Flavonoids, chalcones and phenyl-propanoids in apple and pear flowers

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Summary: The presence of phloretin-glycosides in the hypanthium and pistil of apple and pear flowers can be verified. Thin layer chromatography is a reliable method for detecting phloretin, gained by acidic hydrolysis. The dominance of phloretin was equally characteristic for flowers in apple ('Sampion', 'Freedom') and pear ('Beurré Bosc', 'Conference') cultivars treated with various bioregulators (Biomit, Bion 50WG, Regalis), no significant difference could be found visually as compared to control samples. Chlorogenic acid and caffeic acid was detected in all apple and pear samples, rutin was present only in pear, and hyperoside was found only in a few apple samples.

Key words: apple, pear, *Erwinia amylovora*, phloretin, chlorogenic acid, phytoalexin, thin layer chromatography

Introduction

On the effect of microbial attacks, genes manifested in the synthesis of specific, phenolic substances, are expressed. Such substances are phytoalexins, as well as various plant phenolics, phenyl-propanoids and poly-phenolic substances (*Goodman et al.*, 1986). Discussion of this huge phytochemical area is out of the scope of the present paper, only the significance of substances important for apple and pear will be underlined.

Phenylpropionic acids (e.g. cinnamomic acid, caffeic acid, p-coumaric acid) are common as intermediers, frequently occurring in flowering plants. From their estherised acid-derivatives chlorogenic acid can also commonly be found in certain plant families, being present not only in sunflower and potato, but also in apple and pear. According to our pilot studies it can be well detected by TLC in the flower organs of pear and apple, and in small quantities also in the nectar of both fruit species.

Due to their plant biological significance and manifold role, the most important group of phenolics is composed by flavonoids (*Packer*, 2001), especially the pigments occurring in flowers and fruits. They can be found mainly in the vacuoles in the form of glycosides, being dissolved in water. The structure of more than 2000 flavonoids is known in vascular plants. According to their oxidative state, most yellow (flavone) and red or blue (anthocyanin) pigments are yielded by these heterocyclic phenyl-benzopyran substances (= phenyl-croman or flavane). The key amino acid of biosynthesis is phenylalanine, out of which trans-cinnamic acid is derived by the catalytic activity of phenylalanine-ammonia-lyase. Trans-cinnamic acid is connected by condensation to the cyclic triketid evolving from 3 malonyl-CoA. The biosynthesis is catalysed by the chalcone synthase/chalcone isomerase enzyme couple. This highly important enzyme reaction enables the evolving acyclic chalcone to form a flavonoid frame by turning into a heterocyclic form. With further hydroxylation – with the involvement of Cyt P-450 monooxygenases and NADPH-dependent dioxygenase type of flavonol synthases – various hydroxylated derivatives may come into being. With the involvement of NADPH reductases, reduced forms arise (*Stafford*, 1991).

Among the hydroxylated and methylated derivatives there are several antiviral, antibacterial, antimycotic and antioxidant compounds. Many of them are scavengers, able to neutralise superoxide and hydroxyl free radicals, thus protecting the membrane.

The most frequent flavonol is quercetin and its glycoside, rutin. Kaempferol, hyperoside, luteolin, myricetin, morin and apigenin occur in several plant species. Today the gene expression of several flavonol synthases is being studied, e.g. the synthesis of quercetin-3-glycosides in the developing grape berries (*Downey et al.*, 2003). The decomposition of flavonoid aglycones takes place along synthesis of non-toxic compounds (e.g. 4-hydroxi-phenyl-glyoxylic acid, phloroglucinol carboxylic acid).

Flavonoids possessing an outstanding antibiotic character can have special importance from the viewpoint of resistance biology. In order to preserve our environment, inducing the endogenous synthesis of effective protective substances, harmless for primary consumers, should be promoted in plant protection, instead of using xenobiotics and persistent pesticides. Among plant growth regulators the practical usage of prohexadione-Ca has gained a significant role recently, especially in fruits like apple and pear. Its effective mechanism has given an impetus also to its theoretical research.

It is known that salicylic acid is an endogenous signal molecule, having an important role in controlling disease

resistance (*Raskin*, 1995). Moreover, *Heyens et al.* (2002) tried to prove that in apple rootstocks salicylic acid is an endogenous factor in the susceptibility to fire blight infections. Its antibacterial and antimycotic feature is well known, just like that of thymol, gained from thymes, being twenty times as much effective as phenol (*Bruneton*, 1995).

Tomás-Barberán et al. (1993) confirmed with analytical data that apple fruit is rich in dihydrochalcones. Later on *Tomás-Barberán & Clifford* (2000) reported that among food resources – especially fruits – not only flavanons, but also the most important precursors of flavonoids, chalcones and dihydrochalcones, can be found in a stable form in great amounts. In most plant species, however, the activity of the previously mentioned chalcone isomerase (CHI) follows that of chalcone synthase (CHS), as it is the case in apple, too. Phloretin, which is the aglycone of the dihydrochalcone-glycoside called phloridzin, turns into a flavanon called naringenin. Naringenin is hydroxylated on ring B with the mediation of flavanone-3'-hydroxylase (F3'H), thus forming eriodictyol. In another biochemical step eriodyctiol is further hydroxylated on the effect of flavanone-3-hidroxylase (FHT), forming dihydroquercetin, and then the well-known quercetin. Quercetin derivatives, responsible for the coloration of the pome fruit pericarp, contribute to protection against oxidative light stress and diverse pathogens.

Synthetic bioregulators (like growth regulators), inducing disease-related changes in the metabolism of secondary metabolic products, may play a role concerning prophylactic control of diseases. Recently, among plant growth regulators the practical usage of Regalis (with a.i.: prohexadione-Ca) has gained convincing importance.

Prohexadione-Ca, which is an inhibitor of 2-oxoglutarate dependent dioxygenases, acts as an anti-gibberellin growth retardant (*Rademacher*, 2000) and also reduces fire blight and some fungal diseases of apple and pear. This beneficial side effect is related to changes in metabolism of the flavonoids and phenyl-propanoids occurring in apple and pear leaves.

Prohexadione-Ca induces an alternative flavonoid pathway, the formation of the uncommon flavan 4-ol *luteoforol* and 3-deoxycatechin *luteoliflavan*, but causes a decrease of catechins, procyanidins and flavonols (*Roemmelt et al.*, 2003).

The formation of 3-deoxycatechins is due to the channelling of intermediates unusual for pome fruits, which requires the presence of a *flavanone 4-reductase* (FNR) activity. Other crop plants like strawberry, grapevine, cranberry, cherry, peach, plum, elder and kiwi fruit react in the same way to the application of prohexadione-Ca. By cloning of the corresponding cDNAs and studies with the heterologously expressed enzymes it was shown that the *dihydroflavanone 4-reductase* (DFR) enzymes of grapevine, cranberry and strawberry posses also FNR activity. Conclusively, these plants have the potential to form *3-deoxy-flavonoids* (*Gosch et al.*, 2003).

An important practical result is that prohexadione-Ca treatment decreased the *Erwinia amylovora* infection in apple flowers, similarly to streptomycin treatment (*Bubán et al.*, 2003). This fact is in correlation with the in vitro studies of *Roemmelt et al.* (2003), according to which the inhibitory effect of 250 ppm phloretin and naringenin is significantly exceeded by that of luteoforol, which acts an inhibitor even in 30 ppm concentration.

The present studies are closely related to the floral biological examinations carried out at the Department of Botany, University of Pécs, together with the Research and Extension Centre for Fruitgrowing, Újfehértó. The aim of this study was to gain insight into the fenoloid composition of the hypanthium and the pistil in apple and pear flowers, since the pathogen infects the flowers by penetrating through the nectary (located on the surface of the hypanthium), often covered by the secreted nectar, which can serve as a nutrient source for the bacteria. The examinations tried to find an answer to the question, whether the strongly antibacterial luteoforol or its precursors, synthesised by a metabolic pathway modified by the environment-friendly prohexadione-Ca, are present in the hypanthium and pistil of the flowers.

Material and methods

Samples and compounds

Samples: apple flower samples were collected in the high density experimental orchard of the Research and Extension Centre for Fruitgrowing, Újfehértó whereas pear flower samples were collected in a commercial orchard in Györgytarló, in April 2003. Flowers have previously been treated with various bioregulators (details below). The hypanthium together with the pistil was used for phytochemical studies, following the removal of sepals, petals and stamina. Following this preparation, hypanthia with pistils were immediately put into flasks containing pure methanol. The weight of each sample and the volume of methanol was recorded (e.g. Beurré Bosc untreated 10,83 g/34 ml). Samples were kept in refrigerator until further processing.

Products used:

BION 50WG:	a.i.: acibenzolar-S-methyl, a plant activator, inducing systemic acquired resistance to fire blight
Treatments:	10 g/100 litres: April 24, 28 and May 5, 2003
Regalis:	a.i.: prohexadione-Ca 10%, a bioregulator developed by the BASF Corporation (Germany)
Treatments:	179 g/100 litres: April 28 and May 19 Additive: Dash HC 100 ml/100 litres

Biomit Plussz (Ponton Ltd, Hungary): a leaf fertiliser containing macro-, meso- and microelements, as well as

more than 60 plant extracts, supposed to increase the resistance against diseases by the complete and/or harmonic supply of nutrient elements
Treatments: 2.0 litres/100 litres: April 24, 28 and May 5

Pear samples: 'Beurré Bosc' (untreated), 'Beurré Bosc' (BION 50WG treatment), 'Conference' (untreated), 'Conference' (BION 50WG treatment)

Apple samples: 'Sampion' (untreated), 'Sampion' (Regalis treatment), 'Sampion' (Biomit Plussz treatment), 'Sampion' (BION 50WG treatment), 'Freedom' (untreated), 'Freedom' (Regalis treatment), 'Freedom' (Biomit Plussz treatment), 'Freedom' (BION 50WG treatment)

Tests used for TLC: phloretin: (3-[4-hydroxyphenyl]-1-[2,4,6-trihydroxyphenyl]-1-propanone) (Sigma), naringenin: (4',5,7-trihydroxyflavanone) (Sigma), isoliquirtigenin: (4,2',4'-trihydroxychalcone) (Sigma), eriodictyol (Roth), rutin (Fluka), chlorogenic acid (Roth), hyperoside (Merck), caffeic acid (Serva)

Developing agent used in TLC: Naturstoff-polyethylene glycol reagent: Solution A: 1.0 g diphenylboric acid β-ethyl-amino ester dissolved in 100.00 ml methanol; solution B: 5.0 g polyethylene glycol 4000 dissolved in 100.00 ml ethanol, then solutions A and B were mixed.

Extraction

An acidic hydrolysis was applied in order to detect chalcone aglycones. Turbo extraction (Polytron, Switzerland) with methanol was carried out for 30 sec at room temperature. Following extraction, samples were filtered through cotton balls. The rest of the sample remaining on the filter was washed two more times with the amount of methanol used for extraction. 2 N sulphuric acid was added to the filtrate, their volumes being the same, then it was placed on a hot water-bath for 30 min. Following cooling, the extracts were separated three times with 50.0 ml ethylacetate in a separatory funnel, then the united phases containing ethylacetate were washed in a separatory funnel with 50.0 ml distilled water until free of acid. Then the extracts were filtered through a filter containing ca. 3 g anhydrous sodium sulphate (dehydration). Vacuum-distillation (Büchi, Switzerland) was started with reduced (245 mbar) pressure, then pressure was gradually decreased by decreasing the amount of the solvent. The extract was taken up in 5.00 ml ethylacetate. These stock solutions were used for thin layer chromatography.

Thin layer chromatographic detection

Detection of chalcones

Plate: 10×20 cm Silica gel 60 F254 TLC (Merck) aluminium sheet

Authentic test compounds: phloretin (Sigma), naringenin (Sigma), isoliquirtigenin (Sigma), eriodictyol (Roth)

10 µl of the samples and 1 µl of the tests was applied to the plates by glass capillaries.

Mobile phase: chloroform-acetone-formic acid (75:16.5:8.5)

Before sample application 20 ml of the mobile phase was poured into the chromatographic chamber (23 × 22.5 × 8 cm) (Camag), and it was saturated for 20 min. Then the plates were placed into the chromatographic chamber. Following separation, plates were dried at room temperature. Plates developed with Naturstoff-polyethylene glycol reagent were dried at room temperature, then put into a heating chamber at 105 °C for 5 min. Chromatograms were evaluated in UV light at 365 nm.

Detection of fenoloids

Plate: 10×20 cm Silica gel 60 F254 TLC (Merck) aluminium sheet

Authentic test compounds: rutin (Fluka), chlorogenic acid (Roth), hyperoside (Merck), caffeic acid (Serva)

10 µl of the samples and 1 µl of the tests was applied to the plates by glass capillaries.

Mobile phase: ethylacetate-formic acid-glacial acetic acid-distilled water (100:11:11:27)

Separation of samples, developing and detecting of the plates was done similarly to chalcones.

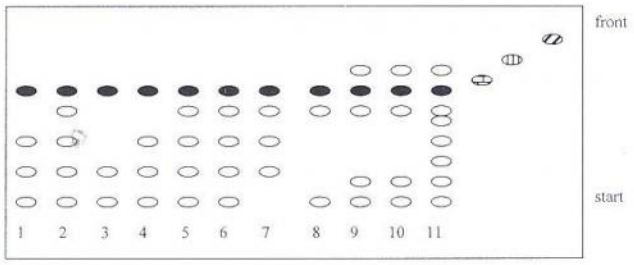
Results and discussion

On the basis of the chromatograms it was stated that at ca. Rf = 0.6, at the same height as the phloretin test, the spot of this compound can be well distinguished in the samples *(Fig. 1.)*. According to visual evaluation, phloretin content in apple samples is approximately three times as high as in pear samples. The elaborated eluent system, which can be considered as optimal for separation, makes future quantitative measurements possible from several samples. Thus the optimalisation of the hydrolytic method for extracting the phloretin-aglycone was successful.

In the samples no naringenin (Rf = 0.7), a derivative of phloretin, could be identified. Eriodictyol, which is formed out of naringenin in vivo, could neither be detected in the plant samples treated with prohexadione-Ca, although luteoforol, acting as a phytoalexin, could be synthesised from this on the inductive effect of bacterial infection.

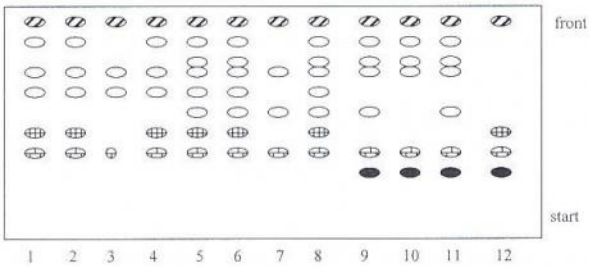
Isoliquirtigenin (Rf = 0.75) was applied only as a test, its presence in the samples could not be expected.

Of the fenoloids rutin (Rf = 0.4) could be detected only in pear samples *(Fig. 2.)*. Chlorogenic acid (Rf = 0.45) was present in both apple and pear samples, although its amount was less in the hypanthium and pistil of the apple cultivars 'Freedom' and 'Sampion', treated with Biomit Plussz. Hyperoside (Rf = 0.6) could be detected only in a few apple cultivars/treatments ('Freedom' untreated, 'Freedom' Regalis, 'Freedom' Bion, 'Sampion' untreated, 'Sampion' Regalis, 'Sampion' Bion), and it was missing from pear samples. The hypanthium and pistil of cultivars treated with Biomit Plussz was not characterised by the presence of



1: Freedom untreated, 2: Freedom Regalis, 3: Freedom Biomit, 4: Freedom Bion, 5: Sampion untreated, 6: Sampion Regalis, Sampion Biomit Plussz, 8: Sampion Bion, 9: Beurré Bosc untreated, 10: Conference Bion, 11: Beurré Bosc Bion, ●: phloretin, ⊕: naringenin, ·III·: isoliquirtigenin, ▨: eriodyctiol

Figure 1. The chromatogram of phloretin and some unidentified flavonoids in flowers of apple and pear cultivars



1: Freedom untreated, 2: Freedom Regalis, 3: Freedom Biomit Plussz, 4: Freedom Bion, 5: Sampion untreated, 6: Sampion Regalis, 7: Sampion Biomit Plussz, 8: Sampion Bion, 9: Beurré Bosc untreated, 10: Conference Bion, 11: Beurré Bosc Bion, 12: tests: ● rutin, ⊕: chlorogenic acid, ⊕: hyperoside, ▨: caffeic acid

Figure 2. The chromatogram of fenoloids detected in flowers of apple and pear cultivars

hyperoside. Caffeic acid (Rf = 0.9) was detected in all samples.

According to our preliminary studies the presence of phloretin-glycosides in the hypanthium and pistil of apple and pear flowers can be verified. Thin layer chromatography is a reliable method for detecting phloretin, gained by an acetic hydrolysis. So the stable presence of this essential dihydrochalcone can ensure the biosynthesis of subsequent flavonoids, but it can also serve as the initial compound of the alternative flavonoid pathway, induced by the dioxygenase-inhibitor prohexadione-Ca. The presence of naringenin and luteoforol, synthesised in a special pathway, could not be proved so far from the hypanthium and pistil of the flowers, although it could have been expected especially following the treatment with prohexadione-Ca. It should be emphasised, however, that according to *Roemmelt et al.* (2003) the antibacterial activity of phloretin can be proved in vitro, although it does not come up with that of luteoforol. The presence of phloretin is supposed to be important for natural protection.

The dominance of phloretin was equally characteristic for flower samples (hypanthium and pistil) treated with various products, no significant difference could be found visually as compared to control samples.

According to TLC studies, minor components (phenylpropanoids, flavonoids) occur only in small amounts in the hypanthium and pistil of apple and pear flowers. There are no or only minor differences between the samples. Examinations should be extended with further standards, since it cannot be stated with certainty that – especially due to the effect of the prominent prohexadione-Ca – no phytoalexin-like luteoliflavans (3-deoxy-catechins) are synthesised in apple and pear flowers.

Acknowledgements

The authors would like to thank Tamás Bubán, scientific consultant at the Research and Extension Centre for Fruitgrowing, Újfehértó, for initiating this research. We are grateful to Lajos Botz, associate professor at the Pharmaceutical Institute, Faculty of Medicine, University of Pécs, for his advice in the phytochemical work. We are also thankful to Mária Mészáros-Horváth for her technical assistance.

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