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Can Prohexadione-Ca Induce Antimicrobial Flavonoids in Rose?

K. Schlangen[1)], C. Gosch[2)], S. Roemmelt[3)], J. Knott[2)], T. C. Fischer[2)], D. Treutter[3)], G. Forkmann[2)], K. Stich[1)] and H. Halbwirth[1)]

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

Summary

Rose (*Rosa* x *hybrida*), a popular and widely spread garden plant, is infected by numerous plant diseases, which are mainly caused by fungal and even by bacterial pathogens such as *Erwinia amylovora*, the causal agent of fire blight. Recently, prohexadione-Ca, a 2-oxoglutarate specific inhibitor originally developed as a growth retardant, was reported to influence the flavonoid composition of apple and pear leaves and, thus, to increase resistance against fire blight and other diseases. It was supposed that this approach could also mediate resistance in roses. As the first step, the induction of the antimicrobial 3-deoxyflavonoid in rose leaves after prohexadione-Ca application was investigated in a time course experiment. Due to the presence of disturbing phenolic compounds impeding the preparation and testing of enzymes, enzyme preparation procedures and assays had to be adapted and optimised for rose leaves. Standard enzyme assays are described for phenylalanine ammonia lyase (PAL), chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR) and flavanone 4-reductase (FNR). Cloning and heterologous expression showed that the DFR of rose exhibits FNR activity as well. Treatment of rose leaves with prohexadione-Ca led to the inhibition of FHT and to the formation of the 3-deoxyflavonoid luteoliflavan. Likewise, induction effects on PAL, CHS and DFR/FNR activity were also observed. In general, it could be shown that rose leaves produce 3-deoxyflavonoids after prohexadione-Ca treatment and, therefore, this method can be considered as a potential protection strategy for roses that will be studied phytopathologically in a next step.

Zusammenfassung

Kann Prohexadion-Ca bei Rosen die Bildung von antimikrobiellen Flavonoiden herbeiführen? Die Rose (*Rosa* x *hybrida*), eine beliebte und weit verbreitete Zierpflanze, kann von vielen Pflanzenkrankheiten befallen werden. Die meisten werden von Pilzen verursacht, es gibt aber auch bakterielle Erkrankungen wie z.B. Feuerbrand, der von *Erwinia amylovora* hervorgerufen wird. Es wurde beschrieben, dass die Behandlung mit Prohexadion-Ca den Flavonoidgehalt in Apfelblättern beeinflusst und die Resistenz gegenüber Feuerbrand erhöht. Da Flavonoide generell eine wichtige Rolle bei der Pathogen-Abwehr spielen, wurde die Flavonoidbiosynthese in Rosenblättern, insbesondere die auftretenden Veränderungen nach Behandlung mit Prohexadion-Ca, im Zeitverlauf untersucht. Aufgrund des hohen Gehaltes an phenolischen Inhaltsstoffen, welche die Präparation und Untersuchung der Enzyme stören, wurden Enzymaufarbeitung und Enzymtests für die Rosenblätter optimiert. Standardenzymtests für die Phenylalaninammoniumlyase (PAL), Chalkonsynthase/Chalkonisomerase (CHS/CHI), Flavanon 3-hydroxylase (FHT), Dihydroflavonol 4-reduktase (DFR) und Flavanon 4-reduktase (FNR) werden beschrieben. Durch Klonierung und heterologe Expression der Rosen-DFR konnte gezeigt werden, dass diese auch FNR-Aktivität besitzt. Die Behandlung von Rosenblättern mit Prohexadion-Ca führte zu einer Inhibition der FHT-Aktivität und zur Akkumulation des 3-Deoxyflavonoids Luteoliflavan. Gleichzeitig konnte eine Induktion der PAL, CHS und der DFR/FNR-Aktivität beobachtet werden. Zusammenfassend konnte gezeigt werden, dass die Rose nach Prohexadion-Ca-Behandlung 3-Deoxyflavonoide bilden, und dass diese Methode somit als potentielle Pflanzenschutzstrategie für die Rose betrachtet werden kann. Im nächsten Schritt sollen phytopathologische Untersuchungen durchgeführt werden.

Key words. 3-deoxyflavonoids – dihydroflavonol 4-reductase (DFR) – fire blight (*E. amylovora*) – flavanone 4-reductase (FNR) – induced resistance – luteoliflavan – polyphenol rich tissue

Introduction

Recently, it was shown that the growth retardant prohexadione-Ca, a structural analogue of 2-oxoglutarate, inhibits the 2-oxoglutarate dependent dioxygenase flavanone 3-hydroxylase (FHT), a core enzyme in the flavonoid pathway. This inhibition causes the accumulation of the flavanone eriodictyol. Studies on pome fruit trees revealed that the accumulated eriodictyol is further transferred to 3-deoxyflavonoids that could be correlated with an enhanced resistance against the bacterial disease fire blight (*Erwinia amylovora*) and the fungal disease scab (*Venturia inaequalis*) (RADEMACHER 2000; ROEMMELT et al. 2002 ROEMMELT et al., this issue).

The key step in the formation of 3-deoxyflavonoids, the reduction of flavanones to flavan 4-ols, is catalysed by flavanone 4-reductase (FNR) (STICH and FORKMANN 1988). In apple, pear and maize as well as some further plant species, the dihydroflavonol 4-reductase (DFR), commonly catalysing the reduction of dihydroflavonols to flavan 3,4-diols, also exhibits FNR activity (FISCHER et al. 2003; HALBWIRTH et al. 2003; GOSCH et al., this issue). Thus, both, the inhibition of FHT and the presence of FNR activity are necessary for the formation of 3-deoxyflavonoids.

As pome fruits, roses belong to the Rosaceae family. Due to their beauty and popularity, roses are widespread ornamental plants, which can be found in nearly all gardens and public parks. Moreover, roses are the top cut flowers in ornamental crop production. Unfortunately, cultivation of roses is threatened by a number of fungal diseases like powdery mildew (*Podosphaera pannosa*), blackspot (*Diplocarpon rosae*) or rust (*Phragmidium* sp.). Therefore, it was investigated, whether the treatment of rose leaves with prohexadione-Ca also leads to the formation of antimicrobially active 3-deoxy-flavonoids that could be accompanied by an enhanced resistance against these pathogenic fungi. Furthermore, as a member of the Rosaceae family, roses may also be affected by fire blight (BERGER 2001). Although commercial losses are negligible, the spread of fireblight via roses and some other rosaceous species might be facilitated due to their ubiquitous presence in fields, gardens and parks. Whereas biosynthesis of flavonoids has been extensively studied in various ornamental plants (FORKMANN and HELLER 1999), very few reports are available on enzymatic investigations in rose petals (ELSHERIF 2000), and flavonoid biosynthesis in rose leaves has not been studied so far. This might be due to the fact, that roses accumulate large amounts of tanning agents and other phenolic compounds in petals and leaves (LAMAISON et al. 1991; ELSHERIF 2000) which impede enzyme preparation and testing by standard methods.

Thus, for enzymatic studies on prohexadione-Ca treated and untreated leaves of rose, it was necessary, to optimise methods for the demonstration of flavonoid enzymes in the respective protein preparations. Due to the key role in 3-deoxyflavonoid formation, special emphasis was stressed on whether or not the DFR of rose leaves also exhibits FNR activity. This was achieved by cloning of the corresponding cDNA and subsequent studies with the heterologously expressed enzyme. In order to get a more detailed insight into the action of prohexadione-Ca in rose leaves, changes in flavonoid formation and enzyme activities were recorded over a time period of four days on shoots treated with prohexadione-Ca.

Material and Methods

List of Abbreviations

ANS: anthocyanidin synthase, BSA: bovine serum albumine, CHS: chalcone synthase, CHI: chalcone isomerase, DIECA: diethyldithiocarbamic acid, DFR: dihydroflavonol 4-reductase, DHK: dihydrokaempferol, DHQ: dihydroquercetin, ERI: eriodictyol, FHT: flavanone 3-hydroxylase, F3´H: flavonoid 3'-hydroxylase, FLS: flavonol synthase, FNR: flavanone 4-reductase, NAR: naringenin, PA: phenylalanine, PAL: phenylalanine ammonia lyase, PEG: polyethylenglycol, PVP: polyvinylpyrrolidon

Plant material

The studies were performed on the red flowering *Rosa* x *hybrida* cultivar 'Kardinal'. For enzymatic investigations and RNA preparation, young leaves were harvested, shock frozen in liquid nitrogen and stored at –80 °C until usage. For analyses of phenolic compounds, the leaves were lyophilised.

Prohexadione-Ca treatment of roses

Three year old pot grown plants were kept in a greenhouse with conventional glass, which excludes most UV from sunlight. All flowering shoots of 50 plants were removed in May 2002. After the establishment of new and still non-flowering shoots, more than 200 randomly distributed shoots were labelled for prohexadione-Ca treatment or as a control. Half of the shoots were sprayed with 250 ppm prohexadione-Ca in water, the control shoots were sprayed with water. The treatments were made in the morning, after the leaf samples for the 0 hour time had been taken. After spraying, plants were shaded until the 4-hour sampling to prevent premature drying and poor uptake of the applied prohexadione-Ca. Subsequently, samples were always taken at the same time in the morning. For each sample, 20 first leaves (just opening and pale red), randomly distributed among the plants, were harvested, pooled, frozen in liquid nitrogen and kept at –80 °C until analysis. Samples were taken at 0 and 4 hours and at 1, 2 and 4 days after treatment. Before and during the experiment, no phytosanitary measures were undertaken. Weather conditions were sunny and uniform after treatment and during the whole sample collection period.

Chemicals (Materials)

For spray application of prohexadione-Ca, the respective commercial product Regalis® (BASF AG, Ludwigshafen, Germany) was used. (2-^{14}C)-Malonyl-coenzyme A (55 mCi/mmol) was purchased from Amersham International (Freiburg, Germany). Substrates were synthesised as described (GOSCH et al., this issue).

Buffers

Extraction buffer A: 0.7 M KP_i buffer, pH=8.0, containing 0.4 M sucrose, 1 mM $CaCl_2$ and 30 mM EDTA, was boiled for 10 min. After cooling down under nitrogen atmosphere 1.5 % PEG 20000, 0.3 M sodium ascorbate, 50 mM cysteine, 5 mM DIECA, and 0.1 % BSA were added.

Extraction buffer B: 0.1 M Tris-HCl, pH=7.5 was boiled for 10 min. After cooling down under nitrogen atmosphere 10 % sucrose, 1.5 % PEG 1500, 0.1 M sodium ascorbate and 25 mM $CaCl_2$ were added.

Enzyme preparations

All steps were performed at 4 °C.

Preparation A: The procedure described from CLAUDOT and DROUET (1992), was modified as follows: 0.5 g rose leaf tissue was homogenised with 0.5 g polyclar AT in liquid nitrogen. 5 ml ice cold extraction buffer (A) and 0.5 g Dowex 1x2 were added to the homogenate and the mixture was stirred on ice under nitrogen atmosphere for 15 min and filtered through glass wool. The filtrate was centrifuged for 20 min at 38000 x g.

Preparation B: The preparation was performed according to ELSHERIF (2000). 0.5 g fresh weight rose leaf tissue were homogenised with 0.5 g polyvinylpolypyrrolidone in liquid nitrogen. 5 ml ice cold extraction buffer B were added to the homogenate and then centrifuged for 20 min at 10000 x g. The supernatant was stirred with 0.5 g Dowex 1x2 on ice under nitrogen atmosphere for 15 min and then centrifuged for 5 min at 10000 x g.

Aliquots of the clear supernatants were passed through a gel chromatography column (Sephadex G 25) with a bed volume of 1 ml to remove low molecular weight compounds.

Enzyme assays

Enzyme assays were performed according to HALBWIRTH et al. (2002), using the assay conditions optimised for rose leaves (Table 1). For determination of FHT inhibition in prohexadione-Ca treated leaves, enzyme extracts were prepared without a gel chromatography step, which would have removed prohexadione-Ca. Enzymatic data were calculated based on the protein content, which was determined by a modified Lowry procedure (SANDERMANN and STROMINGER 1972), using bovine serum albumine as a standard.

Cloning of the DFR-cDNA

The cloning of the DFR from *Rosa* mRNA and subsequent heterologous expression in yeast was performed as described (GOSCH et al., this issue). RT-PCR primers for the non-coding 5´-region (including the start codon and the first G of the second codon) and 3´-region were derived from the public GenBank database sequence of *Rosa* DFR (TANAKA et al. 1995, accession number D85102). Finally, the primers 5´-CTTAGATTAATCTCCAAGCAATGG-3´ (forward) and 5´-GAGATACAAATGTTACCAATACTG-3´ (reverse) were used. Cycling conditions for the PCR were 1.5 min at 94 °C, 30 x (30 sec at 94 °C, 1 min at 55 °C, 2 min at 72 °C), 7 min at 72 °C.

Analyses of phenolic compounds

Phenolic compounds were extracted from lyophilised and pulverised material using methanol and analysed by rp-HPLC as described (GOSCH et al., this issue). Quantification was performed using response factors from standards previously isolated (procyanidin B2, procyanidin C1) or purchased from Roth, Karlsruhe (catechin, epicatechin, eriodictyol 7-glucoside, rutin). Luteoliflavan was calculated as catechin, procyanidin B5 as B2, the trimeric procyanidin E-B5 and the tetrameric E4 as C1, unknown flavanones as eriodictyol 7-glucoside and flavonols as rutin.

Results

Enzymatic Studies

Standard procedures (see FORKMANN and HELLER 1999; HALBWIRTH et al. 2002) did not result in enzyme preparations showing any activity. In contrast, with preparation methods A and B the activities of chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT), dihydroflavonol 4-reductase

Table 1. Optimised conditions for assays of the main enzymes of the flavonoid pathway in rose leaves.

	Prep	Final assay volume	Assay buffer	Substrates	Cofactors	pH-optimum	T.optimum	Total protein amount
PAL	B	100 µl	0.1 M H_3BO_3 + 0.4 % Na-asc.	PA	none	8.5	30 °C	16 µg
CHS/ CHI	A	100 µl	0.1 M KP_i + 0.4 % Na-asc.	p-Cou-CoA	none	7.0	30 °C	56 µg
FHT	A	100 µl	0.1 M Tris + 0.4 % Na-asc.	NAR	0.5 mM 2-OGT 0.1 mM $FeSO_4$	7.5	30 °C	8 µg
DFR	B	50 µl	0.1 M KP_i + 0.4 % Na-asc.	DHQ	5 mM NADPH	6.25	30 °C	14 µg
FNR	B	50 µl	0.1 M KP_i + 0.4 % Na-asc.	ERI	5 mM NADPH	6.25	30 °C	56 µg

Abbrev.: Prep.: Preparation; T: Temperature, Na-asc.: Sodium ascorbate, p-Cou-CoA: p-Coumaroyl-CoA, 2-OGT: 2-Oxoglutarate, KP_i: K_2HPO_4/KH_2PO_4

(DFR) and flavanone 4-reductase (FNR) could be demonstrated. In case of CHS/CHI, DFR and FNR, higher activities were measured with preparation A, whereas FHT activity was higher with preparation B. However, PAL activity was present only in preparation B. Flavonoid 3'-hydroxylase (F3´H) and anthocyanidin synthase (ANS) activity could not be detected. Flavonol synthase (FLS) was only observed with enzyme preparation B, but the activity was very low.

PAL, CHS/CHI, FHT, DFR and FNR were characterised in detail. The conditions for the respective standard enzyme assays are presented in Table 1.

Cloning and studies on substrate specificity of the heterologously expressed rose DFR

The DFR was further studied by a molecular biological approach to test for an FNR activity, the key activity for 3-deoxyflavonoid formation. Therefore, the DFR expressed in the leaves of cv. 'Kardinal' rose was cloned as a cDNA and used for heterologous expression in yeast. Incubation of the dihydroflavonol DHQ with the heterologously expressed enzyme in the presence of NADPH showed the formation of a single product, which was identified as leucocyanidin (DFR activity, Table 2). Apart from the sequence similarity of the cloned cDNA to the known DFR sequence from rose, this verified the cDNA as a functional DFR. Incubation of the flavanone eriodictyol with the heterologously expressed enzyme in the presence of NADPH led to the formation of the corresponding flavan 4-ol luteoforol. Thus, it could be shown that the DFR of rose leaves possesses FNR activity as well (Table 2). However, the conversion rate and the pH-optimum of the FNR reaction were lower than for the DFR reaction with DHQ as a substrate.

Table 2. Half-quantitative substrate specificity of the heterologously expressed DFR of rose.

Substrate	ERI	DHQ
Conversion rate	++	+++
pH-optimum	5.5	6.0

++: medium +++: high

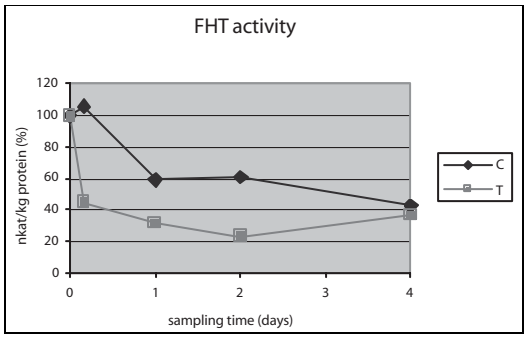


Fig. 1 Time course of FHT activity in rose leaves. Abbr.: C: control, T: prohexadione-Ca treatment.

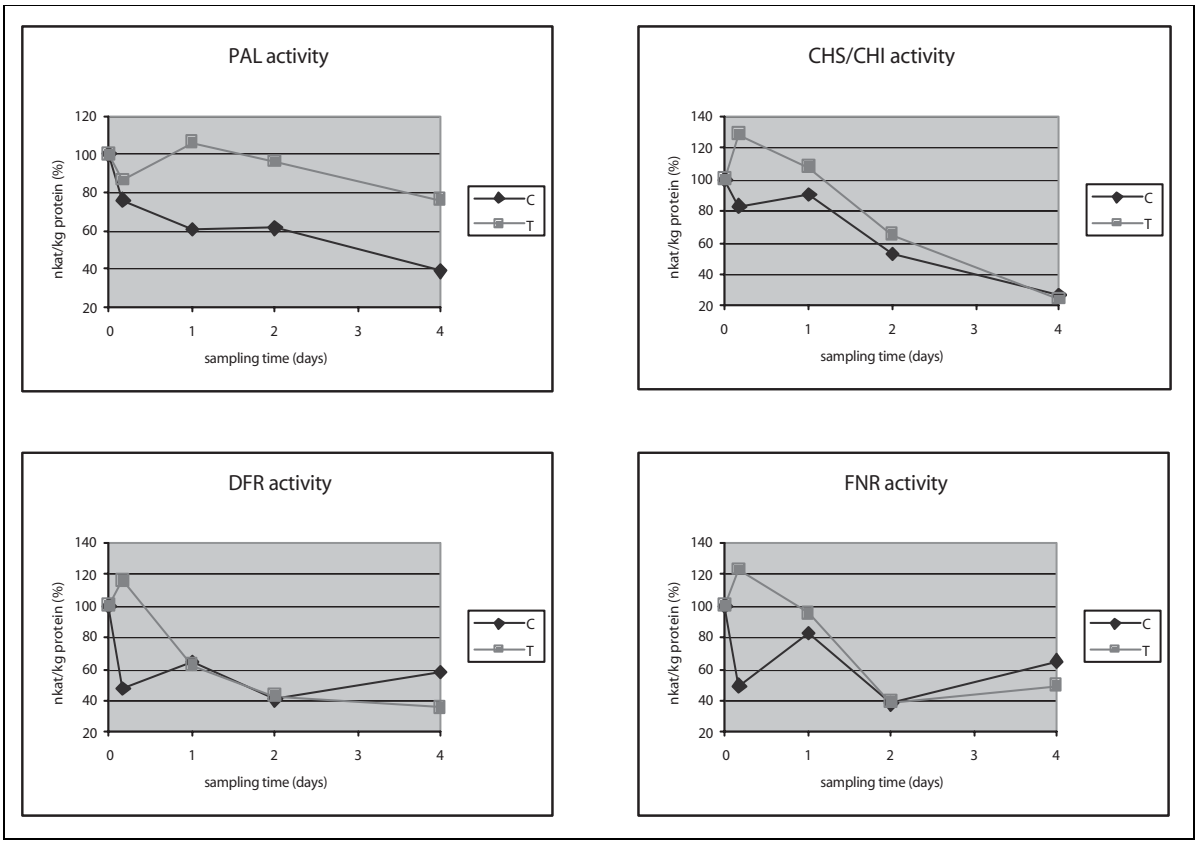


Fig. 2 Time course of PAL, CHS/CHI, DFR and FNR activities in rose leaves. Abbr.: C: control, T: Prohexadione-Ca treatment.

Time course studies on enzyme activities and flavonoid contents

As for some other important crop plants (GOSCH et al., this issue), luteoliflavan was detected after prohexadione-Ca application on rose leaves. The effect of prohexadione-Ca on flavonoid biosynthesis in rose leaves was studied in detail by following the induced changes for a time period of four days as compared to untreated leaves. For time course studies, activities at time '0 hours' were taken as 100 % and activities at the different sampling times were calculated in relation to this. The treatment of prohexadione-Ca led to a reduction of enzyme activity of FHT (Fig. 1). This is due to the presence of prohexadione-Ca inhibiting the enzyme and reflects the situation *in vivo*. When the enzyme was prepared with removal of prohexadione-Ca by gel chromatography, there was only a slighter inhibition observed (data not shown, see discussion). The time course studies of the other enzymes showed, that there was an induction of PAL, CHS/CHI, DFR and FNR activities after application of prohexadione-Ca. In case of PAL, an induction of enzyme activity during the whole observation period of four days after treatment could be detected. For CHS/CHI, DFR and FNR the induction was only observed during the first day after treatment (Fig. 2).

Simultaneously to FHT inhibition, accumulation of luteoliflavan was observed (Fig. 3a) in treated leaves. Luteoliflavan is not formed under natural conditions. Moreover, the catechin content slightly increased in prohexadione-Ca treated leaves compared to the control (Fig. 3b). However, the FHT inhibition did not alter the content of procyanidins (Fig. 3b). In contrary, the content of flavonols clearly decreased after the application (Fig. 3c).

Discussion

The multiple functions of flavonoids, in particular their contribution to pathogen resistance, have been extensively studied and are reviewed in text books (GEIBEL et al. 1994; FEUCHT and TREUTTER 1999). Furthermore, the flavonoid composition of various rose hybrids es-

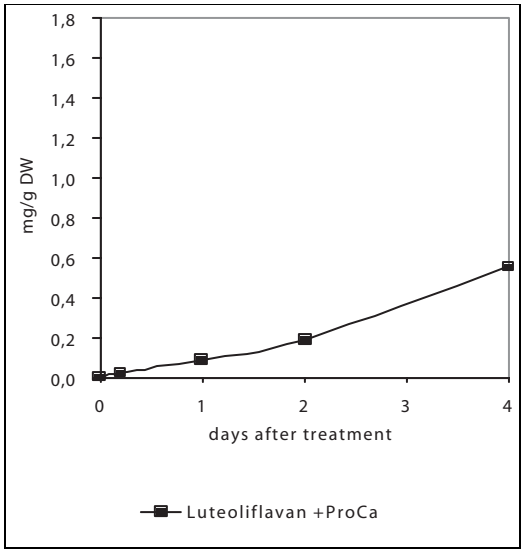


Fig. 3a

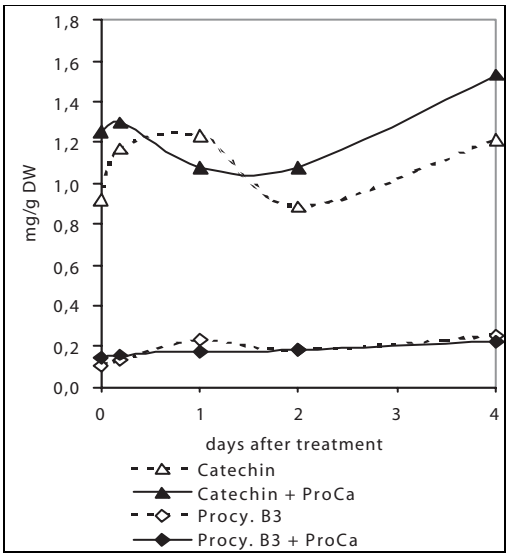


Fig. 3b

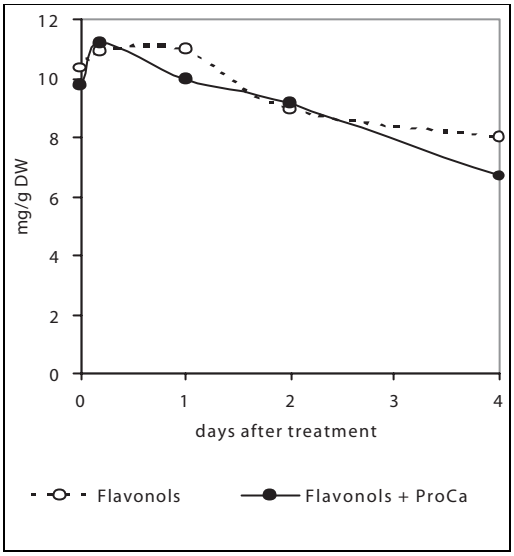


Fig. 3c

Fig. 3 a–c. Time course of concentrations of flavonoids in rose leaves treated with prohexadione-Ca.

sential for flower colour was investigated in detail (BIOLLEY and JAY 1993; BIOLEY et al. 1993, 1994; ELSHERIF 2000). However, few reports are available on enzymatic studies in roses (ELSHERIF 2000; KNOTT 2001). Since standard methods (see FORKMANN and HELLER 1999; HALBWIRTH et al. 2002) did not result in active enzyme preparations, procedures had to be adapted and optimised for the detection of flavonoid enzymes in rose leaves. There are only few protocols available for the detection of flavonoid enzymes from tissues with high contents of disturbing phenolic compounds, such as hydrolysable tannins occurring in roses (CLAUDOT and DROUET 1992; DELLUS et al. 1997).

Generally, interfering phenols are deactivated by addition of polyphenol adsorbing agents as well as of oxidation protectants such as PVP, PEG or ascorbate to the extraction buffer, thus minimising interactions between polyphenols or their oxidation products and proteins (LOOMIS and BATTAILE 1966; LOOMIS 1969; BRITSCH and GRISEBACH 1984; DELLUS et al. 1997). For instance, BRITSCH and GRISEBACH (1984) showed, that the presence of Dowex 1X2 and ascorbate under nitrogen flow during tissue homogenisation avoided oxidation and, thus, losses of CHS activity of parsley (*Petroselinum hortense*) cell cultures. CHS activity of enzyme preparations from walnut barks is one example where the presence of PEG (1.5 % w/v) and insoluble PVP (10 % w/v) turned out to be essential (CLAUDOT and DROUET 1992). Furthermore, a stabilising effect of bovine serum albumin was observed (SCHRÖDER et al. 1979; CLAUDOT and DROUET 1992), which seems to be due to lowered protease activity and protection of the catalytic centre of the enzymes (HINDERER et al. 1983). The addition of sucrose, calcium chloride, cysteine, EDTA and DIECA to the extraction buffer also led to increased enzyme activity.

Enzyme preparations from rose leaves according to CLAUDOT and DROUET (1992) showed high activities of CHS, DFR and FNR, whereas FHT activity was rather low. Consequently, reduction of the EDTA concentration from 50 mM to 30 mM and an increase of the Fe^{2+} (one of the cofactors for dioxygenases such as FHT and FLS) concentration from 0.1 mM to 30 mM resulted in enzyme preparations with moderate FHT activity (preparation A). However, no PAL activity could be measured. Enzyme preparations performed according to ELSHERIF 2000 (preparation B) showed the activities of PAL as well as of CHS, FHT, DFR/FNR and FLS. In case of FHT, conversion rates were considerably higher, whereas the activities of CHS, DFR and FNR were much lower than in assays with enzyme preparation A. FLS activity could be detected as well, but the conversion rates were generally very low. Therefore, in time course studies, preparation A was used for measurements of CHS, DFR and FNR activity and preparation B for PAL and FHT activity.

The time course study on the treated rose leaves was performed to provide insight into the prohexadione-Ca effect on flavonoid enzyme levels and flavonoid contents. The main question was, whether luteoliflavan as an indicator of induced 3-deoxyflavonoids including the antimicrobial luteoforol is found in rose too, and how the induction proceeds.

Treatment with prohexadione-Ca clearly influenced flavonoid biosynthesis in rose leaves. The most striking observation was the accumulation of luteoliflavan as described in pome fruits. The general decrease of flavonol concentration in the control leaves may be explained by an ontogenetic decline, which was also described for apple (TREUTTER 2002). The additional clear decrease in prohexadione-Ca treated leaves was not unexpected, due to inhibition of both FHT and a second 2-oxoglutarate dependent dioxygenase FLS, that is catalysing flavonol synthesis. The catechin content showed increasing tendency after day 1. This may be explained by the fact that catechin biosynthesis has only to pass one bottleneck, the FHT reaction. Moreover, FLS and ANS, another 2-oxoglutarate dependent dioxygenase, should also be inhibited. Both enzymes catalyse reactions branching from the catechin biosynthesis and consume catechin precursors. Without this competition, these precursors can mainly be used for catechin formation. Furthermore, the increase of catechin may also be a consequence of the stimulated activity of PAL, which provides more flavonoid precursors.

Generally, the effect of prohexadione-Ca treatment on the secondary metabolism of rose leaves was not as pronounced as in apple (ROEMMELT et al., this issue). This is confirmed by the enzymatic data. In rose leaves, induction of PAL activity could be observed for at least four days after treatment. For CHS/CHI, DFR and FNR activities, however, a pronounced induction was observed only during the first day. In contrast to PAL, as an enzyme of general phenylpropanoid biosynthesis, CHS, CHI and DFR/FNR are flavonoid specific enzymes and may be regulated differently. Trials to measure FHT without the inhibiting prohexadione-Ca were performed by enzyme preparations of time course samples using gel chromatography. A weaker inhibition was observed by this means. However, these results should be considered with care, since enzyme bound prohexadione-Ca could co-purify with the FHT enzyme. Such an effect had been observed with FHT bound 2-oxoglutarate before (FORKMANN, unpublished).

Summarising, it can be stated, that after prohexadione-Ca application formation of 3-deoxyflavonoids takes place in rose leaves, as judged by the occurrence of the conversion product luteoliflavan as an indicator. The time course showed the progressive accumulation of luteoliflavan; the effect lasts at least several days. The biosynthesis of 3-deoxyflavonoids can readily be explained by the FNR activity of the rose leaf DFR, as verified by heterologous expression. Thus, prohexadione-Ca induction of resistance against rose pathogens should be possible. Therefore, in a next step, phytopathological investigations with powdery mildew, blackspot and rust have to be performed.

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Addresses of authors: K. Schlangen, K. Stich and H. Halbwirth (corresponding author), Institute of Technical BioScience, Technical University of Vienna, Vienna, Austria, e-mail: hhalb@mail.zserv.tuwien.ac.at, C. Gosch, J. Knott, T. C. Fischer and G. Forkmann, Chair of Floriculture Crops and Horticultural Plant Breeding, Department of Plant Sciences, Technical University of Munich, Freising, Germany and S. Roemmelt and D. Treutter, Unit of Fruit Science, Department of Plant Sciences, Technical University of Munich, Freising, Germany.