Journal of Plant Diseases and Protection, **115** (6), 247–251, 2008, ISSN 1861-3829.

Ontogenetic variation of catechin biosynthesis as basis for infection and quiescence of *Botrytis cinerea* in developing strawberry fruits

Entwicklungsabhängige Catechin-Biosynthese als Basis für Infektion und Quieszenz von *Botrytis cinerea* in Erdbeerfrüchten

I. Puhl & D. Treutter*
Center of Life and Food Sciences Weihenstephan, Technische Universität München, Fachgebiet für Obstbau, Freising, Germany
* Corresponding author, e-mail dieter.treutter@wzw.tum.de

Received 3 September 2008; accepted 15 September 2008

Summary

The accumulation of catechin derived procyanidins is regarded as one fundamental factor inhibiting the growth of the pathogenic fungus *Botrytis cinerea* in immature strawberry fruits – a phenomenon referred to as quiescence after invasion at bloom usually ending during fruit ripening. For evaluation of that hypothesis an attempt was made to modify the concentration of flavanols in developing strawberry fruits by treatments with an inhibitor of 2-oxoglutarate-dependent dioxygenases which is known to affect the flavonoid pathway. The accumulation of novel flavonoids identified as luteoliflavan and eriodictyol 7-glucoside and enhanced levels of catechin were found when green fruits were treated. The effect of the bioregulator varies during fruit ontogenesis with a steady increase after flowering to the stage of small green fruits and a rapid decrease thereafter. It became obvious that young fruits just at flowering do not accumulate flavanols to a sufficient level for preventing primary receptacle infection.

Key words: *Botrytis cinerea*, catechin, eriodictyol 7-glucoside, luteoliflavan, prohexadione-Ca, strawberry

Zusammenfassung

Die Akkumulation von Catechin und Proanthocyanidinen in unreifen Erdbeerfrüchten wird als ein Faktor der vorübergehenden Resistenz gegenüber *Botrytis cinerea*, dem Erreger der Grauschimmelfäule, angesehen und dafür verantwortlich gemacht, dass der Pilz nach dem Befall der Blüte bis zur Fruchtreife in einem Ruhezustand verharrt. Zur Bewertung dieser Hypothese wurde ein experimenteller Ansatz gewählt, bei dem die Konzentration von Flavanolen in sich entwickelnden Erdbeerfrüchten durch Behandlung mit einem Inhibitor von 2-Oxoglutarat abhängigen Dioxygenasen modifiziert werden sollte. Nach der Behandlung junger, grüner Früchte wurde die Anreicherung neuartiger Flavonoide, Luteoliflavan und Eriodictyol -7-glucosid, sowie von Catechin festgestellt. Der Effekt der Bioregulator-Behandlung variierte jedoch während der Fruchtentwicklung und hatte die größte Auswirkung bei kleinen grünen Früchten. Zur Zeit der primären Besiedelung der Blüten durch den Pilz ist der junge Blütenboden jedoch nur unzureichend zur Flavanol-Biosynthese befähigt.

Stichwörter: *Botrytis cinerea*, Catechin, Erdbeere, Eriodictyol 7-glucoside, Luteoliflavan, Prohexadion-Ca

1 Introduction

Strawberry (*Fragaria* × *ananassa*) is one of the main fruit crops in Germany. Most varieties are highly susceptible to grey mould caused by the fungus *Botrytis cinerea*. This pathogen invades via floral parts into the bottom of the receptacle where the mycelium remains quiescent until fruit ripening (Scherer 1982; Bristow et al. 1986; Jersch et al. 1989; Boff et al. 2003). Grey mould symptoms occur only in ripe, red-coloured fruits. Thus, the choice of the flowers as the favoured tissue for fungal invasion as well as the latency of the pathogen in green fruits can be regarded as the critical points in *B. cinerea* development. Jersch et al. (1989) observed strawberry proanthocyanidins to be responsible for resistance of immature fruits. Proanthocyanidins in the strawberry consist of catechin units (Ishimaru et al. 1995) which is a main flavonoid in strawberries (Ishimaru et al. 1995; Törrönen and Määttä 2002; Breitfellner et al. 2002; 2003; Auger et al. 2004; Seeram et al. 2006; Wulf et al. 2008) and it possesses antimicrobial properties (Scalbert 1981; Yamamoto et al. 2000). Strawberry cultivars with higher concentrations of free catechin were more resistant to *B. cinerea* (Hébert et al. 2001). Several authors also found a positive correlation between resistance to *B. cinerea* and the concentration of proanthocyanidins (DiVenere et al. 1998; Jersch et al. 1989; Hébert et al. 2001).

For further elucidation of that role of catechins it is of interest either to inhibit the flavanol biosynthesis or to increase their amount in ripe fruits. Recently, the bioregulator prohexadione-Ca was described as a useful tool for modification of flavonoid biosynthesis (Römmelt et al. 2003; Halbwirth et al. 2002; 2003). Prohexadione-Ca is a structural mimic of 2-oxoglutarate and according to this property it is able to inhibit dioxygenase enzymes which require 2-oxoglutarate as cosubstrate (Rademacher 2000). One prominent dioxygenase of the flavonoid pathway is the flavanone 3-hydroxylase (FHT) which is involved in the biosynthesis of catechin precursors (Fig. 1, Forkmann and Heller 1999). In apple leaves, a reduced accumulation of catechin was found after treatment with the bioregulator (Römmelt et al. 2003). Other experiments, however, revealed an enhanced biosynthesis of flavanols in several plants triggered by prohexadione-Ca treatment (Gosch et al. 2003; Puhl et al. 2008; Fischer et al. 2006). Both possible effects of the bioregulator turn its application to a tool which may help to understand the role of flavanols in strawberry fruits with respect to defence of *B. cinerea*.

Therefore, we treated strawberry flowers and fruits with prohexadione-Ca and analysed their composition of phenolics in order to gain insight into biosynthetic activity with respect to the flavan-3-ols (catechin and proanthocyanidin B_3).

2 Material and methods

2.1 Plant material

For the trial potted strawberry plants of the cv. Elsanta, and the breeding lines II/29 and 91/13/3 bred by H. Schimmelpfeng (Technische Universität München, Weihenstephan) were used. Two different treatments were chosen: control (water treatment) and a prohexadione-Ca treatment (200 ppm). Two prohexadione-Ca treatments were performed with one day waiting time between the applications. Two days after

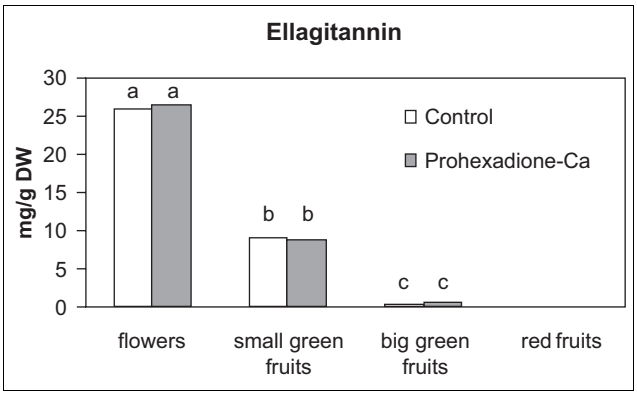

Fig. 1: Concentrations (mg g^{-1} dry weight) of ellagitannin in prohexadione-Ca treated and untreated strawberry fruits at different developmental stages. Different letters indicate significant differences at the 95% level using Mann-Whitney-test.

the last treatment fruits/flowers were collected and assigned to the respective developmental stages (full flower, small green fruits, big green fruits, and ripe fruits). The control plants were harvested in the same way.

From the flowers only the receptacle area was collected. All samples to be tested were frozen in liquid nitrogen directly after sampling, stored at −20°C and, finally, were lyophilized.

2.2 Extraction of phenolic compounds

For the extraction of phenolic compounds, the lyophilized material from all developmental stages except ripe fruits was ground in a ball mill. The fine powder (100 mg) was extracted with 500 µl methanol (100%) containing flavone (Roth, Karlsruhe, Germany) as an internal standard for 30 min in a cooled water bath during sonication. The extracts were centrifuged for 10 min × 10000 g at 4°C and the supernatant was directly used for analytical HPLC.

Ripe fruits were homogenised using an Ultra-Turrax homogeniser. For extraction of 1 g fruits a volume of 10 ml methanol containing 0.05 mg/ml of flavone as an internal standard was used. After centrifugation 500 µl of the supernatant were taken and the solvent was evaporated. The residue was redissolved in 100 µl methanol, centrifuged and used for HPLC-analysis.

2.3 HPLC analysis

Phenolic compounds were separated on a column (150 × 4 mm) prepacked with Hyperclone ODS, 3 µm particle size, following a stepwise gradient, using mixtures of solvent A (formic acid, 5% in water) and solvent B (methanol) from 95:5 (A/B) to 10:90 (A/B) with a flow rate of 0.6 ml/min using the following gradient: 0–5 min, 0–2,5% B, 5–15 min 2,5% B, isocratic, 15–30 min, 2,5–5% B; 30–40 min isocratic 5% B, 40–65 min, 5–10% B; 65–105 min, 20–30% B; 105–165 min, 30–40% B, 165–180 min, 40–50% B; 180–195 min, 50–90% B; 195–215 min isocratic 90% B.

Eriodictyol 7-glucoside and ellagitannin were detected at 280 nm, whereas the flavanols (catechin and luteoliflavan) and proanthocyanidin B_3 were estimated at 640 nm after post-column derivatisation with DMACA (Treutter 1989).

Peak identification was conducted by their UV absorbance spectra and by comparison with authentic standards: eriodictyol 7-glucoside (Roth), catechin (Roth), procyanidin, B_3 (previously isolated, Feucht et al. 1996) and luteoliflavan (formerly isolated, Römmelt et al. 2003). Quantification was performed using response factors of standards. Luteoliflavan was calculated as catechin, proanthocyanidin B_3 as procyanidin B_2, and ellagitannin as ellagic acid.

2.4 Acid hydrolysis

For acid hydolysis the compound corresponding to the proposed ellagitannin peak was collected from several HPLC runs. To the collected fractions an equal volume of 1 N methanolic hydrochloric acid was added and the solution hydrolised for 20 minutes in a boiling water bad. Thereafter the sample was extracted three times with ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness redissolved in methanol and used for further identification.

2.5 Statistics

For statistical analysis, a pair wise comparison was performed by the non-parametrical Mann-Whitney-test using "Minitab 14 – statistical software". Data were analysed according to varieties but different effects were not observed (data not shown). Thus, they were combined as repetitions. Values in figures followed by different letters are significantly different (significance level 95%).

3 Results and discussion

3.1 Identification of phenolics compounds

In the strawberry fruits catechin and procyanidin B_3 were identified as the main flavan-3-ols according to Wulf et al. (2008). Young fruits exhibited another big peak with an absorbance maximum at 252 nm. This compound was purified by several HPLC runs. The combined fractions were hydrolysed in hydrochloric acid which resulted in ellagic acid. This component is therefore identified as an ellagitannin.

As a consequence of the prohexadione-Ca treatment two novel flavonoids were found: eriodictyol 7-glucoside and luteoliflavan. They were identified by comparison with previously isolated standards (Römmelt et al. 2003).

3.2 Concentration of phenolic compounds during fruit development

As formerly described by Halbwirth et al. (2006) flavonoid biosynthesis of strawberry fruits is integrated into the programme of fruit development. During flowering a high concentration of ellagitannin was found in the receptacle which declines rapidly during fruit growth (Fig. 1) whereas maximum accumulation of catechin and its dimer procyanidin B_3 takes place in green fruits (Fig. 2). Treatment of flowers and fruits with the dioxygenase inhibitor increased the concentration of catechin in small immature fruits (Fig. 2) and induced the accumulation of the novel compounds eriodictyol 7-glucoside and luteoliflavan (Fig. 3, 4). The latter can be explained by the inhibition of FHT activity (Halbwirth et al. 2002; Fischer et al. 2003) which results in a tailback at the flavanone level and in a switch of dihydroflavonol reductase (DFR) activity toward flavanone reductase (FNR) (Halbwirth et al. 2002; Fischer et al. 2003; Almeida et al. 2007), respectively. FNR is only active if flavanones are available (Gosch et al. 2003; Fischer et al. 2003). In untreated plants, the non-disturbed FHT passes the flavanone rapidly away into dihydroquercetin (Forkmann and Heller 1999) (Fig. 4).

The increase in catechin concentration seems to be contradictory to the occurring FHT bottleneck. However, similar observations were made on apple (Fischer et al. 2006) and grapevine (Puhl et al. 2008). In those tissues this could be

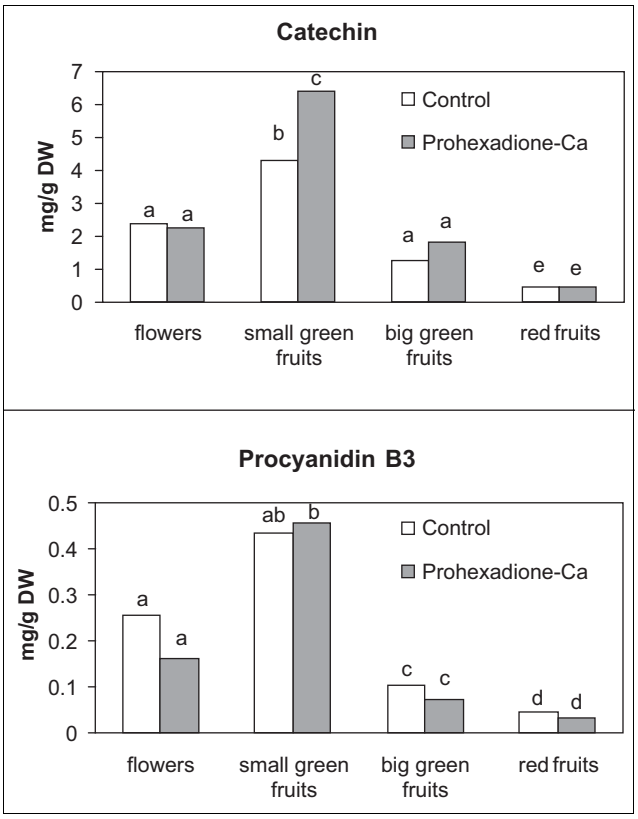

Fig. 2: Concentrations (mg g^{-1} dry weight) of catechin and procyanidin B3 in prohexadione-Ca treated and untreated strawberry fruits at different developmental stages. Different letters indicate significant differences at the 95% level using Mann-Whitney-test.

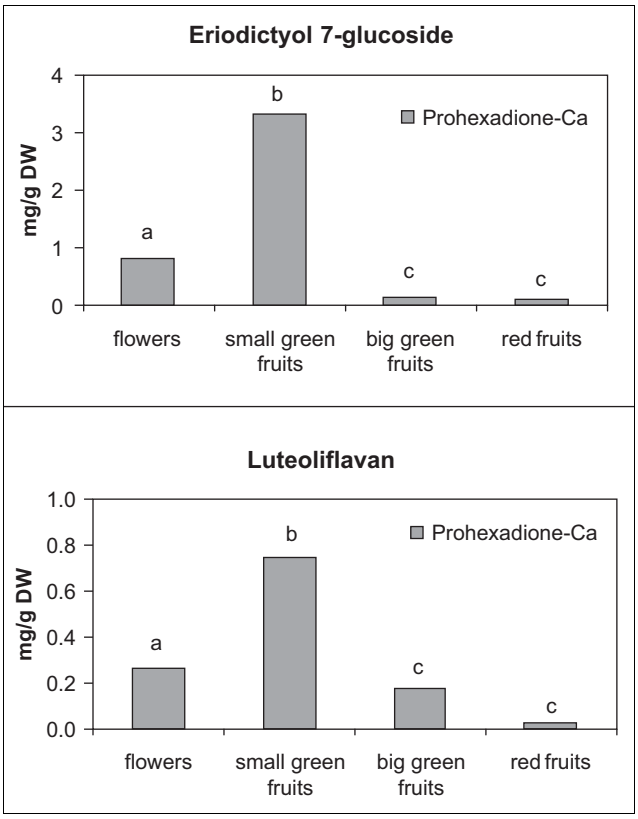

Fig. 3: Concentrations (mg g^{-1} dry weight) of the novel flavonoids eriodictyol 7-glucoside and luteoliflavan in prohexadione-Ca treated strawberry fruits at different developmental stages. Different letters indicate significant differences at the 95% level using Mann-Whitney-test.

explained by an additional strong inhibition of the flavonol synthase which also is a 2-oxoglutarate dependent dioxygenase and thus an excess supply of substrates for the remaining FHT activity was assumed. Another possible explanation may be the observed general inducing effect of prohexadione-Ca on the expression of PR-protein related genes (Bini et al. 2008). An enhanced level of PAL transcripts as well as the corresponding enzyme activity was previously described in apple (Fischer et al. 2006). The bioregulator induced enhancement of the catechin concentration occurs only in young strawberry fruits (Fig. 2). It must therefore be assumed that flavanol biosynthesis is highly active in this developmental stage. This is confirmed by the strong accumulation of eriodictyol 7-glucoside and luteoliflavan in the same tissues (Fig. 3). The weak response during flowering and late ripening stages corresponds to a weak activity of the flavanol pathway. For ripe fruits weak activities of PAL and CHS were previously described (Halbwirth et al. 2006).

3.3 Conclusion

It is obvious that young fruits just at flowering do not accumulate flavanols to a level which may be sufficient for preventing primary receptacle infection. This may be the reason why *B. cinerea* has chosen the flowering stage for invading into the fruit. The increasing catechin and proanthocyanidin concentrations during further fruit development may restrict fungal growth according to the hypothesis of Jersch et al. (1989).These results on the biosynthetic activity raise the question if the constitutive concentrations of catechin and proanthocyanidins in the receptacle are responsible for restricting the fungus in its quiescence stage. In leaves of strawberries and several fruit trees of the *Rosaceae* infected by different fungi an induced accumulation of flavan-3-ols (catechins and proanthocyanidins) was observed around the infection zone where fungal growth stops (Treutter and Feucht 1990; Feucht and Treutter 1999). Beside the concentration of constitutively formed metabolites, a rapid biosynthesis of antifungal flavanols just at the infection site may also account for successful defence of pathogens in young strawberry fruits.

Acknowledgements

The authors gratefully acknowledge the Bayerisches Staatsministerium für Umwelt, Gesundheit und Verbraucherschutz for financial support of the study.

Literature

Almeida, J.-R.-M., E. D'Amico, A. Preuss, F. Carbone, C.-H. Ric de Vos, B. Deiml, F. Mourques, G. Perotta, T.-C. Fischer, A.-G. Bovy, S. Martens, C. Rosati, 2007: Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (*Fragaria* × *ananassa*). Arch. Biochem. Biophys. **465**, 61-71.

Auger, C., N. Al-Awwadi, A. Bornet, J.-M. Rouanet, F. Gasc, G. Cros, P.-L. Teissedre, 2004: Catechins and procyanidins in Mediterranean diets. Food Res. Int. **37**, 233-245.

Bini, F., A. Ragaini, C. Bazzi, 2008: Resistance responses in induced by the plant growth retardant prohexadione-Ca in apple against scab infections. Ann. Appl. Biol. **152**, 19-27.

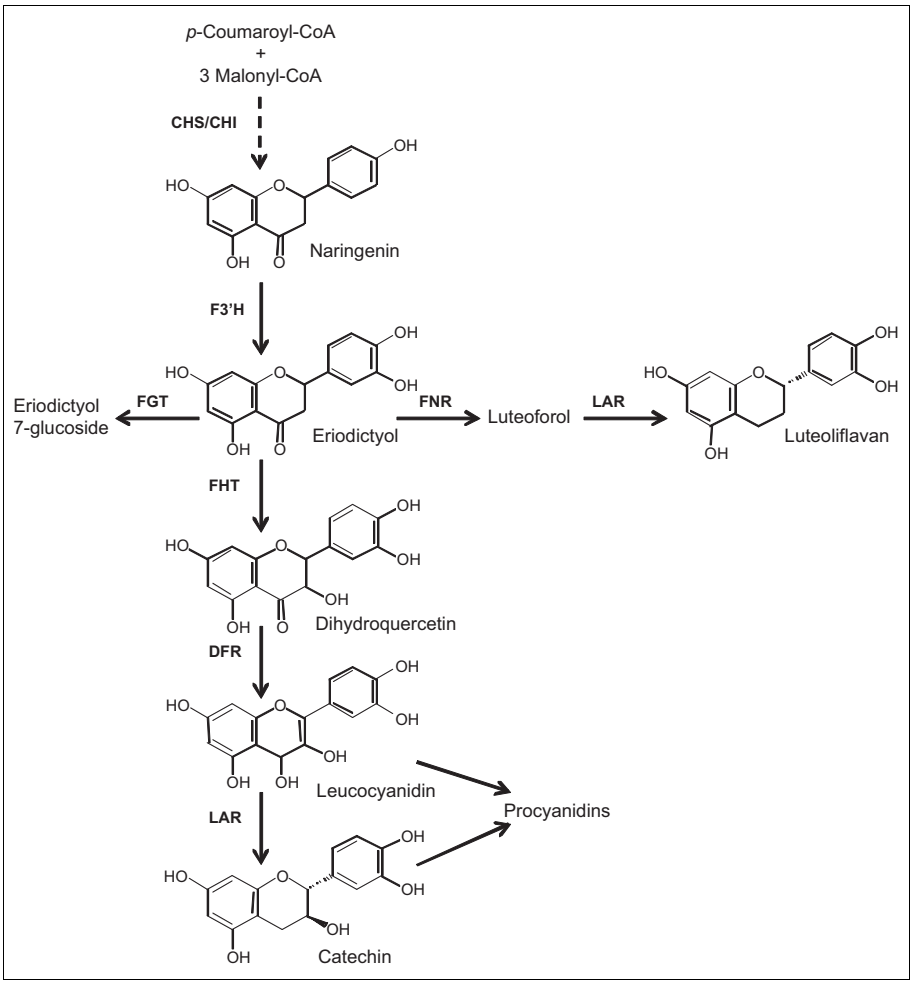

Fig. 4: Simplified scheme of the flavonoid pathway in strawberry fruits showing the biosynthesis of catechin, luteoliflavan and eriodictyol 7-glucoside.
Enzymes involved: CHS, chalcone synthase; CHI, chalcone/flavanone isomerase; F3'H, flavonoid 3'-hydroxylase; FGT, flavonoid glucosyl transferase; FNR, flavanone 4-reductase; LAR, leucoanthocyanidin reductase; FHT, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase.

Breitfellner, F., S. Solar, G. Sontag, 2002: Effect of gamma irradiation on flavonoids in strawberries. Eur. Food Res. Technol. **215**, 28-31.

Breitfellner, F., S. Solar, G. Sontag, 2003: Radiation induced chemical changes of phenolic compounds in strawberries. Radiat. Phys. Chem. **67**,497-499.

Bristow, P.-R., R.-J. McNicol, B. Williamson, 1986: Infection of strawberry flowers by *Botrytis cinerea* and its relevance to grey mould development. Ann. Appl. Biol. **109**, 545-554.

Boff, P., J. De Kraker, M. Gerlagh, J. Köhl, 2003: The role of petals in development of grey mould in strawberries. Fitopatol. Brasil. **28**, 076-083.

DiVenere, D., V. Linsalata, A. Ippolito, F. Nigro, P. Arcuti, V. Lattanzio, 1998: Endogenous phenolics, ripening and susceptibility of strawberry fruits (*Fragaria* × *ananassa* Duch.) to post harvest diseases. In: Polyphenols Communication 98, XIX International Conference on Polyphenols, Lille, France, 1-4 September, pp. 459-460.

Feucht, W., D. Treutter, E. Christ, 1996: Flavanols in grapevine: in vitro accumulation and defence reactions in shoots. Vitis **35**, 113-118.

Feucht, W., D. Treutter, 1999: The role of flavan-3-ols and proanthocyanidins in plant defence. In: S. Inderjit, K.-M.-M. Dakshini, C.-L. Foy (eds.): Principles and Practices in Plant Ecology, pp. 307-338. CRC Press, Boca Raton, FL, USA.

Fischer, T.-C., H. Halbwirth, B. Meisel, K. Stich, G. Forkmann, 2003: Molecular cloning, substrate specificity of the functionally expressed dihydroflavonol 4-reductases from *Malus domestica* and *Pyrus communis* cultivars and the consequences for flavonoid metabolism. Arch. Biochem. Biophys. **412**, 223-230.

Fischer, T.-C., H. Halbwirth, S. Römmelt, E. Sabatini, K. Schlangen, C. Andreotti, F. Spinelli, G. Costa, G. Forkmann, D. Treutter, K. Stich, 2006: Induction of polyphenol gene expression in apple (*Malus* × *domestica*) after the application of a dioxygenase inhibitor. Physiol. Plant. **128**, 604-617.

Forkmann, G., W. Heller, 1999: Biosynthesis of flavonoids. In: U. Sankawa (ed.): Comprehensive Natural Products Chemistry, vol. 1, pp. 714-748. Elsevier, Amsterdam, The Netherlands.

Gosch, C., I. Puhl, H. Halbwirth, K. Schlangen, S. Römmelt, C. Andreotti, G. Costa, T.-C. Fischer, D. Treutter, K. Stich, G. Forkmann, 2003: Effect of prohexadione-Ca on various fruit crops: flavonoid composition and substrate.specificity of their dihydroflavonol 4-reductases. Eur. J. Hort. Sci. **683**, 144-151.

Halbwirth, H., T.-C. Fischer, B. Meisel, W. Kampan, W. Rademacher, G. Forkmann, K. Stich, 2002: Biochemical and molecular biological investigations with respect to induction of fire blight resistance in apple and pear by transiently altering the flavonoid metabolism with specific enzyme inhibitors. Acta Hort. **590**, 485-492.

Halbwirth, H., T.-C. Fischer, S. Roemmelt, F. Spinelli, K. Schlangen, S. Peterek, E. Sabatini, C. Messina, J.-B. Speakman, C. Andreotti, W. Rademacher, C. Bazzi, G. Costa, D. Treutter, G. Forkmann, K. Stich, 2003: Induction of antimicrobial 3-deoxyflavonoids in pome fruit trees controls fire blight. Z. Naturforsch. **58c**, 765-770.

Halbwirth, H., I. Puhl, U. Haas, K. Jezik, D. Treutter, K. Stich, 2006: Two-phase flavonoid formation in developing strawberry (*Fragaria* × *ananassa*) fruit. J. Agric. Food Chem. **54**, 1479-1485.

Hébert, C., M.-T. Charles, L. Gauthier, C. Willemot, S. Khanizadeh, J. Cousineau, 2001: Strawberry proanthocyanidins: Biochemical markers for *Botrytis cinerea* resistance and shelf-life predictability. Acta Hort. **567**, 659-662.

Ishimaru, K., T. Omoto, I. Asai, K. Ezaki, K. Shimomura, 1995: Taxifolin 3-arabinoside from *Fragaria* × *ananassa*. Phytochemistry **40**, 345-347.

Jersch, S., C. Scherer, G. Huth, E. Schlösser, 1989: Proanthocyanidins as basis for quiescence of *Botrytis cinerea* in immature strawberry fruits. J. Plant Dis. Protect. **96**, 365-378.

Puhl, I., F. Stadler, D. Treutter, 2008: Alterations of flavonoid biosynthesis in young grapevine (*Vitis vinifera* L.) leaves, flowers, and berries Induced by the dioxygenase inhibitor Prohexadione-Ca. J. Agric. Food Chem. **56**, 2498-2504.

Rademacher, W., 2000: Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. Plant Mol. Biol. **51,** 501-531.

Römmelt, S., N. Zimmermann, W. Rademacher, D. Treutter, 2003: Formation of novel flavonoids in apple (Malus × domestica) treated with the 2-oxoglutarate-dependet dioxygenase inhibitor prohexadione-Ca. Phytochemistry **64**, 709-716.

Scalbert, A., 1981: Antimicrobial properties of tannins. Phytochemistry **30**, 3875-3883.

Scherer, C., 1982: Rolle von Proanthocyanidinen bei der Stadienresistenz von Erdbeerfrüchten gegenüber *Botrytis cinerea* Pers. PhD thesis, University of Gießen.

Seeram, N.-P., H. Rupo Lee, H.-S. Scheuller, D. Heber, 2006: Identification of phenolic compounds in strawberries by liquid chromatography electrospray ionization mass spectroscopy. Food Chem. **97**, 1-11.

Törrönen, R., K. Määttä, 2002: Bioactive substances and health benefits of strawberries. Acta Hort. **56**, 797-803.

Treutter, D., 1989: Chemical reaction detection of catechins and proanthocyanidins with 4-dimethylaminocinnamaldehyde. J. Chromat. **467**, 185-193.

Treutter, D., W. Feucht, 1990: Accumulation of flavan-3-ols in fungus-infected leaves of Rosaceae. Z. Pflanzenk. Pflanzen. **97**, 634-641.

Wulf, J.-S., S. Rühmann, I. Regos, I. Puhl, D. Treutter, M. Zude, 2008: Analyses of phenolic compounds in strawberry fruits (*Fragaria* × *ananassa*) by chromatographic and non-destructively applied laser-induced fluorescence spectroscopy. J. Agric. Food Chem. **56**, 2875-2882.

Yamamoto, M., S. Nakatsuka, H. Otani, K. Kohimoto, S. Nishimura, 2000: (+)-Catechin acts as an infection-ihibiting factor in strawberry leaf. Phytopathology **90**, 595-599.