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Effect of N-Nutrition in Apple on the Response of its Secondary Metabolism to Prohexadione-Ca Treatment

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Summary

The response of *in vitro* grown apple plantlets onto the treatment with the growth retardant prohexadione-Ca is demonstrated with regards to their secondary metabolism and under the influence of N nutrition. Reported prohexadione-Ca induced changes of the flavonoid composition were the formation of the uncommon 3-deoxycatechin luteoliflavan, a decrease of catechins, procyanidins and flavonols, which was also observed in our experiments on 'Royal Gala' and 'Golden Delicious'. The extent of effects, however, was markedly modified by N-nutrition of the plantlets. In general, high N-nutrition reduces the amount of total phenolic compounds in apple leaves whereas low N supply favours the accumulation of flavonoids and the formation of resistance related compounds after prohexadione-Ca treatment.

Zusammenfassung

Einfluss der N-Ernährung auf Veränderungen des Sekundärstoffwechsels nach Behandlung von Apfelbäumen mit Prohexadion-Ca. Die Veränderungen des Sekundärstoffwechsel von *in vitro*-kultivierten Apfel-Sprosskulturen nach Behandlung mit dem Wachstumsregulator Prohexadion-Ca und unter dem Einfluss der N-Ernährung werden demonstriert. Erwartete Effekte bezüglich der Flavonoidgehalte waren die Bildung des für den Apfel ungewöhnlichen 3-Deoxycatechins Luteoliflavan sowie eine Reduktion der Konzentrationen von Catechinen, Procyanidinen und Flavonolen, die in den vorgestellten Experimenten an 'Royal Gala' und 'Golden Delicious' auch beobachtet wurden. Das Ausmaß der Effekte wurde aber stark durch die N-Ernährung modifiziert. Im Allgemeinen reduziert hohe N-Versorgung die Konzentration der phenolischen Verbindungen insgesamt, wohingegen eine maßvolle N-Ernährung die Akkumulation von Flavonoiden begünstigt und auch die Bildung der durch Prohexadion-Ca-Behandlung induzierten Verbindungen, die mit Resistenzreaktionen korrelieren, unterstützt.

Key words. Flavonoids – procyanidins – dihydrochalcones – flavonols – phenylpropanoids – 3-deoxycatechin – luteoliflavan

Introduction

Secondary metabolites, namely phenylpropanoid derivatives including hydroxycinnamic acids, dihydrochalcones and flavonoids, often have been mentioned to be involved in resistance against various diseases (GEIBEL et al. 1994; MAYR et al. 1997). Efforts have therefore been undertaken to stimulate the formation of these compounds in target tissues, such as apple leaves, with the aim of resistance induction (MAYR et al. 1995; Michalek 1996). Recently, the application of the 2-oxoglutarate dependent dioxygenase inhibitor prohexadione-Ca has been found to channel flavonoid metabolism towards the 3-deoxyflavan pathway (ROEMMELT et al., this issue) which in several plants is obviously related to resistance against various diseases (RADEMACHER and KOBER, this issue; BAZZI et al., this issue) These beneficial effects of an induced or, more in general, of a changed phenylpropanoid metabolism, however, can only be achieved if the pathway is supplied with sufficient precursor metabolites. The biosynthesis of phenylpropanoids depends on PAL (phenylalanine ammonia lyase) activity, which is the key enzyme. It converts the amino acid phenylalanine to cinnamic acid and thereby initiates the biosynthesis of hydroxycinnamic acids and the flavonoids derived from them. It has been postulated by MARGNA (1977) and intensively discussed by JONES and HARTLEY (1999) that PAL activity may be regulated by substrate supply. PAL represents the switch between primary and secondary metabolism since the amino acid is also a building stone of proteins. The primary metabolism is enhanced by high nitrogen nutrition at the expense of phenylpropanoid formation, which has been shown for several plants (RUEHMANN et al. 2002; KESKI-SAARI and JULKUNEN-TIITO 2003). The aim of N-fertilisation of fruit crops is to improve growth and fruit yield and sophisticated fertilisation measures were successfully developed in the past. High N-fertilisation, however, decreases the formation of secondary metabolites and thereby enhances disease susceptibility, as was shown for apple by RUEHMANN et al. (2002). In a cost-benefit model calculation, it could

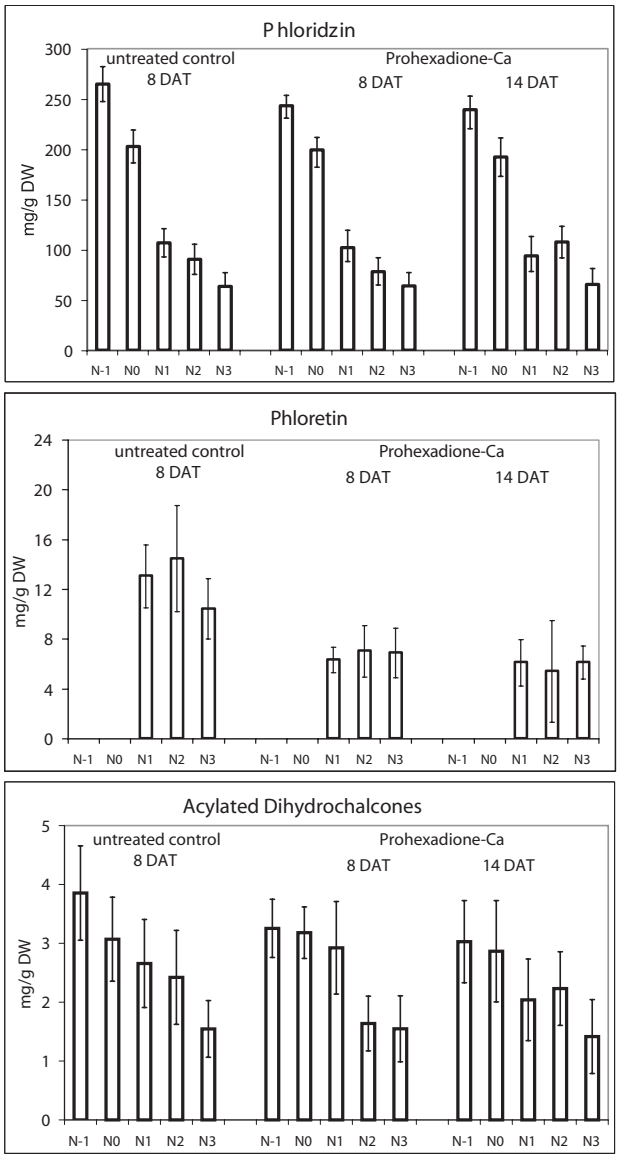


Fig. 1 Content of phloretin derivatives in young leaflets of *in vitro* grown apple plants of 'Royal Gala' 8 and 14 days after treatment (DAT) with prohexadione-Ca cultivated on agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). The vertical line represents the standard deviation of 4 replicates.

recently be shown that under high N conditions apple trees may favour growth at the expense of defensive compounds (GAYLER et al. 2003).

Prohexadione-Ca is a growth retardant acting as an anti-gibberellin (RADEMACHER and KOBER, this issue). It may therefore be expected that its application on plants may switch the utilisation of phenylalanine towards phenylpropanoid formation. The study presented here aimed at clarifying whether the treatment of *in vitro* grown apple plantlets with prohexadione-Ca will lead to accumulation of phenylpropanoids even in the case of high N-nutrition. The behaviour of luteoliflavan, which is the marker compound of prohexadione-Ca induced resistance (ROEMMELT et al. 2002, 2003), was thereby of particular interest.

Material and Methods

The apple plantlets were grown as shoot cultures without roots on modified Murashige/Skoog-media (LUX-ENDRICH et al. 2000) under growing conditions described earlier (RUEHMANN et al. 2002) with 3 different amounts of nitrogen and 3 % sucrose for the 'Royal Gala' experiment and with 5 % sucrose for the time course study with 'Golden Delicious'. The nitrogen supplements of the media were the following:

N–1: 0 g l^{-1} NH_4NO_3, 0.95 g l^{-1} KNO_3;
N0: 0 g l^{-1} NH_4NO_3, 1.90 g l^{-1} KNO_3;
N1: 1.65 g l^{-1}, NH_4NO_3, 1.90 g l^{-1} KNO_3;
N2: 3.3 g l^{-1} NH_4NO_3, 1.90 g l^{-1} KNO_3;
N3: 4.95 g l^{-1} NH_4NO_3, 1.90 g l^{-1} KNO_3

Prohexadione-Ca was sprayed at a concentration of 100 ppm (active ingredient) 3 weeks after establishing the culture of the apple cultivars 'Royal Gala' and 'Golden Delicious'. 20 shoots per variant were harvested for analyses. For phenol analyses the 4 youngest leaflets of 5 shoots each were combined and analysed as mixed samples. Phenolic compounds were extracted and analysed by HPLC as described by RUEHMANN et al. (2002).

Experiment with 'Royal Gala'

Shoots were harvested for analysis of phenolic compounds at day 8 and day 14 after treatment (DAT) with four replicates each consisting of 5 plantlets. Due to the limiting quantity of plantlets, untreated controls were available only at day 8 after treatment (DAT).

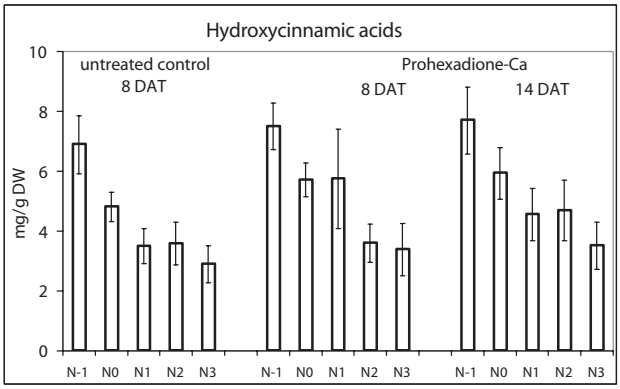
Time course study with 'Golden Delicious'

A more detailed study was performed in a time course experiment with 5 sampling dates after treatment with prohexadione-Ca. At each harvest date, 20 plantlets were combined and their young leaves were analysed as a mixed sample. Untreated controls were taken at day 3 and day 14.

Results

It is obvious from the data shown in Fig. 1–3 for 'Royal Gala' plantlets that high N supply reduces the total amount of phenolic compounds in the young leaves. This is particularly represented by the dihydrochalcone phloridzin (Fig. 1) which decreases from 25 % to about 5 % per dry weight. A similar decrease, but on a lower level, show flavonols (Fig. 2), monomeric flavan-3-ols and procyanidins (Fig. 3) whereas the tendency for hydroxycinnamic acids (Fig. 2) and acylated dihydrochalcones (Fig. 1) is not as pronounced. The dihydrochalcone aglycone phloretin, however, appears only in the variants N1, N2 and N3 (Fig. 1). After treatment with prohexadione-Ca the formation of luteoliflavan is induced (Fig. 3). This compound also follows the decreasing tendency with increasing N supply. Prohexadione-Ca slightly enhances the concentration of hydroxycinnamic acids (Fig. 2) and of monomeric flavan-3-ols (Fig. 3), while phloretin is reduced (Fig. 1).

The growth curves obtained in a time course study with 'Golden Delicious' (g fresh weight per plantlet, given in Fig. 4) show the growth reducing effects of N



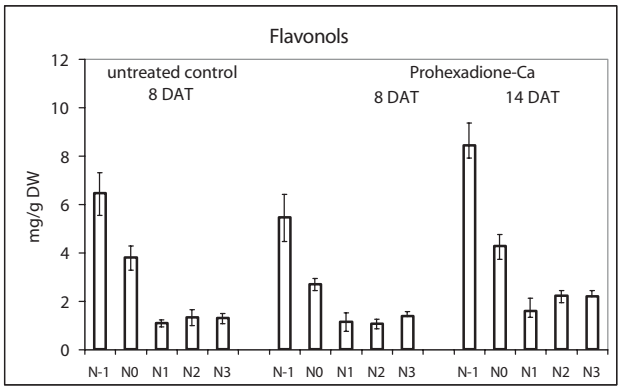


Fig. 2 Content of hydroxycinnamic acids and flavonols in young leaflets of *in vitro* grown apple plants of 'Royal Gala' 8 and 14 days after treatment (DAT) with prohexadione-Ca cultivated on agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). The vertical line represents the standard deviation of 4 replicates.

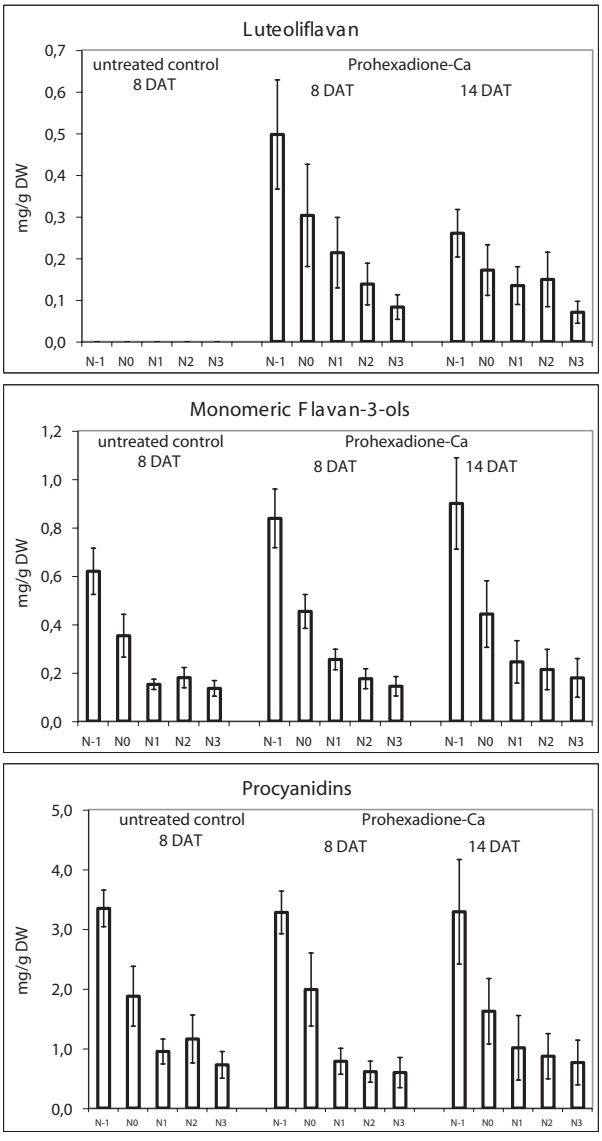


Fig. 3 Content of flavans in young leaflets of *in vitro* grown apple plants of 'Royal Gala' 8 and 14 days after treatment (DAT) with prohexadione-Ca cultivated on agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). The vertical line represents the standard deviation of 4 replicates.

deficiency and of the prohexadione-Ca treatment. The growth retardant was most effective in the variants N2 and N3. As shown for 'Royal Gala'. high N-supply reduces the concentrations of phloridzin which remains on a rather constant level during the period of study (Fig. 5). After Prohexadione-Ca treatment it was only slightly reduced in some cases. The other dihydrochalcones estimated behaved differently (Fig. 6). The acylated dihydrochalcones and the 3-hydroxyphloridzin were not influenced whereas the phloretin exhibited increasing values when N-supply was high. The phloretin was also influenced by the prohexadione-Ca treatment. Its concentration decreased in the variants N1, N2 and N3 with most pronounced effects at day 3 after treatment. The hydroxycinnamic acids were not affected by prohexadione-Ca treatment but were reduced by high N-nutrition (Fig. 7). During the time course the concentrations of the hydroxycinnamic acids remained rather stable. Rather constant levels was also shown for the flavonols in the low N variants (Fig. 8) but were generally reduced with increasing N in the medium. The prohexadione-Ca treatment tended to increase flavonols to some extent in the variants N1, N2 and N3. The monomeric flavan-3-ols exhibited considerable high amounts only when N was deficient (N–1, Fig. 9). The effect of prohexadione-Ca on the level of monomeric flavan-3-ols was influenced by the N-nutrition: N-deficiency (N–1) led to reduction at day 14 whereas in variants N1 and N2 an increase was found at day 14. The oligomeric derivatives of the flavan-3-ols, the procyanidins (Fig. 10) exhibited a similar behaviour. The 3-deoxycatechin luteoliflavan was only detected in plantlets treated with prohexadione-Ca but in very low concentrations (Fig. 11). Nevertheless, highest amounts occurred in the low N variant.

Discussion

The growth retardant prohexadione-Ca could reduce the fresh weight production of *in vitro* grown apple plantlets to a certain extent (Fig. 4). This, however, did not enhance the total phenolic content since the main compound, the phloridzin, was not affected (Fig. 5). In contrary, the flavan-3-ols and procyanidins, which are correlated to resistance of apple against scab (MAYR et

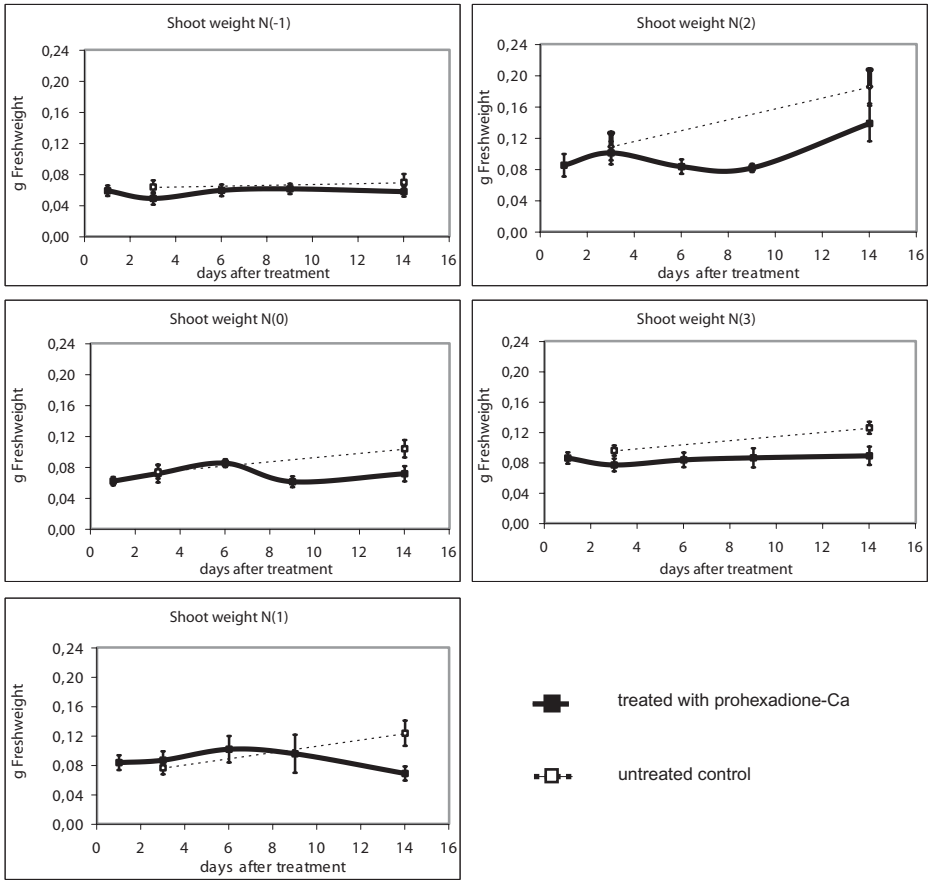


Fig. 4 Fresh weight of *in vitro* grown apple plantlets of the cv. 'Golden Delicious' during 14 days with and without treatment with prohexadione-Ca under the influence of various agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). The vertical line represents the standard deviation of 20 replicates.

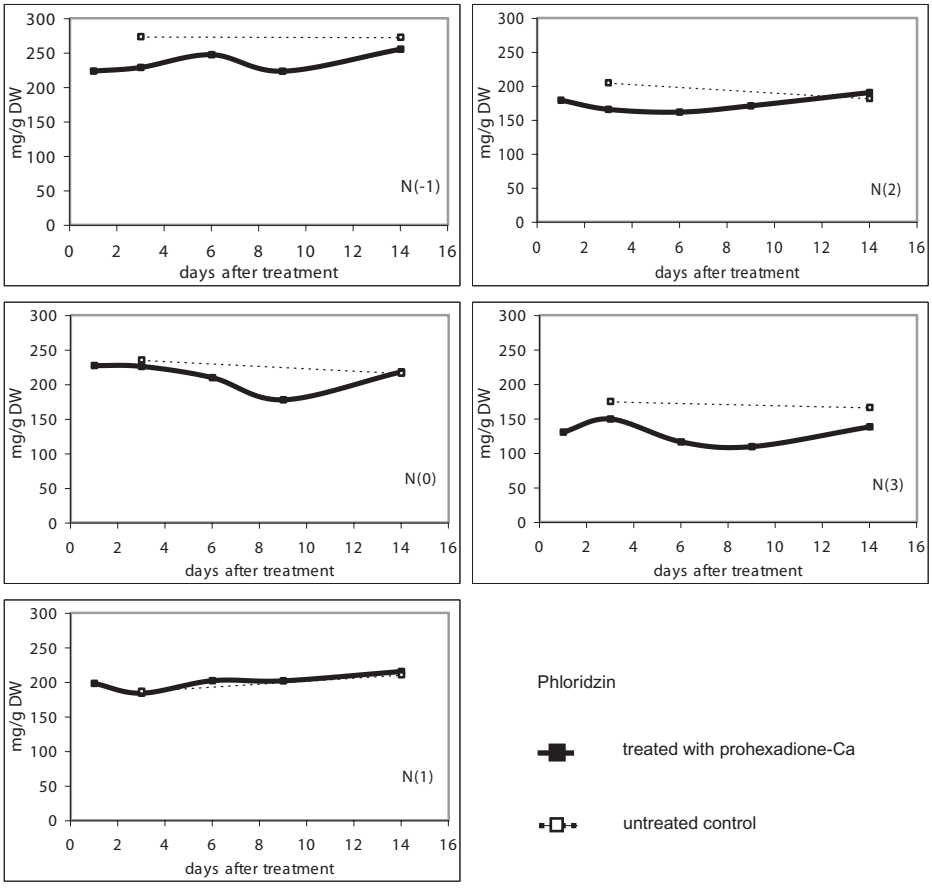


Fig. 5 Concentration of phloridzin in the young leaflets of *in vitro* grown apple plantlets of the cv. 'Golden Delicious' during 14 days with and without treatment with prohexadione-Ca under the influence of various agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). Each data point represents the analysis of a mixed sample consisting of 20 shoot tips.

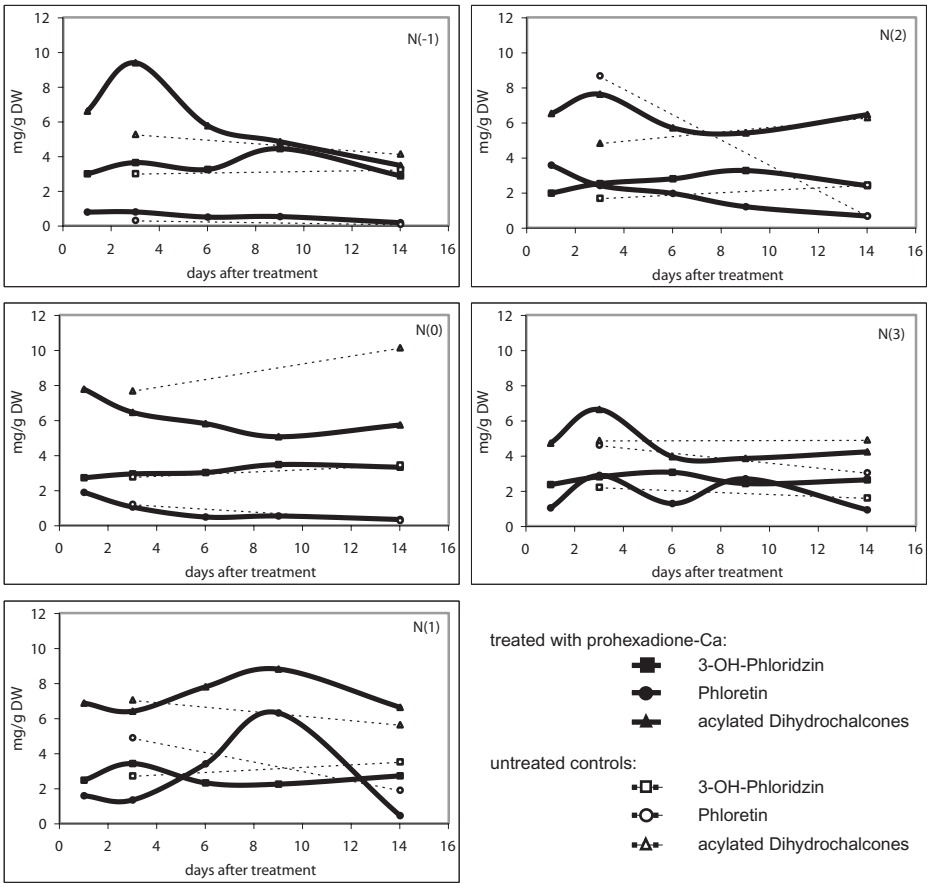


Fig. 6 Concentration of other dihydrochalcones in the young leaflets of *in vitro* grown apple plantlets of the cv. 'Golden Delicious' during 14 days with and without treatment with prohexadione-Ca under the influence of various agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). Each data point represents the analysis of a mixed sample consisting of 20 shoot tips.

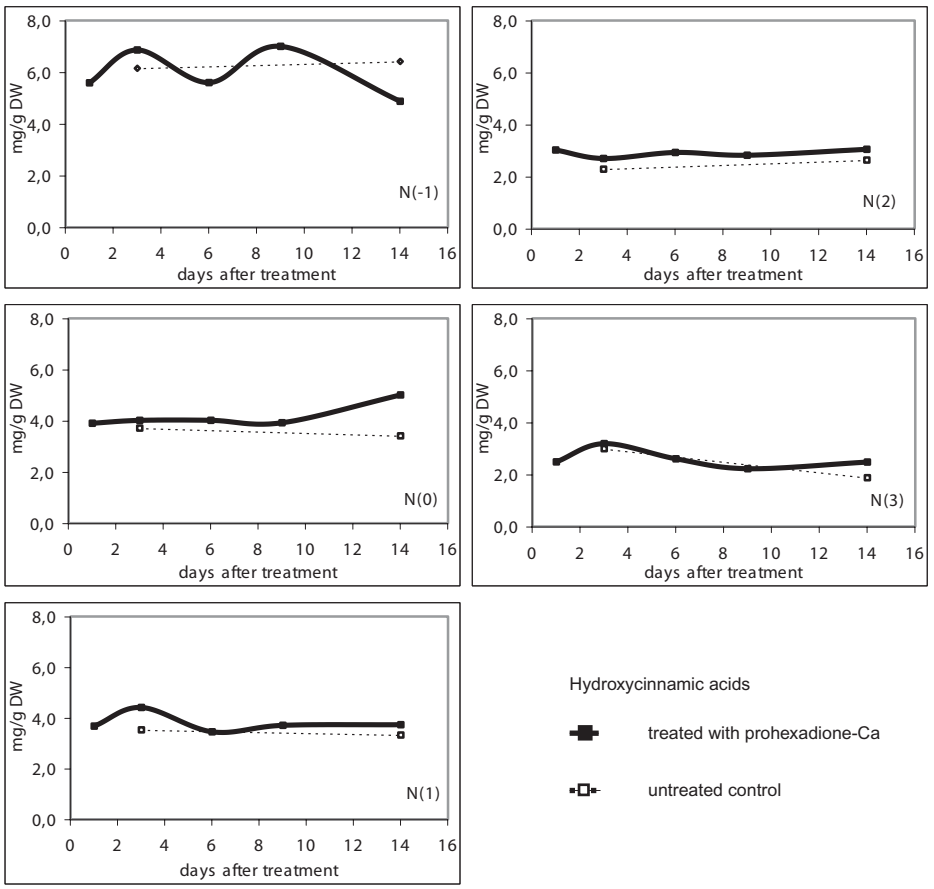


Fig. 7 Concentration of hydroxycinnamic acids in the young leaflets of *in vitro* grown apple plantlets of the cv. 'Golden Delicious' during 14 days with and without treatment with prohexadione-Ca under the influence of various agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). Each data point represents the analysis of a mixed sample consisting of 20 shoot tips.

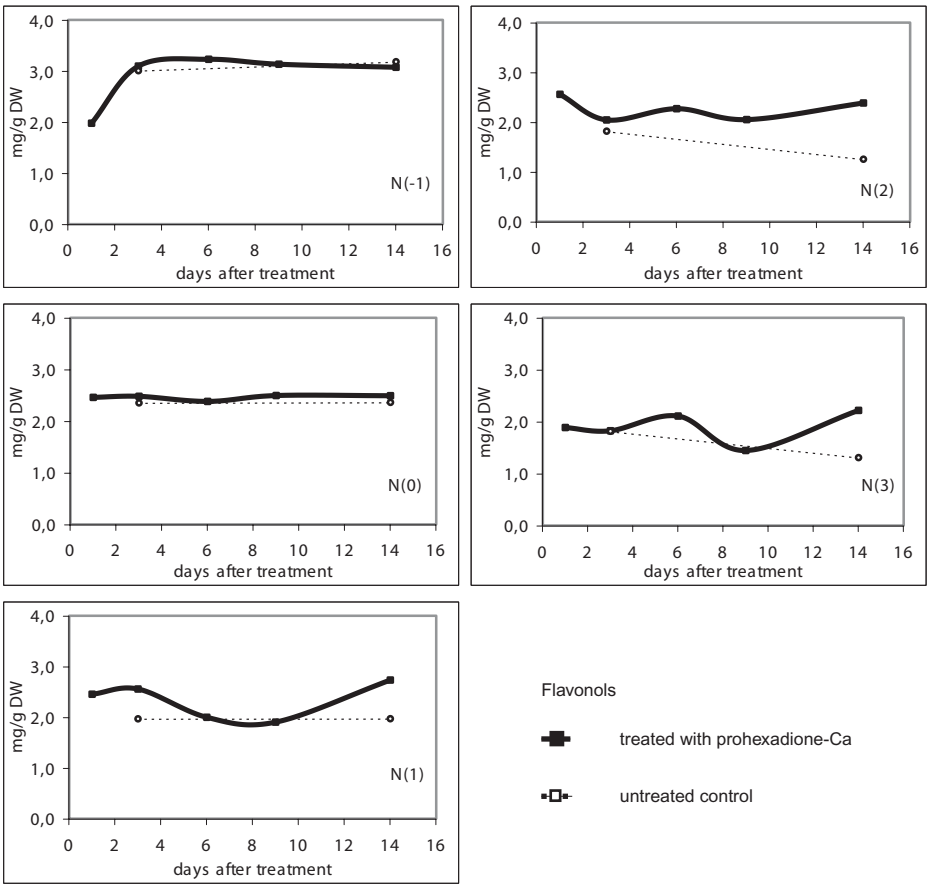


Fig. 8 Concentration of flavonols in the young leaflets of *in vitro* grown apple plantlets of the cv. 'Golden Delicious' during 14 days with and without treatment with prohexadione-Ca under the influence of various agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). Each data point represents the analysis of a mixed sample consisting of 20 shoot tips.

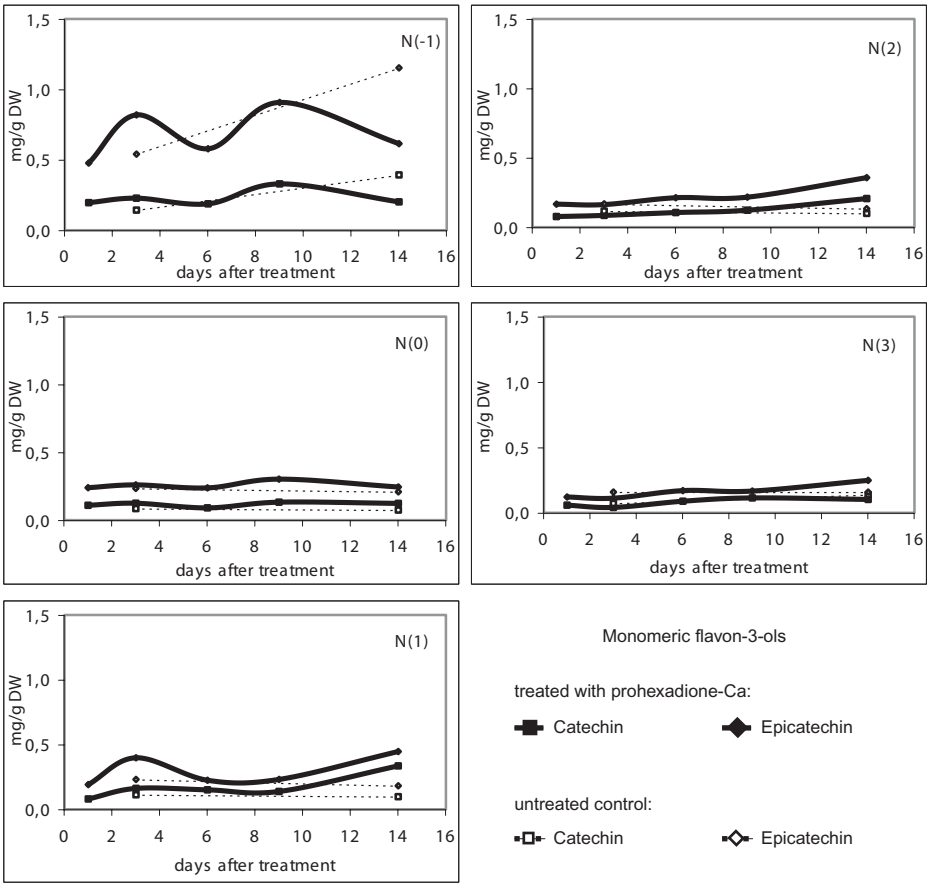


Fig. 9 Concentration of monomeric flavan-3-ols in the young leaflets of *in vitro* grown apple plantlets of the cv. 'Golden Delicious' during 14 days with and without treatment with prohexadione-Ca under the influence of various agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). Each data point represents the analysis of a mixed sample consisting of 20 shoot tips.

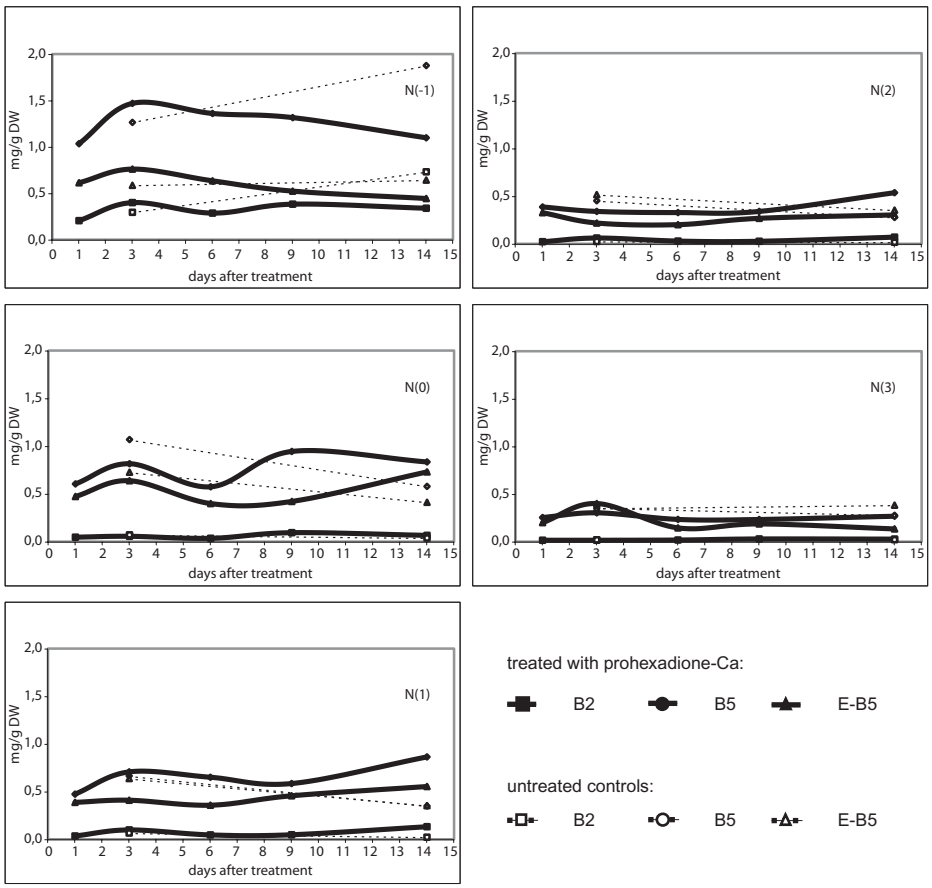


Fig. 10 Concentration of procyanidins in the young leaflets of *in vitro* grown apple plantlets of the cv. 'Golden Delicious' during 14 days with and without treatment with prohexadione-Ca under the influence of various agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). Each data point represents the analysis of a mixed sample consisting of 20 shoot tips.

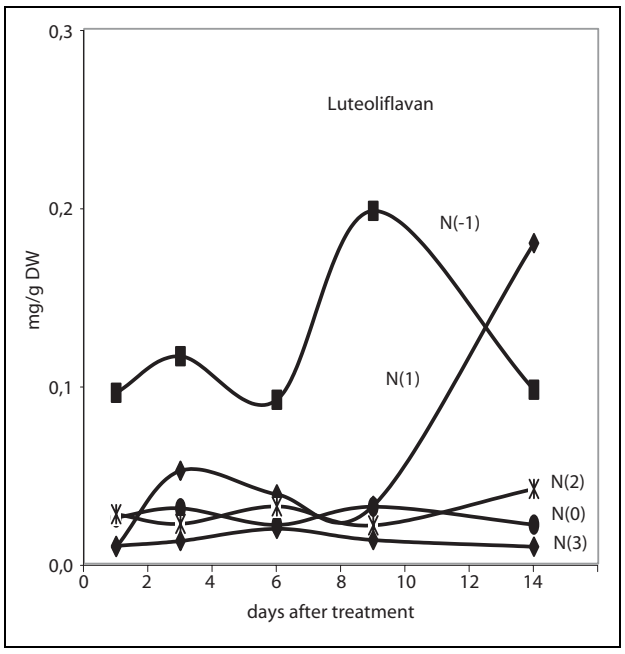


Fig. 11 Concentration of luteoliflavan in the young leaflets of *in vitro* grown apple plantlets of the cv. 'Golden Delicious' during 14 days after treatment with prohexadione-Ca under the influence of various agar media containing increasing amounts of inorganic nitrogen (N–1, N0, N1, N2, N3). Each data point represents the analysis of a mixed sample consisting of 20 shoot tips.

al. 1997), increased in 'Royal Gala' (Fig. 3) and in 'Golden Delicious' (Fig. 9 and 10). This observation does not confirm theories, which explain the competition between growth and secondary metabolism as a competition for carbon (HERMS and MATTSON 1992; JONES and HARTLEY 1999). If the carbon channelling towards the phenylpropanoids and flavonoids is decided at the level of the key enzyme PAL, it will be expected that all flavonoids behave in the same way. The changes in the flavonoid composition after treatment with the growth retardant cannot be explained by a simple carbon channelling towards the phenylpropanoid/flavonoid pathway. Even when growth of the plantlets is reduced the general effect of high N-nutrition is predominating. As described earlier (RUEHMANN et al. 2002), high N-fertilisation decreases the concentration of nearly all phenolic compounds in the young leaves of apple with the exception of phloretin (Fig. 1 and 6). This dihydrochalcone aglycone may be correlated to growth since its concentration is decreased after prohexadione-Ca treatment.

The main effects of prohexadione-Ca must be explained by the inhibition of 2-oxoglutarate dependent dioxygenases (RADEMACHER and KOBER, this issue) which also concerns some enzymes of the flavonoid pathway, namely the FHT (flavanone 3-hydroxylase) and the FLS (flavonol synthase). As a consequence of FHT inhibition, apple leaves form the 3-deoxycatechin luteoliflavan (Fig. 3 and 11) which was previously identified by ROEMMELT et al. (2003). When flavanones are directed towards the 3-deoxyflavan pathway they will be lacking as substrates for the formation of fla-

van-3-ols, proanthocyanidins and flavonols. Therefore a decrease of these flavonoids may be expected after prohexadione-Ca treatment which was found several times (ROEMMELT et al. 2003; GOSCH et al., this issue; SCHLANGEN et al., this issue) and was also confirmed in the N–1 variant of the 'Golden Delicious' experiment. Increasing amounts of flavan-3-ols (Fig. 9), procyanidins (Fig. 10) and flavonols (Fig. 8), however, were found in the high N variants. This coincides with the reduced growth and, thus, may be explained by concomitant better availability of carbon skeletons for PAL and also for CHS (chalcone synthase) in these treatments.

When regarding prohexadione-Ca as a resistance inducing agent which decreases incidence of fire blight and scab in apple trees (ROEMMELT et al. 2002; RADEMACHER and KOBER, this issue) and considering the role of 3-deoxyflavans thereby, it must be noticed that environmental conditions such as N nutrition will notably modify the efficiency of the treatment. This was shown for luteoliflavan (Fig. 3 and 11) which can be taken as a marker for prohexadione-Ca action. The data show that moderate N-nutrition of the shoots enhances the response of the leaves to the dioxygenase inhibitor as indicated by the highest luteoliflavan content in the N–1 variant. This is probably due to a favoured biosynthesis of phenylpropanoids in moderately fertilised and moderately growing apple trees (RUEHMANN et al. 2002). This points to the influence of growth conditions on the resistance inducing capacity of prohexadione-Ca. Only in such tissues where the biosynthesis of phenylpropanoids is well working, the accumulation of luteoliflavan can be stimulated. If this compound is the active component or at least a marker for resistance, the defence against fire blight and scab will be affected by the nutritional status and growth conditions. The unexpected increase of flavan-3-ols and procyanidins as well as of flavonols has to be mentioned and may also contribute to the beneficial effects of prohexadione-Ca. This, however, can only take place under physiological conditions favouring the formation of these flavonoids with a sufficient availability of carbon skeletons and energy as a prerequisite. Predictions for the efficiency of prohexadione-Ca treatments with regards to resistance induction must therefore be aware of environmental impacts.

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