic stoma position (guard cells sunken to half of the epidermal cells) occurs more frequently (Fig. 5).

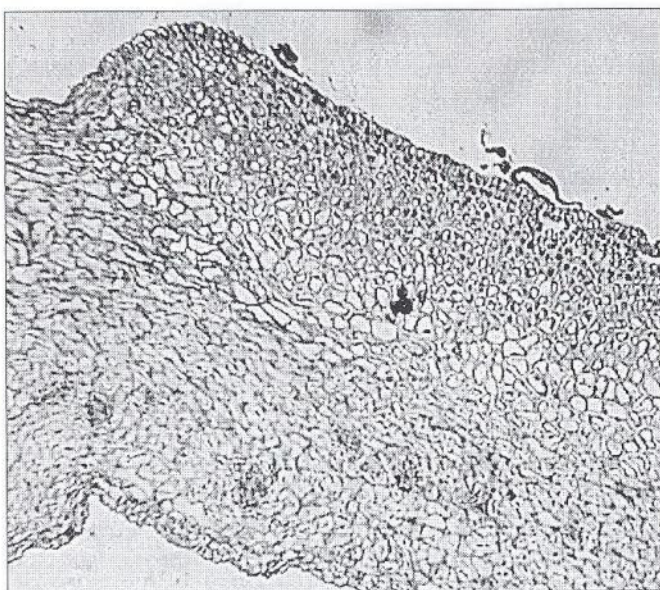


Figure 1. Apical region of the nectary in pear cv. 'Bötermő Nyári Kálmán'

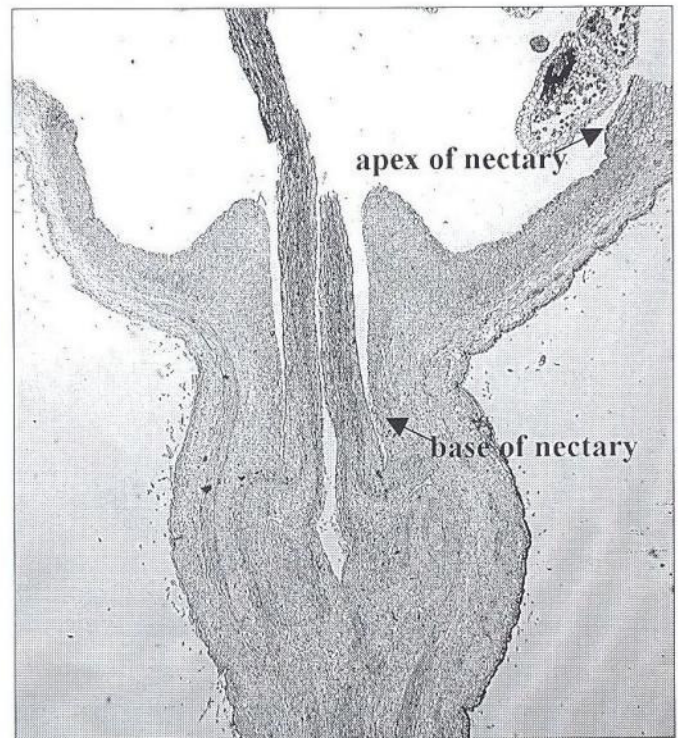


Figure 2. Automorphic nectary of cv. 'Hardenpont'

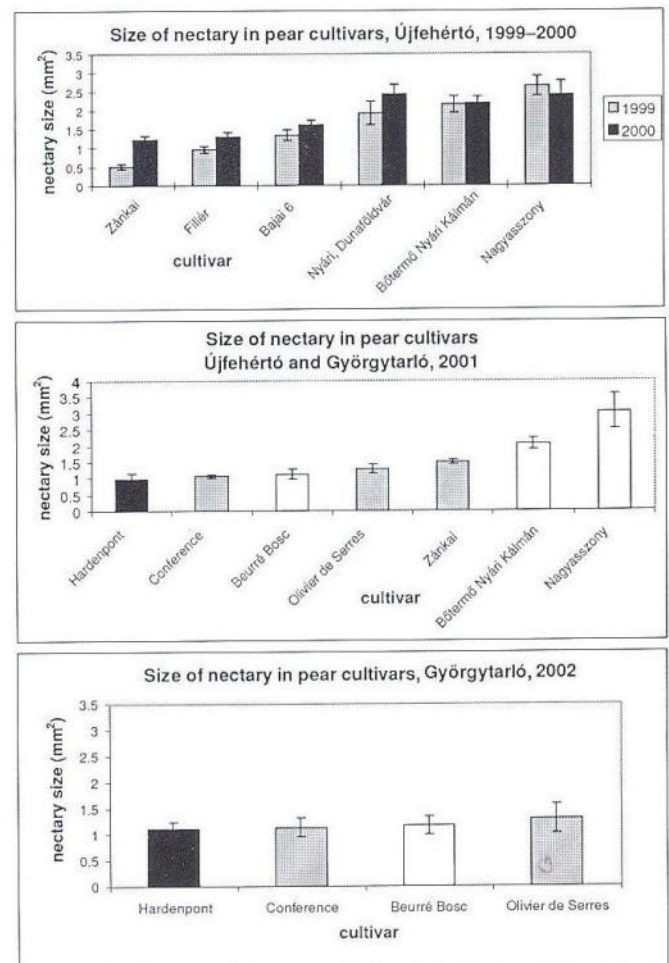


Figure 3. Nectary size of variously susceptible pear cultivars

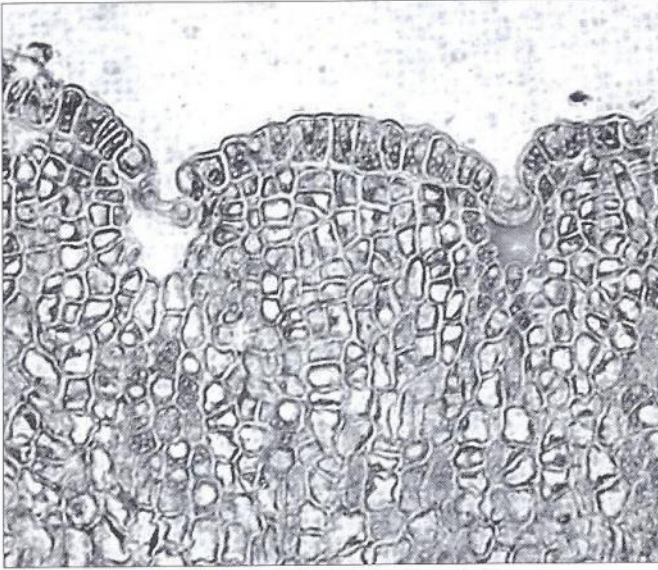


Figure 4. Strongly xeromorphic nectary stomata of the susceptible cv. 'Hardenpont'

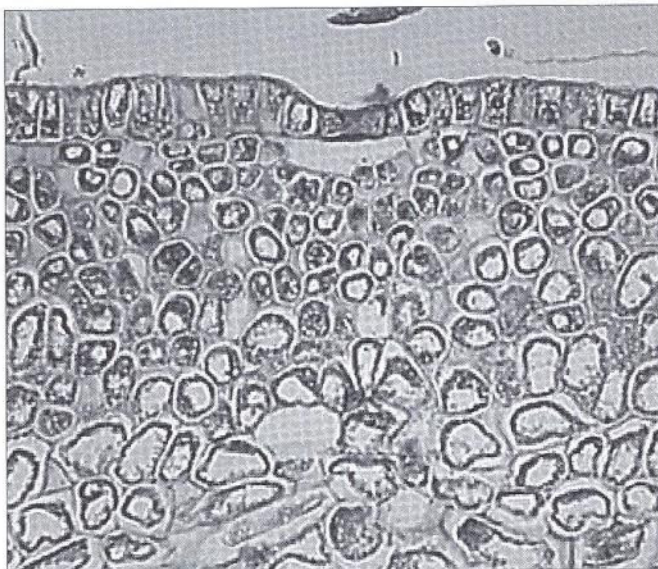


Figure 5. Stomatal guard cells sunken to half of epidermal cells in cv. 'Nagyasszony'

The size of nectar chambers, located below stomatal guard cells, is usually larger in less susceptible cultivars than in highly susceptible ones (Fig. 6), except for year 2002, when the nectar-storing cavity was the largest in the most susceptible cultivar, 'Hardenpont'. Non-susceptible pear cultivars from the gene bank in Újfehértó possessed spacious nectar chambers (Fig. 7) in all years of study, their size was 2 or 3 times as large as that of susceptible cultivars, often exceeding $500 \mu\text{m}^2$ (Fig. 6).

Daily nectar production

Volume and refraction of the nectar produced during 24 hours was compared in the flowers of a tolerant ('Beurré

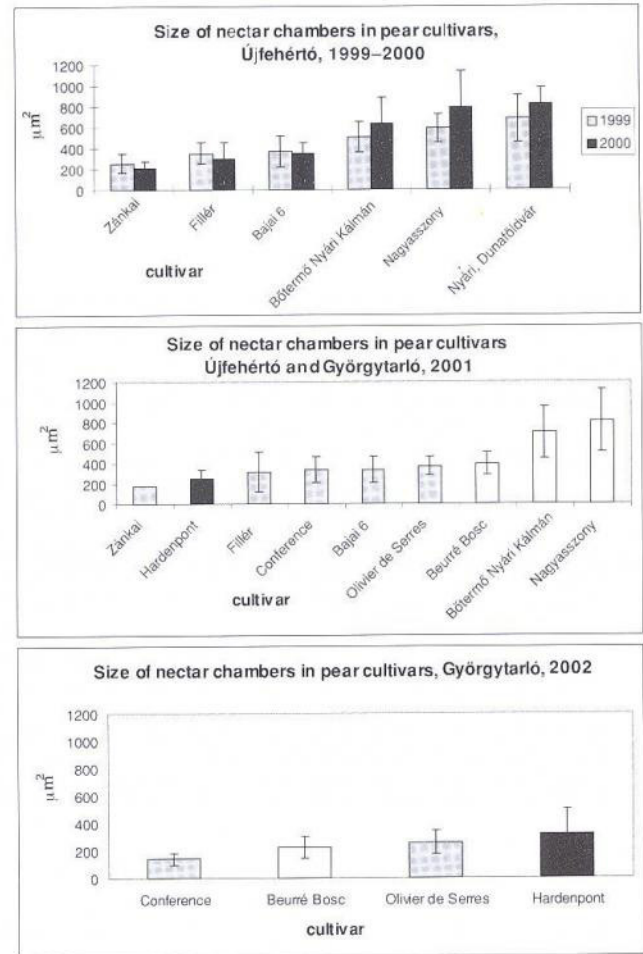


Figure 6. Nectar chamber size of variously susceptible pear cultivars

Bosc') and a susceptible pear cultivar ('Conference') in 2003. Flowers in cv. 'Conference' produced five times as much nectar as those in 'Beurré Bosc' (Fig. 8). Nectar refraction was higher in the tolerant cultivar than in the susceptible one. Higher amounts of nectar and lower refractions, typical in the susceptible cultivar, ensure more favourable conditions for *E. amylovora* than smaller amounts of more concentrated nectar in the tolerant cultivar.

Nectar composition

Similarly to the majority of pear cultivars (Wykes 1952, Farkas et al. 2002), the floral nectar of 'Beurré Bosc' and 'Conference' was hexose-dominant, i.e. the secretory product contained only glucose and fructose in measurable amounts. The amount of fructose was higher than that of glucose, their ratio being 5.5:4.5.

The nectar of 'Beurré Bosc' contained higher amounts of both glucose and fructose than the secretory product of 'Conference' (Fig. 9). Thus, in accordance with refraction data obtained in field measurements (24 h nectar production), densitometric evaluation confirmed that the nectar of the tolerant pear cultivar is more concentrated, making conditions less favourable for the pathogen.



Figure 7. Spacious nectar chambers in cv. 'Bótermő Nyári Kálmán'

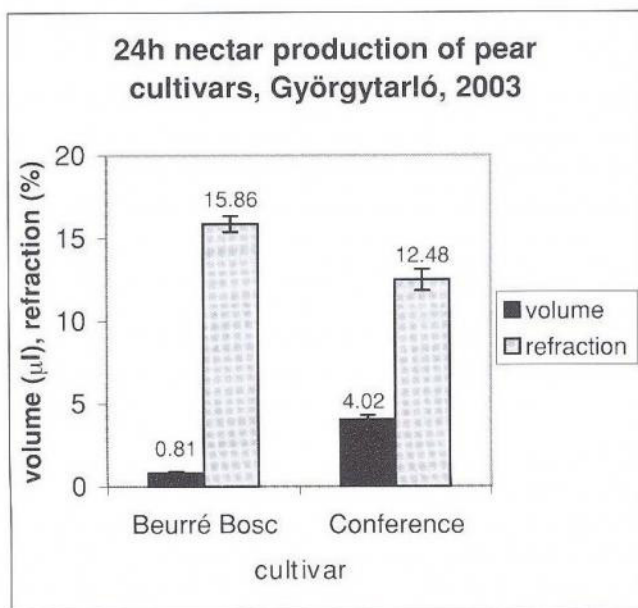


Figure 8. 24h nectar production of a susceptible and a tolerant pear cultivar

Phenolic compounds of flowers

In 2002 all flower samples were characterised by the dominance of chlorogenic acid, a phenyl-propionic acid derivative that can take part in the defense mechanism of fruit trees. On the basis of mean values (all floral organs included), the highest amount was detected in the most tolerant cultivar ('Beurré Bosc'), which contained approximately twice as much chlorogenic acid in each flower part as the most susceptible one ('Hardenpont', Fig. 10).

Concerning its distribution in various floral organs, the least chlorogenic acid was always found in the anthers, the most in the pistil, except for 'Olivier de Serres', where the corolla contained the highest amount of this compound.

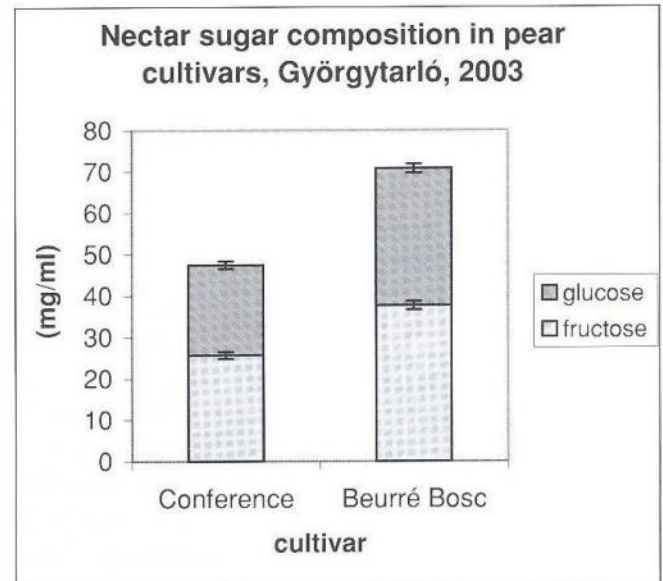


Figure 9. Nectar sugar composition of a susceptible and a tolerant pear cultivar

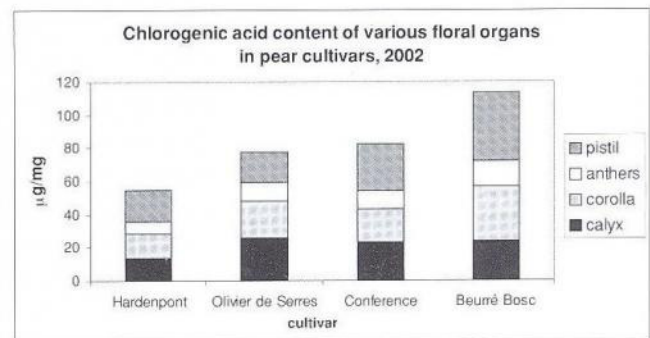


Figure 10. Chlorogenic acid content of various floral parts in pear cultivars

Besides chlorogenic acid, the most important flavonoids, rutin and hyperoside, could also be detected in all floral organs of 'Beurré Bosc' and 'Hardenpont', as well as in the calyx and corolla of 'Conference' and 'Olivier de Serres', while no hyperoside was found in the reproductive organs of the latter two cultivars (data not shown).

Further studies

Following the detailed description of nectary structure, further studies are needed to confirm that some phytochemical characteristics of the flowers (amount, concentration and composition of nectar, the presence or absence of secondary metabolites) can influence the degree of susceptibility to *E. amylovora* in pear cultivars.

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