Europ.J.Hort.Sci., **68** (3). S. 129–136, 2003, ISSN 1611-4426. © Verlag Eugen Ulmer GmbH & Co. Stuttgart

Effect of Dioxygenase Inhibitors on the Resistance-related Flavonoid Metabolism of Apple and Pears: Chemical, Biochemical and Molecular Biological Aspects

S. Roemmelt[1)], T. C. Fischer[2)], H. Halbwirth[3)], S. Peterek[1)], K. Schlangen[3)], J.-B. Speakman[4)], D. Treutter[1)], G. Forkmann[2)] and K. Stich[3)]

([1)]Unit of Fruit Science, Department of Plant Sciences, Technical University of Munich, Freising, Germany, [2)]Chair of Floriculture Crops and Horticultural Plant Breeding, Department of Plant Sciences, Technical University of Munich, Freising, Germany, [3)]Institute of Technical BioScience, Technical University of Vienna, Vienna, Austria and [4)]BASF Agricultural Center, Limburgerhof, Germany)

Summary

The plant growth regulator prohexadione-Ca, which is an inhibitor of 2-oxoglutarate dependent dioxygenases, acts as an anti-gibberellin and also reduces fire blight and scab of apple and pear. This beneficial side effect is related to a transient alteration of the flavonoid metabolism in apple and pear target tissues. This paper reviews the changes of the composition of flavonoids and phenylpropanoids occurring in leaves treated with prohexadione-Ca. An alternative flavonoid pathway is described leading to the formation of 3-deoxycatechins, which are uncommon in apple and pear. As biochemical prerequisites for this change in metabolism the substrate specificity of key enzymes is demonstrated.

Zusammenfassung

Wirkung von Dioxygenase-Inhibitoren auf den Flavonoidstoffwechsel bei Apfel und Birne: Chemische, biochemische und molekularbiologische Aspekte. Der im Obstbau als Antigibberellin eingesetzte Wachstumsregulator Prohexadion-Ca hemmt 2-Oxoglutarat abhängige Enzyme. Als positiver Nebeneffekt von Prohexadion-Ca-Behandlungen wird bei Apfel und Birne eine erhöhte Widerstandsfähigkeit gegenüber Feuerbrand und Schorf beobachtet, was auf einer vorübergehenden Veränderung des Flavonoidstoffwechsels beruht. In diesem Übersichtsbeitrag werden die Veränderungen der Zusammensetzung der Flavonoide und Phenylpropanoide dargestellt. Die Behandlung von Apfel- und Birnenblättern führt zur Bildung der für diese Pflanzen ungewöhnlichen 3-Deoxycatechine über einen alternativen Biosyntheseweg. Als biochemische Voraussetzung für diesen Weg wird die Substratspezifität von Schlüsselenzymen dokumentiert.

Key words. *Malus* – *Pyrus* – prohexadione-Ca – *Erwinia amylovora* – dihydroflavonol 4-reductase – flavanone 4-reductase – luteoliflavan

Introduction

Orchard trials revealed that trees pre-treated with the plant growth regulator prohexadione-Ca, an inhibitor of 2-oxoglutarate dependent dioxygenases (RADEMACHER 2000), were significantly less affected by fire blight (ROEMMELT et al. 1999; YODER et al. 1999). Moreover, it could be shown that apple trees were even less susceptible towards infection by scab, which is caused by the fungal pathogen *Venturia inaequalis* (RADEMACHER et al. 1999). However, prohexadione-Ca is absolutely inactive as a bactericide or as a fungicide (RADEMACHER and KOBER, this issue). Furthermore, other plant growth regulators with similar action on the plant habit (e.g. daminozide, chlormequat chloride, paclobutrazol) do not affect the susceptibility to such pathogens. Therefore, it has been hypothesised that prohexadione-Ca may induce an accumulation of distinct metabolites which would, directly or indirectly, make plants less susceptible against pathogens. It is known that leaves of apple trees with low susceptibility towards infection by scab often accumulate high amounts of phenolic compounds, in particular flavonoids, at the infection site (TREUTTER and FEUCHT 1990; MAYR et al. 1997).

Since prohexadione-Ca is known to act as a structural mimic of 2-oxoglutarate (RADEMACHER 2000) inhibiting 2-oxoglutarate dependent dioxygenases we hypothesised as follows: In the flavonoid pathway, four enzymes have been classified as 2-oxoglutarate dependent dioxygenases, flavanone 3-hydroxylase (FHT), flavonol synthase (FLS), anthocyanidin synthase (ANS) and flavone synthase I (FNS I). These enzymes could be inhibited competitively by prohexadione-Ca, which may lead to marked changes in the flavonoid composition of apple and pear via backward accumulation of precursors, feed back activation or repression and over-

flow reactions. This review intends to give a synopsis of the chemical, biochemical and molecular coherence related to the action of dioxygenase inhibitors such as prohexadione-Ca and trinexapac-ethyl.

Biosynthesis of flavonoids in apple and pear

The enzymes involved in flavonoid biosynthesis are well established and the corresponding genes have been cloned from many plants (FORKMANN and HELLER 1999). In apple and pear leaves (Fig. 1), the main enzymes of the flavonoid biosynthesis were demonstrated and the respective assays were optimised (HALBWIRTH et al. 2002). Phenylalanine ammonia-lyase (PAL) provides the link between primary metabolism and the phenylpropanoid pathway. The enzyme catalyses the reaction from phenylalanine to trans-cinnamate, which is transformed by two further enzymes to 4-coumaroyl-CoA. The key enzyme of the flavonoid pathway is chalcone synthase (CHS) catalysing the condensation of three molecules malonyl-CoA with 4-coumaroyl-CoA. The reaction leads to the formation of naringenin chalcone which is transferred by chalcone isomerase (CHI) to naringenin. B-ring hydroxylation of naringenin, catalysed by flavonoid 3´-hydroxylase (F3´H), leads to the formation of eriodictyol, which is further converted by flavanone 3-hydroxylase (FHT) and by dihydroflavonol 4-reductase (DFR) to leucocyanidin. The latter is the biosynthetic intermediate in the formation of the 3-hydroxyflavonoid catechin, the reduction is catalysed by leucoanthocyanidin reductase (LAR). Alternatively anthocyanidin synthase (ANS) oxidises the leucocyanidin to cyanidin, which then may be glycosylated to the anthocyanin or be reduced by anthocyanidin reductases to epicatechin. The latter reaction was recently described (XIE et al. 2003). Both flavan 3-ols, catechin and epicatechin, are found as monomeric subunits of procyanidins.

Unexpected accumulation of diverse flavonoids and phenylpropanoids

Phenolic compounds of apple leaves were intensively studied with sensitive and selective HPLC-methods using UV-detection as well as chemical reaction detection (TREUTTER 1989; MAYR et al. 1997). The combination of the common UV-detection mode with online post-column derivatisation revealed the occurrence of novel compounds (ROEMMELT et al. 2003) such as the 3-deoxycatechin luteoliflavan and further flavans which are not yet completely identified (Fig. 2; Table 1). Additionally, some flavanones accumulated after prohexadione-Ca treatment. These were identified as naringenin 7-O-glucoside, eriodictyol 7-O-glucoside, 6´´-*O*-*trans*-*p*-coumaroyl-eriodictyol 3´-O-glucoside (Fig. 3; Table 2). Furthermore, some phenylpropanoids have been isolated and identified as novel compounds, such as *cis*-*p*-coumaroylglucose, *trans*-*p*-coumaroylglucose, 4-*O*-*cis*-*p*-coumaroyl-phloridzin and 4-*O*-*trans*-*p*-coumaroyl-phloridzin (Fig. 3; Table 2).

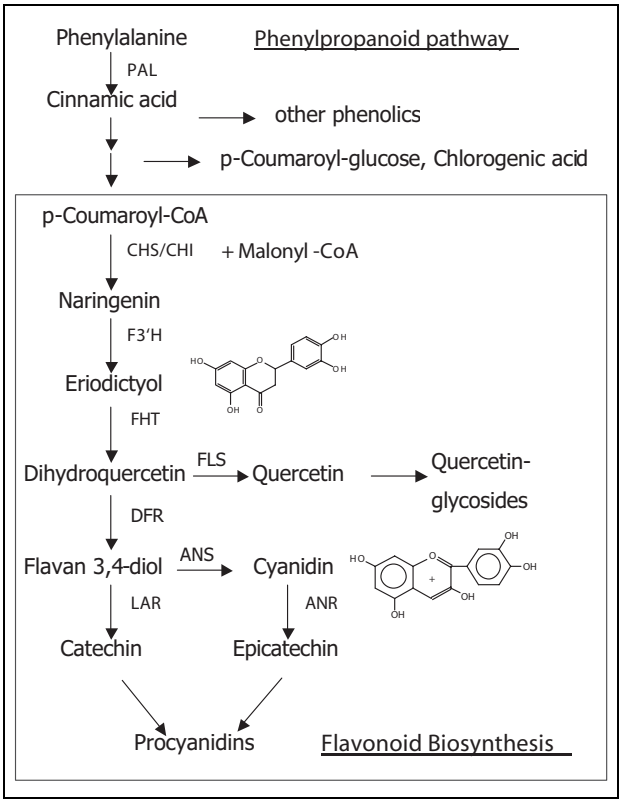


Fig. 1 Main flavonoid pathway in pome fruit trees.

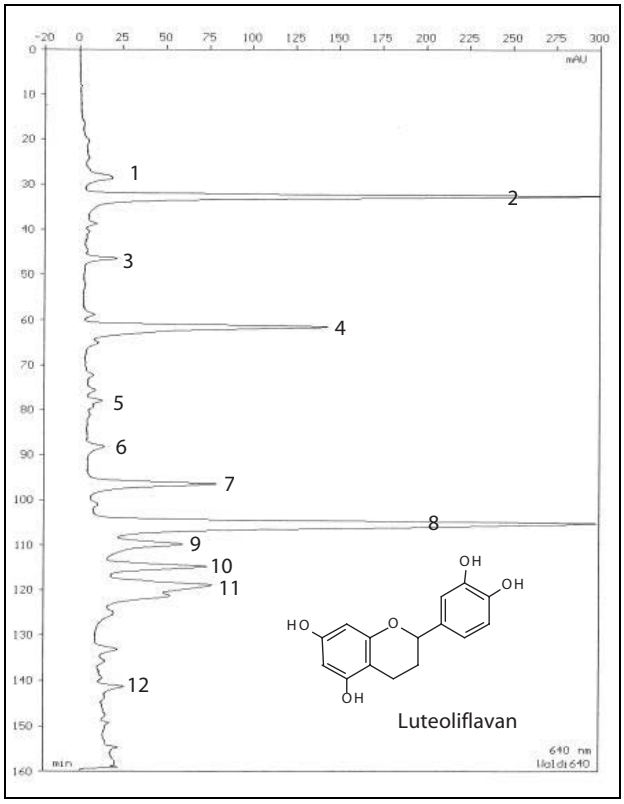


Fig. 2 HPLC-separation of flavans extracted from apple leaves with chemical reaction detection (640 nm) using dimethylaminocinnamaldehyde as selective reagent. For peak identification, see Table 1.

Table 1. Structural identification of the phenolic compounds in apple leaves detected at 640 nm after separation by rp-HPLC and post column derivatisation.

Peak	Trivial name	Structure	Retention time (min)
1	Procyanidin B1	Epicatechin-(4ß→8)-Catechin	28.7
2	Catechin	2,3 trans-Flavan-3-ol	32.6
3	Procyanidin B2	Epicatechin-(4ß→8)-Epicatechin	46.5
4	Epicatechin	2,3 cis-Flavan-3-ol	61.5
5	F7*		78.0
6	F8*		88.2
7	F9*		96.5
8	Luteoliflavan*	3-Deoxycatechin	105.1
9	F11*		109.8
10	F12*		114.8
11	Procyanidin B5	Epicatechin-(4ß→6)-Epicatechin	119.0
12	Procyanidin E-B5	Epicatechin-(4ß→8)-Epicatechin-(4ß→6)-Epicatechin	141.3

*induced by prohexadione-Ca treatment

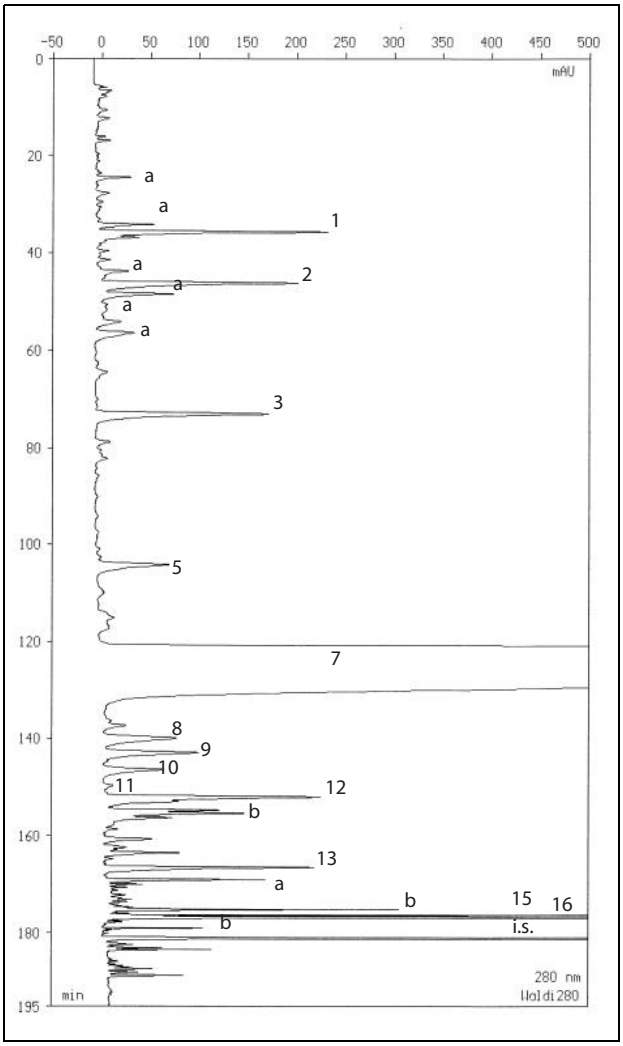


Fig. 3 HPLC-separation of phenolic compounds from apple leaves with common UV-detection at 280 nm. For peak identification, see Table 2. a = not identified hydroxycinnamic acid derivatives; b = not identified dihydrochalcones.

Several studies revealed that as soon as four hours after treatment of apple shoots with prohexadione-Ca, the formation of luteoliflavan (3-deoxycatechin) can be observed (ROEMMELT et al. 1999), which does not normally occur in apple tissue (TREUTTER 2001). Therefore, the occurrence of luteoliflavan is a suitable marker of prohexadione-Ca action on the plant's metabolism.

There are characteristic changes of the pattern of flavonoids and phenylpropanoids of apple leaves (cv. 'Golden Delicious') treated with prohexadione-Ca (250 ppm) (Fig. 4). The most pronounced accumulation was found for luteoliflavan at day 12 after prohexadione-Ca treatment (Fig. 4a). Simultaneous with the luteoliflavan formation, the flavanone glycosides eriodictyol 7-glucoside, its acylated derivative and naringenin 7-glucoside could be detected (Fig. 4a, b). The concentration of hydroxycinnamic acids also increased (Fig. 4b). These accumulations took place at the expense of constitutive compounds, such as procyanidins and flavonols (Fig. 4c). Such induced changes in the flavonoid composition of the leaves correlate with a reduced susceptibility against the bacterium *Erwinia amylovora,* which was found in a comparable experiment with the apple rootstock clone 'M9' (Fig. 4d). It was postulated that these changes in flavonoid composition are responsible for the observed pathogen resistance induced by prohexadione-Ca treatment.

In vitro-testing of antimicrobial activity of the isolated phenolic compounds

Extracted and purified compounds were tested together with related flavonoids and hydroxycinnamic acids on their effect on the growth of *E. amylovora* (Table 3). 4-*O*-*cis*-*p*-Coumaroyl-phloridzin inhibited bacterial growth at concentrations of 1000 ppm and 500 ppm. Growth inhibition was also found for the aglycones *p*-coumaric acid (1000 ppm) and naringenin (in all concentrations tested), while their glycosides *p*-coumaroyl-glucose and naringenin 7-glucoside did not show any effect. Phloretin shows the strongest inhibition of all extracted compounds (250 ppm). Luteoforol, which is

Table 2. Structural identification of the phenolic compounds in apple leaves detected at 280 nm after separation by rp-HPLC.

Peak	Trivial name	Structure	Retention time (min)
1		p-Coumaroylglucose	36.9
2	Chlorogenic acid	5´Caffeoylquinic acid	47.0
3		trans-p-Coumaric acid	74.5
4		Eriodictyol-7-O-glucoside*	92.8
5		3-Hydroxyphloridzin	106.3
6	Prunin	Naringenin-7-O-glucoside	116.9
7	Phloridzin	Phloretin 2´-O-glucoside	125.3
8	Hyperin	Quercetin 3-O-galactoside	144.5
9	Isoquercitrin	Quercetin 3-O-glucoside	147.1
10	Rutin	Quercetin 3-O-rutinoside	150.5
11	Avicularin	Quercetin 3-O-arabinofuranoside	153.2
12	Quercitrin	Quercetin 3-rhamnoside	156.1
13		Phloretin	167.6
14		6´´-O-trans-p-Coumaroyl-eriodictyol 3´-glucoside*	171.9
15		4-O-cis-p-Coumaroyl-phloridzin	176.5
16		4-O-trans-p-Coumaroyl-phloridzin	176.9

*induced by prohexadione-Ca treatment

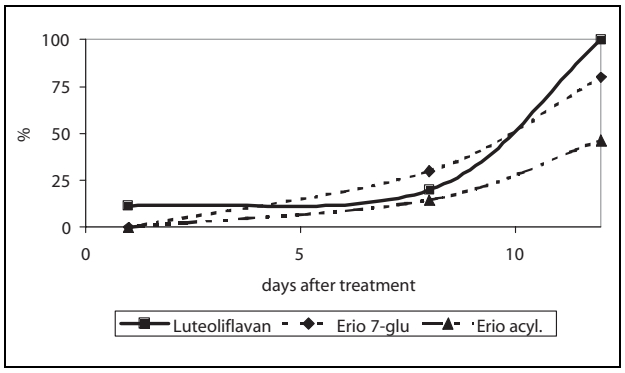


Fig. 4a Accumulation of the newly formed flavonoids (relative to their maximum concentrations which are reached at day 12, 15, 20, respectively) in apple leaves after treatment with prohexadione-Ca (250 ppm): 3-deoxycatechin luteoliflavan, flavanones eriodictyol 7-O-glucoside (Erio 7-glu) and 6"-*O-trans-p*-coumaroyl-eriodictyol 3'-O-glucoside (Erio acyl.).

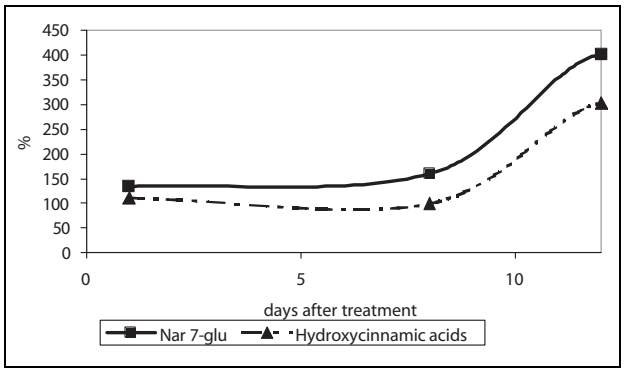


Fig. 4b Accumulation of constitutive phenolic compounds (untreated control = 100 %) in apple leaves after treatment with prohexadione-Ca (250 ppm): hydroxycinnamic acids, the flavanone naringenin 7-O-glucoside (Nar 7-glu).

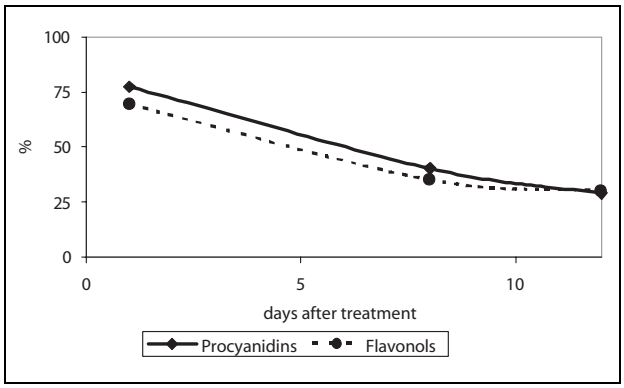


Fig. 4c Decreasing levels (untreated control = 100 %) of procyanidins and flavonols after treatment of apple leaves with prohexadione-Ca (250 ppm).

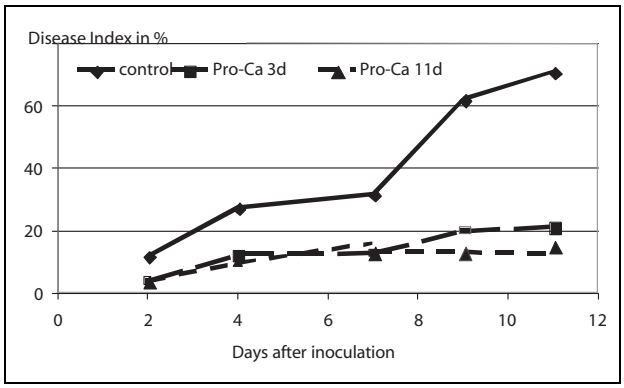


Fig. 4d Effect of prohexadione-Ca (Pro-Ca) on fire blight (diesease index) of apple rootstock clone 'M9' treated 3 or 11 days (d), respectively, before inoculation.

Table 3. Effect of various phenolic compounds on the growth of *E. amylovora* in vitro (with exception of luteoforol, according to ROEMMELT et al. 2002).

	1000 ppm	500 ppm	250 ppm	30 ppm
Luteoliflavan	–	–	–	n.t.
Eriodictyol 7-glucoside	–	–	–	n.t.
6"-*O-trans-p*-Coumaroyl-eriodictyol 3'-O-glucoside	–	–	–	n.t.
Naringenin 7-O-glucoside	–	–	–	n.t.
p-Coumaroylglucose	–	–	–	n.t.
4-*O-cis-p*-Coumaroyl-phloridzin	+	+	–	n.t.
4-*O-trans-p*-Coumaroyl-phloridzin	–	–	–	n.t.
Eriodictyol	–	–	–	n.t.
Naringenin	+	+	+	n.t.
p-Coumaric acid	+	–	–	n.t.
Phloretin	+	+	+	n.t.
Caffeic acid	–	–	–	n.t.
Luteoforol	n.t.	n.t.	n.t.	+
Chlorogenic acid	–	–	–	n.t.

– = no effect; + = growth inhibition; n.t. = not tested

an intermediate of the formation of 3-deoxycatechin, could not be isolated from the leaves. Therefore, it was chemically synthesised for *in vitro* testing against *E. amylovora*. The assays conducted so far demonstrated that even concentrations as low as 30 ppm luteoforol had a strong bactericidal effect after one day of incubation.

Biochemical prerequisites

Apart from the common pathway leading to the various classes of 3-hydroxyflavonoids a few plant species exhibit an additional pathway leading to the formation of 3-deoxyflavonoids (except the flavanones). First studies were performed on *Sinningia cardinalis* and *Columnea hybrida*, where 3-deoxyanthocyanins serve as flower pigments (STICH and FORKMANN 1988a, 1988b). It was shown that the key reaction in the formation of 3-deoxyflavonoids is the reduction of flavanones in position 4 to the corresponding flavan 4-ols, which is catalysed by a flavanone 4-reductase (FNR) activity. However, it remained unclear, whether FNR activity is due to the presence of a novel enzyme or if the reaction is catalysed by the DFR from the common 3-hydroxyflavonoid pathway using beside dihydroflavonols also flavanones as a substrate. Studies on the substrate specificity of the DFR from *Dahlia variabilis* (FISCHER et al. 1988) gave a first hint that the common DFR could possess FNR activity, since the purified enzyme catalysed the reduction of flavanones (Fig. 5), although only to a very low extent. However, the reaction observed with the purified enzyme *in vitro* does not seem to occur under natural conditions, since the formation of 3-deoxyflavonoids has never been observed in *Dahlia* flowers and FNR activity could not be demonstrated in enzyme preparations from *Dahlia* petals. In maize (*Zea mays*), 3-hydroxyanthocyanins and 3-deoxyanthocyanins are formed and may even co-occur in the same tissue. From genetic studies, A1, the structural gene of DFR in maize, was known to be involved in both the formation of 3- hydroxyanthocyanins and 3-deoxyanthocyanins. In biochemical and molecular biological studies it could be proved that the DFR of maize exhibits also FNR activity and that the formation of 3-deoxyanthocyanins in maize depends essentially on the absence or reduction of FHT activity in the respective tissues (HALBWIRTH et al. 2003).

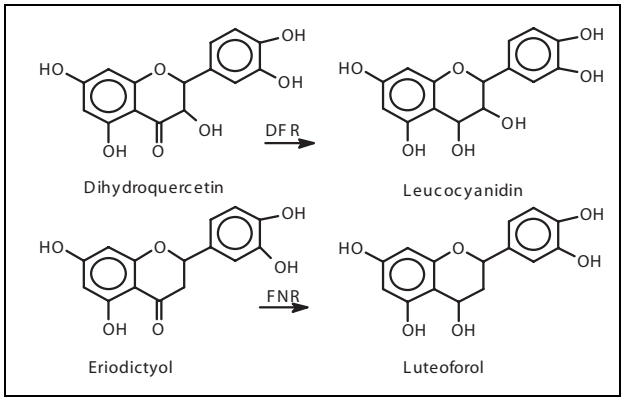


Fig. 5 Reactions catalysed by dihydroflavonol 4-reductase (DFR) and flavanone 4-reductase (FNR).

Under natural conditions, 3-deoxyflavonoids are not formed in apple and pear leaves. An inhibition of FHT by prohexadione-Ca would lead to the accumulation of eriodictyol. The key reaction for the formation of 3-deoxyflavonoids is the conversion of eriodictyol to luteoforol, which could be further converted to luteoliflavan by LAR (Fig. 6). The reduction of eriodictyol is either catalysed by the common DFR showing broad substrate specificity or by an FNR, which was shown to be present in prohexadione-Ca treated apple and pear leaves as well as in untreated controls. Enzymatic stud-

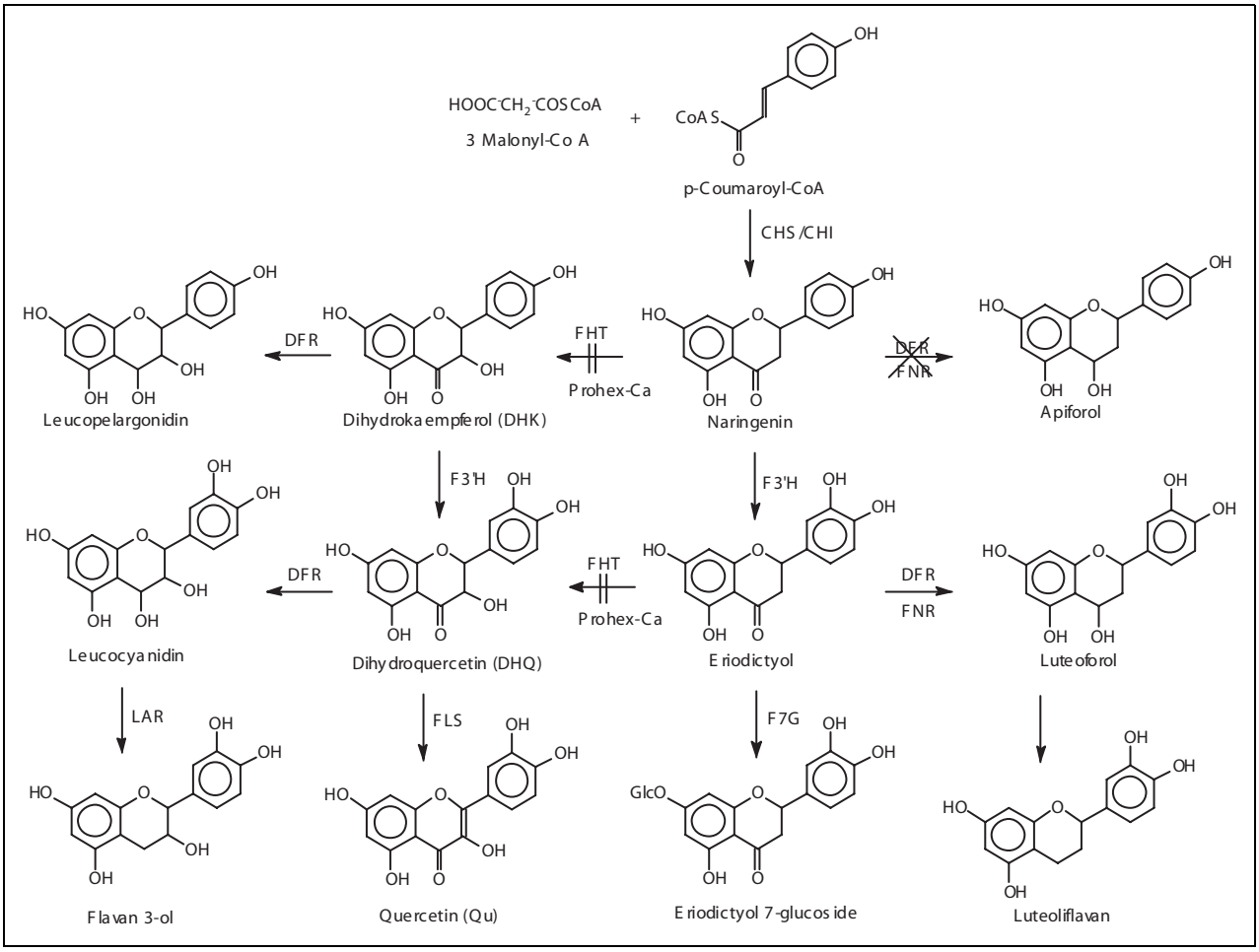


Fig. 6 Comprehensive biosynthetic model explaining the consequences of FHT-inhibition by prohexadione-Ca on flavonoid composition of apple and pear leaves considering the substrate specificity of DFR/FNR.

ies with extracts of apple and pear leaves revealed strong competitive inhibition by prohexadione-Ca *in vitro*. FHT activity was inhibited in treated leaves for several days. This *in vivo* effect gradually decreased five days after treatment, which can be explained by the advancing degradation of prohexadione-Ca. The time course of FHT inhibition correlates with the formation of luteoliflavan (Fig. 7). Furthermore, FNR activity could be shown to be present in prohexadione-Ca treated apple and pear leaves as well as in untreated controls.

Apart from opening the pathway to luteoliflavan, the accumulation of eriodictyol may influence a number of flavonoid enzymes, in particular PAL and CHS, either at the level of gene expression or at the level of enzyme activity, as indicated by the formation of additional flavonoids and simple phenylpropanoids.

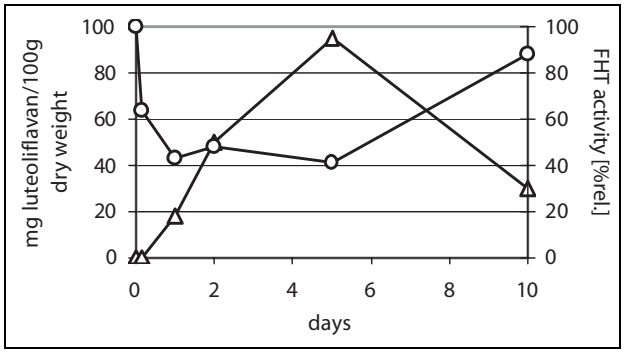


Fig. 7 Effect of prohexadione-Ca on young apple leaves. Time course of FHT activity [as % of the respective control value] (circles) and accumulation of luteoliflavan [mg/100 g dry weight] (triangles) in young apple leaves after treatment with prohexadione-Ca. In the control assays, the inhibitory effect of prohexadione-Ca was compensated by addition of 3.5 mM 2-oxoglutarate.

Molecular biological studies

At first, the molecular biological studies focussed on both the FHT inhibition and the identity of the FNR enzyme. To study the FHT inhibition in vitro, FHT cDNA was cloned from leaves of apple and pear relying on sequence information from two other apple cultivars (DAVIES 1993; LEE et al. 1998). The clones were used for yeast expression of the FHT enzyme and subsequent enzyme studies. Thus the inhibition of the 2-oxoglutarate dependent FHT enzymes by prohexadione-Ca could be confirmed in vitro. Further investigations of other 2-oxoglutarate analogues as FHT inhibitors are in progress. The second important question

was the identity of the FNR enzyme that converts the accumulating eriodictyol to luteoforol, which is probably the immediate intermediate for luteoliflavan formation. Cloning of apple and pear DFR cDNAs, heterologous expression in yeast and enzyme tests showed that both possess FNR activity as well (FISCHER et al. 2003). Thus, the DFR enzyme already present in untreated apple and pear leaves is able to perform the conversion of accumulated eriodictyol to luteoforol after prohexadione-Ca application. However, the kinetic values for dihydroflavonols and flavanones of the DFRs show that dihydroflavonols are the preferred substrates (FISCHER et al. 2003). Under natural conditions with low levels of eriodictyol, products of an FNR reaction are not observed.

A detailed study on the effect of prohexadione-Ca in vivo on the gene expression levels, the corresponding enzyme activities and subsequent changes in flavonoid contents is in preparation.

Conclusions

In summary, the effect of prohexadione-Ca can be explained as follows (Fig. 6): Under natural conditions, FHT and DFR compete for flavanones in a way that only 3,3',4'-hydroxyflavonoids are formed as end products. FHT is present in *Malus* leaves with high activity and converts eriodictyol to the dihydroflavonol dihydroquercetin, the preferred substrate for the DFR activity. As long as FHT provides large amounts of dihydroquercetin, the common 3,3',4'-hydroxyflavonoids are exclusively formed. After treatment with prohexadione-Ca, FHT is inhibited and thus the supply with dihydroquercetin is cut off. In this case naringenin is exclusively converted by F3'H to eriodictyol which accumulates consequently. Without the preferred dihydroflavonols as substrates, the DFR/FNR-enzyme now acts as an FNR and converts eriodictyol to luteoforol, the precursor for the formation of the 3-deoxycatechin luteoliflavan. Thus, 3-deoxyflavonoids are formed in apple and pear leaves only after inhibition of FHT by prohexadione-Ca. Luteoforol acts as the antimicrobial compound. It exhibits its antimicrobial effects against *Erwinia amylovora in vitro* in a concentration as low as 30 ppm. In summary, the mode of action of prohexadione-Ca in fire blight protection in apple and pear could be elucidated. The uncommon 3-deoxyflavonoid luteoforol is induced in the plants by prohexadione-Ca and acts as an antimicrobial agent.

Acknowledgements

These investigations were supported by the European Commission (QLK5-CT-1999-01583).

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Received June 16, 2003 / Accepted June 27, 2003

Addresses of authors: S. Roemmelt, S. Peterek and D. Treutter (corresponding author), Unit of Fruit Science, Department of Plant Sciences, Technical University of Munich, Freising, Germany, T. C. Fischer and G. Forkmann, Chair of Floriculture Crops and Horticultural Plant Breeding, Department of Plant Sciences, Technical University of Munich, Freising, Germany, H. Halbwirth, K. Schlangen and K. Stich, Institute of Technical BioScience, Technical University of Vienna, Vienna, Austria and J.-B. Speakman, BASF Agricultural Center, Limburgerhof, Germany, e-mail: dieter.treutter@wzw.tum.de.