Invited Trends Article

Induction of Antimicrobial 3-Deoxyflavonoids in Pome Fruit Trees Controls Fire Blight

Heidrun Halbwirth[a], Thilo C. Fischer[b], Susanne Roemmelt[c], Francesco Spinelli[d], Karin Schlangen[a], Silke Peterek[c], Emidio Sabatini[d], Christian Messina[e], John-Bryan Speakman[f], Carlo Andreotti[d], Wilhelm Rademacher[f], Carlo Bazzi[e], Guglielmo Costa[d], Dieter Treutter[c], Gert Forkmann[b], and Karl Stich[a*]

[a] Institute of Technical BioScience, Technical University of Vienna, Getreidemarkt 9/173, A-1060 Wien, Austria. Fax: (43) 1588011 7399. E-mail: kstich@mail.zserv.tuwien.ac.at
[b] Chair of Floriculture Crops and Horticultural Plant Breeding, Department of Plant Sciences, Technical University of Munich, Freising, Germany
[c] Unit of Fruit Science, Department of Plant Sciences, Technical University of Munich, Freising, Germany
[d] Department of Arboriculture, University of Bologna, Bologna, Italy
[e] Department of Agroenvironmental Sciences and Technologies, University of Bologna, Bologna, Italy
[f] BASF, Agricultural Center, Limburgerhof, Germany

* Author for correspondence and reprint requests

Z. Naturforsch. **58c**, 765–770 (2003); received August 1, 2003

Fire blight, a devastating bacterial disease in pome fruits, causes severe economic losses worldwide. Hitherto, an effective control could only be achieved by using antibiotics, but this implies potential risks for human health, livestock and environment. A new approach allows transient inhibition of a step in the flavonoid pathway, thereby inducing the formation of a novel antimicrobial 3-deoxyflavonoid controlling fire blight in apple and pear leaves. This compound is closely related to natural phytoalexins in sorghum. The approach does not only provide a safe method to control fire blight: Resistance against different pathogens is also induced in other crop plants.

Key words: Fire Blight (*Erwinia amylovora*), Luteoforol, Enhanced Resistance

Introduction

Whereas fungal diseases can be controlled by use of modern fungicides, control of bacterial diseases is much more difficult. In pome fruit trees, the prevailing cultivars are particularly susceptible to the enterobacterium *Erwinia amylovora*, the causal agent of fire blight. Despite all efforts of restriction, there is a progressive spread of the disease. *E. amylovora* may infect flowers early in the season and later invades shoots (primary and secondary fire blight). The disease threatens developing fruits and may also affect fruit load of the following years by killing fruit spurs, branches and, even worse, the whole tree. It is often mandatory by law to destroy whole orchards in order to avoid further spread of the disease. Resistant cultivars, even if available, do not completely fit commercial demands with respect to fruit quality, yield or other agronomic features. The only effective chemical control is achieved by the use of antibiotics, such as streptomycin (McManus *et al.*, 2002). However, its use is banned in most European countries. After the detection of significant amounts of streptomycin in honey, field treatments had been suspended in Belgium and Germany. Due to the general concerns against the use of antibiotics as crop protectants, alternative and toxicologically as well as environmentally safe strategies are urgently needed. A new strategy, particularly useful for the control of secondary fire blight, involves the use of the plant growth regulator prohexadione-Ca. This contribution summarizes the knowledge on the underlying physiological mechanism and shows that this approach may be used as a general strategy to induce pathogen resistance in various important crop species.

Plant growth regulators

Plant growth regulators are commonly used in commercial farming to reduce superfluous longitu-

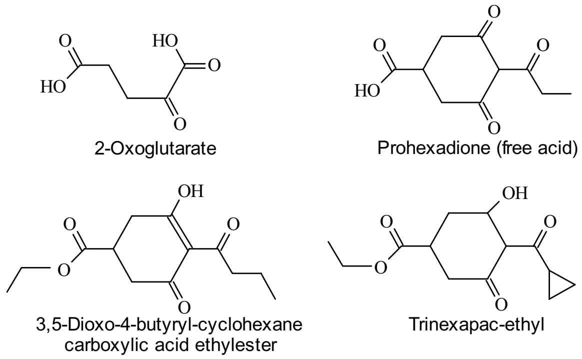

Fig. 1. 2-Oxoglutarate and examples for structural analogues.

dinal shoot growth. Most growth retardants act by inhibiting the biosynthesis of growth-active gibberellic acids (GAs). Several types of such inhibitors are known affecting different steps of the biosynthetic pathway. Acylcyclohexanediones, *e.g.* prohexadione-Ca and trinexapac-ethyl (Fig. 1), block late steps of GA formation. As structural analogues of 2-oxoglutarate, which is the co-substrate of 2-oxoglutarate dependent dioxygenases, they competitively inhibit biosynthetic steps catalyzed by such dioxygenases (Rademacher, 2000). In the biosynthesis of GAs, particularly 3β-hydroxylation is affected thereby inhibiting the formation of highly active GAs from inactive precursors. Prohexadione-Ca is commercially used as a plant growth regulator (trade names: Apogee®, Regalis®) in a number of crop plants including cereal grains, peanuts and pome fruits. The compound shows favorable toxicological and ecotoxicological features, is relatively short-lived and degrades into natural compounds. Trinexapac-ethyl [ethyl 4-cyclopropyl(hydroxy)methylene-3,5-dioxycyclohexane carboxylate] is mainly used in stem stabilization in cereal grains (trade name: Moddus®) and in reduction of shoot growth in turf grasses (trade name: Primo®). Under practical conditions, trinexapac-ethyl (an ester) and prohexadione-Ca (a salt of an acid) display similar degrees of activity when applied in appropriate formulations to graminaceous species. However, in dicots prohexadione-Ca generally is significantly more active than trinexapac-ethyl. Most likely, this indicates that trinexapac-ethyl is easily hydrolyzed into its active acidic form in grasses, whereas this process is not very efficient in dicots (Rademacher, 2000).

Effect on the flavonoid metabolism

Recent trials revealed that pome fruit plants treated with prohexadione-Ca or structurally related compounds, such as trinexapac-ethyl, not only showed reduced shoot growth but were also significantly less affected by fire blight and scab as compared to control trees (Fernando and Jones, 1999; Momol *et al.*, 1999; Roemmelt *et al.*, 1999, 2003a; Yoder *et al.*, 1999; Costa *et al.*, 2001; Maxson and Jones, 2002). This effect was surprising and unexpected, since prohexadione-Ca is absolutely inactive as a bactericide or fungicide (Rademacher, 2000; Rademacher and Bucci, 2002). Furthermore, there were no indications in the pertinent literature that other types of growth regulators, which have long been used in pome fruit production, have such an effect. Chemical analyses of treated shoot tissues revealed the occurrence of flavonoids, which are not commonly formed in apple. In particular, the accumulation of the 3-deoxycatechin luteoliflavan was observed, which was previously unknown in rosaceous species (Rademacher *et al.*, 1998; Roemmelt *et al.*, 1999, 2003b; Ruehmann and Treutter, 2003) (Fig. 2). In many host plants, resistance against a wide range of fungi and bacteria is connected to the presence of phenolic compounds, in particular flavonoids (Snyder and Nicholson, 1990; Feucht *et al.*, 1996; Mayr *et al.*, 1997). However, the flavonoids commonly formed in pome fruit trees are not effective enough against fire blight. Therefore, the induced changes in flavonoid composition, associated with the enhanced resistance, were studied in detail.

In addition to luteoliflavan, some flavanones were accumulated after prohexadione-Ca treatment (Fig. 2). These were identified as naringenin 7-O-glucoside, eriodictyol 7-O-glucoside and 6″-*O-trans-p*-coumaroyl-eriodictyol 3′-O-glucoside (Roemmelt *et al.*, 2003a). Furthermore, a general decrease of the constitutive flavan-3-ols, such as catechin, epicatechin, the procyanidins B2, B3, E.B5, of flavonols and anthocyanidins was observed in leaves treated with prohexadione-Ca (Roemmelt *et al.*, 2003a,b).

Mode of action

Four enzymes of the flavonoid pathway have been classified as 2-oxoglutarate dependent dioxygenases: flavanone 3-hydroxylase (FHT), flavonol

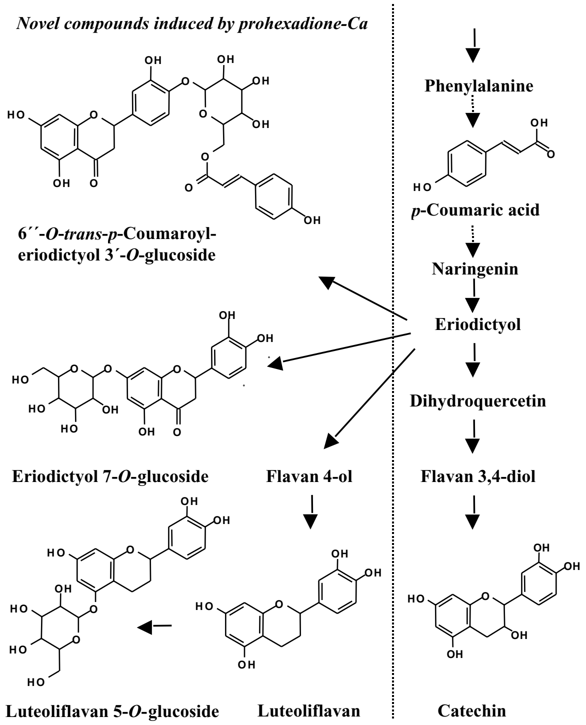

Fig. 2. Novel phenolic compounds in apple.

synthase (FLS), anthocyanidin synthase (ANS) and flavone synthase I (FNS I) (Forkmann and Heller, 1999). These enzymes could be inhibited competitively by analogues of 2-oxoglutarate (such as prohexadione-Ca and trinexapac-ethyl), which may lead to marked changes in the flavonoid composition of apple and pear leaves. It was shown, that by inhibition of a single step in the flavonoid biosynthesis, a novel pathway can be induced. Enzymatic studies with preparations from apple and pear leaves revealed strong competitive inhibition of flavanone 3-hydroxylase (FHT), a 2-oxoglutarate dependent dioxygenase catalyzing the hydroxylation of flavanones to dihydroflavonols (Fig. 3), by prohexadione-Ca *in vitro*. FHT activity was inhibited in treated leaves for several days (Fig. 4). This *in vivo* effect gradually decreased five days after treatment, which can be explained by the advancing degradation of prohexadione-Ca (Roemmelt *et al.*, 2003b). The inhibition by prohexadione-Ca was confirmed by cloning of the FHT cDNA of apple and pear and subsequent enzymatic studies with the heterologously expressed enzymes. Thus, FHT inhibitors like prohexadione-Ca create a transient bottleneck in the flavonoid pathway, which leads to the accumulation of eriodictyol, a common substrate for FHT. Eriodictyol can be glycosylated and/or acylated, but it is mainly metabolized to the 3-deoxycatechin luteoliflavan (Fig. 3).

3-Deoxycatechins belong to the rare class of 3-deoxyflavonoids, which are found only in a few plant species (Styles and Ceska, 1975; Lo *et al.*, 1999; Stich and Forkmann, 1988; Ramesh *et al.*, 2001). The most prominent 3-deoxyflavonoids are the 3-deoxyanthocyanidins, which serve as pigments in flowers of Gesneriaceae (Stich and Fork-

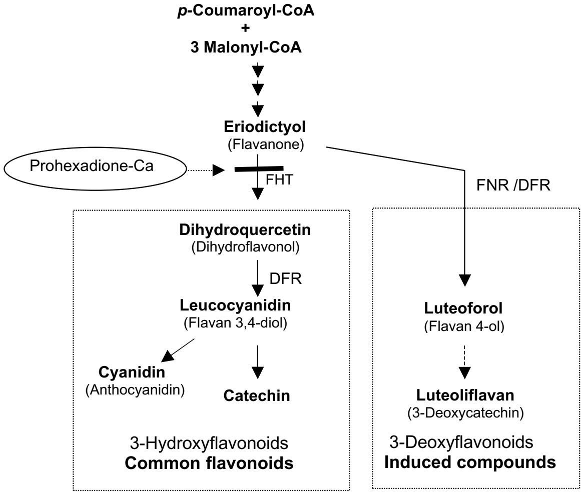

Fig. 3. The flavonoid biosynthesis in apple and pear and the influence of prohexadione-Ca.

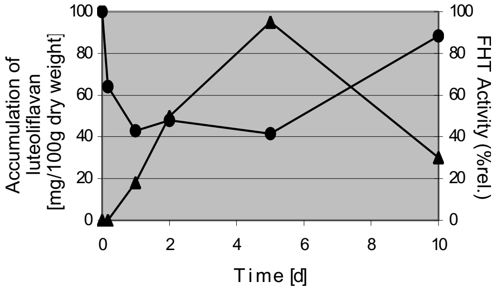

Fig. 4. Effect of prohexadione-Ca on young apple leaves. Time course of FHT activity [as% of the respective control value] (circles) and accumulation of luteoliflavan [mg/100 g dry weight] (triangles) in young apple leaves after treatment with prohexadione-Ca. In the control assays, the inhibitory effect of prohexadione-Ca was compensated by addition of 3.5 mM 2-oxoglutarate.

mann, 1988). In *Sorghum bicolor* 3-deoxyflavonoids act as phytoalexins (Lo *et al.*, 1999), and extracted from *Bridelia crenulata*, they have been reported to inhibit the growth of human pathogenic bacteria (Ramesh *et al.*, 2001). The key step in the biosynthesis of 3-deoxyflavonoids is the reduction of flavanones in position 4 to the corresponding flavan 4-ols (apiforol, luteoforol), which is catalyzed by flavanone 4-reductase (FNR) (Stich and Forkmann, 1988). Therefore, all plants possessing FNR activity have the potential to synthesize 3-deoxyflavonoids. Dihydroflavonol 4-reductase (DFR), an enzyme from the general flavonoid pathway, can exhibit a broad substrate activity and may also act as an FNR (Halbwirth *et al.*, 2003) (Fig. 5). This was also shown for apple and pear by cloning the DFR cDNAs and subsequent studies with the respective heterologously expressed enzymes (Fischer *et al.*, 2003). However, dihydroflavonols are the preferred substrates, compared to flavanones. Therefore, 3-hydroxyflavonoids are exclusively formed under natural conditions. Only after FHT inhibition, flavanones accumulate and are converted to luteoforol by DFR/FNR. Luteoforol *per se* is an unstable intermediate, which is rapidly converted to luteoliflavan (Fig. 3). It also shows strong chemical reactivity, which impedes extraction and analysis.

Since luteoforol could not be isolated from the leaves, it was chemically synthesized (Bate-Smith, 1969) for *in vitro* testing against *E. amylovora* and a large number of different bacterial and fungal pathogens. Relatively low concentrations (0.1 mM to 1 mM) of luteoforol showed strong antimicrobial effects (Fig. 6). Spore germination of *Venturia inaequalis* and other plant pathogenic fungi was even inhibited by concentrations as low as 0.01 mM (Roemmelt *et al.* 2003b). The broad toxic effect is an important feature since it makes selection for resistant pathogen variants highly unlikely. A number of constitutive as well as induced phenolic compounds such as luteoliflavan, flavanone 7-O-glucosides, phloretin derivatives, *p*-coumaroyl-glucose, caffeic acid, chlorogenic acid and 6″-*O*-*trans*-*p*-coumaroyl-eriodictyol 3′-O-glucoside did not show comparable antimicrobial effects *in vitro* as luteoforol did (Roemmelt *et al.*, 2002, 2003a). Thus, it is very likely that luteoforol represents the active principle induced by prohexadione-Ca and related compounds. Due to the relatively high efficacy, even steady state concentrations of luteoforol present in leaves can cause the effects observed. Prohexadione-Ca gives only a transient effect because it disintegrates relatively rapidly (half-life approximately 7–10 days) and is effective only in young shoots. Furthermore, the compound is translocated almost exclusively in the xylem and is, therefore, unlikely to be accumulated in fruits (Rademacher, 2000). Hence, effects on the flavonoid spectrum in fruits are highly unlikely. Analyses of fruits from trees treated with prohexadione-Ca gave proof to this assumption. In total, the strategy described provides a safe and effective control of secondary fire blight.

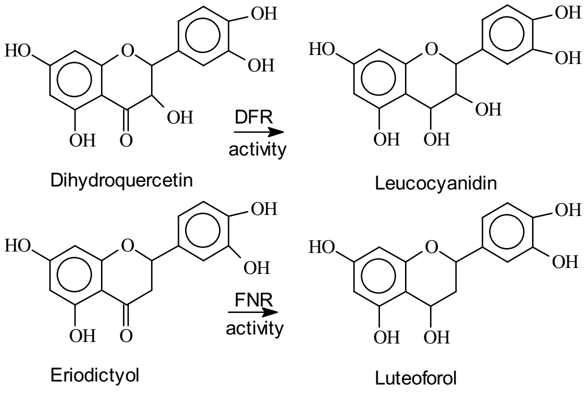

Fig. 5. Reactions catalyzed by the DFR/FNR.

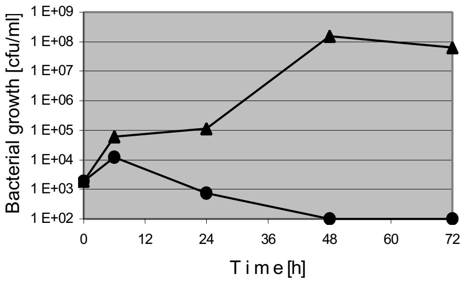

Fig. 6. Effect of luteoforol on *E. amylovora* (strain Ea 1/79, K. Geider). Liquid cultures were incubated with 2 Vol% of a 1 mM solution of luteoforol in ethyl acetate (circles) and with the respective amounts of ethyl acetate serving as controls (triangles). Bacterial population densities were assessed at different times by tenfold serial dilution on Luria agar plates. A similar effect was observed with other *E. amylovora* strains tested [Ea 286 (K. Geider), Ea 1540 (D. V. Dye), Ea 8865 (S. V. Thomson)].

Table I. Overview of FNR activity in various crop species.

Crop species	Luteoliflavan formation after ProCa treatment	DFR/FNR activity measurable	Heterologously expressed enzyme DFR/FNR activity
Grapevine (*Vitis vinifera*)	yes	yes/yes	yes/yes
Strawberry (*Fragaria* × *ananassa*)	yes	yes/yes	yes/yes
Kiwi fruit (*Actinidia* sp.)	yes	yes/yes	n.d.
Peach (*Prunus persica*)	yes	yes/yes	n.d.
Rose (*Rosa hybrida*)	yes.	yes/yes	yes/yes
Cranberry (*Vaccinium macrocarpum*)	yes	yes/yes	yes/yes
Elder (*Sambucus* sp.)	n.d.	yes/yes	n.d.
Plum (*Prunus domestica*)	n.d.	yes/yes	n.d.
Cherry (*Prunus avium*)	n.d.	yes/yes	n.d.

n.d.: not determined.

Effect on further crop plants

The natural formation of 3-deoxyflavonoid phytoalexins represents a principle of resistance which can be found only in a few plant species (Lo *et al.*, 1999; Viswanathan *et al.*, 1996). Likewise, the presence of luteoforol in plant tissues has also been reported only in a few isolated cases (Ramesh *et al.*, 2001; Bate-Smith, 1969; Viswanathan *et al.*, 1996; Bate-Smith and Creasy, 1969). By inhibition of FHT, luteoforol formation can be artificially induced in plants exhibiting FNR activity in the target tissues. The presence of FNR activity can be shown *in vitro* by enzymatic investigations or by formation of luteoliflavan after prohexadione-Ca application. In addition to apple and pear, FNR activity as well as luteoliflavan formation after prohexadione-Ca treatment could also be demonstrated in other important crops such as strawberry, grapevine, kiwi fruit, cranberry, rose and peach so far (Schlangen *et al.*, 2003; Gosch *et al.*, 2003) (Table I). Thus, prohexadione-Ca-triggered formation of 3-deoxyflavonoids with phytoalexin-like activity might control a wide range of fungal and bacterial diseases in several crop plant species.

First phytopathological investigations have shown that application of prohexadione-Ca significantly lowers progression of bacterial wilt (*Ralstonia solanacearum*) and reduces leaf lesions of bacterial speck (*Pseudomonas syringae* pv. *tomato*) and spot (*Xanthomonas vesicatoria*) on tomato plants in the greenhouse (Bazzi *et al.*, 2003a). Furthermore, a valuable protection of grapevine leaves to downy mildew (*Plasmopara viticola*) and grey mould (*Botrytis cinerea*) was observed (Bazzi *et al.*, 2003a,b). However, in other plant pathogen systems prohexadione-Ca was not effective, *e.g.* in controlling cereal downy mildew (*Blumeria graminis*) in wheat, or bacterial spot/canker (*X. arboricola* pv. *pruni*) on plum trees (Bazzi *et al.*, 2003a). In plum, this might be due to the low FNR activity observed in the leaves (Gosch *et al.*, 2003). Further work will be necessary to evaluate the spectrum of potential target plants and respective plant pathogens.

Conclusions

Since antibiotics, such as streptomycin, are progressively restricted from being used against plant pathogens, fruit growers are urged to rely on alternatives. Numerous investigations have clearly demonstrated that products containing acylcyclohexanediones represent a considerable alternative to antibiotics for the control of secondary fire blight (*Erwinia amylovora*) in pears as well as in apples. The underlying physiological mechanism of prohexadione-Ca has been elucidated and seems to be closely related to the natural defense observed in *Sorghum* and sugar cane. Additional experience is needed to fully exploit its potential for the use in several other host-pathogen systems.

Acknowledgement

Part of the investigations were supported by the EU Commission (QLK5-CT-1999-01583).

Bazzi C., Messina C., Tortoreto L., Bini F., Cecca G. S., and Stefani E. (2003a), Investigations on the possible use of abiotic and biotic elicitors in defence-related responses in plants. Eur. J. Hortic. Sci. **68**, 115–122.

Bazzi C., Messina C., Tortoreto L., Stefani E., Bini F., Brunelli A., Andreotti C., Sabatini E., Spinelli F., Costa G., Hauptmann S., Stammler G., Doerr S., Marr J., and Rademacher W. (2003b). Control of pathogen incidence in pome fruits and other horticultural crop plants with prohexadione-Ca. Eur. J. Hortic. Sci. **68**, 108–114.

Bate-Smith E. C. (1969), Luteoforol (3′,4,4′,5,7-pentahydroxyflavan) in *Sorghum vulgare*. Phytochemistry **8**, 1803–1810.

Bate-Smith E. C. and Creasy L. L. (1969), Luteoforol in strawberry leaves. Phytochemistry **8**, 1811–1812.

Costa G., Andreotti C., Bucchi F., Sabatini E., Bazzi C., Malaguti S., and Rademacher W. (2001), Prohexadione-Ca (Apogee(r)): Growth regulation and reduced fire blight incidence in pear. HortScience **36**, 931–933.

Fernando W. G. D. and Jones A. L. (1999), Prohexadione Calcium – a tool for reducing secondary fire blight infection. Acta Hort. **489**, 597–600.

Feucht W., Treutter D., and Christ E. (1996), Shot hole disease in sour cherry: defensive flavanol barriers. J. Plant Dis. Protect. **103**, 279–287.

Fischer T. C., Halbwirth H., Meisel B., Stich K., and Forkmann G. (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.

Forkmann G. and Heller W. (1999), Biosynthesis of flavonoids. Compr. Nat. Prod. Chem. **1**, 713–748.

Gosch C., Puhl I., Halbwirth H., Schlangen K., Römmelt S., Andreotti C., Costa G., Fischer T. C., Treutter D., Stich K., and Forkmann G. (2003), Effect of prohexadione-Ca on various fruit crops: flavonoid composition and substrate specificity of their dihydroflavonol 4-reductases. Eur. J. Hortic. Sci. **68**, 144–151.

Halbwirth H., Martens S., Wienand U., Forkmann G., and Stich K. (2003), Biochemical formation of anthocyanins in silk tissue of *Zea mays*. Plant Sci. **164**, 489–495.

Lo S. K., De Verdier K., and Nicholson R. L. (1999), Accumulation of 3-deoxyanthocyanidin phytoalexins and resistance to *Colletotrichum sublineolum* in *Sorghum*. Physiol. Mol. Plant Path. **55**, 263–273.

Maxson K. L. and Jones A. L. (2002), Management of fire blight with gibberellin inhibitors and SAR inducers. Acta Hort. **590**, 217–223.

Mayr U., Michalek S., Treutter D., and Feucht W. (1997), Phenolic compounds of apple and their relationship to scrab resistance. J. Phytopath. **145**, 69–75.

McManus P. S., Stockwell V. O., Sundin G. W., and Jones A. L. (2002), Antibiotic use in plant agriculture. Phytopathol. **40**, 443–465.

Momol M. T., Ugine J. D., Norelli J. L., and Aldwinckle H. S., (1999), The effect of prohexadione calcium, SAR inducers and calcium on the control of shoot blight caused by *Erwinia amylovora* on apple. Acta Hort. **489**, 601–605.

Rademacher W., Speakman J. B., Evans R. R., Evans J. R., Roemmelt S., Michalek S., Lux-Endrich A., Treutter D., Iturriagogaoitia-Bueno T., and John P. (1998), Prohexadione-Ca – a new plant growth regulator for apple with interesting biochemical features. In: Proceedings of the 25th Annual Meeting of the Plant Growth Regulation Society of America, Chicago, July 7–10, 1998 (Shafer W. E., ed.), pp. 113–118.

Rademacher W. (2000), Growth retardants: Effects on gibberellin biosynthesis and other metabolic pathways. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51**, 501–531.

Rademacher W. and Bucci T. (2002), New plant growth regulators: High risk investment? HortTechnology **12**, 64–67.

Ramesh N., Viswanathan M. B., Saraswathy A., Brindha P., Balakrishna K., and Lakshmanaperumalsamy P. (2001), Antibacterial activity of luteoforol from *Bridelia crenulata*. Fitoterapia **72**, 409–411.

Roemmelt S., Treutter D., Speakman J. B., and Rademacher W. (1999), Effects of prohexadione-Ca on the flavonoid metabolism of apple with respect to plant resistance against fire blight. Acta Hort. **489**, 359–363.

Roemmelt S., Peterek S., Treutter D., Rademacher W., Speakman J. B., Andreotti C., Costa G., Sponza G., Tortoreto L., Bazzi C., Halbwirth H., Zimmermann N., Stich K., and Forkmann G. (2002), Alteration of phenylpropanoid biosynthesis of fruit trees as a tool for enhancement of fire blight resistance. Acta Hort. **590**, 477–484.

Roemmelt S., Zimmermann N., Rademacher W., and Treutter D. (2003a), Unusual flavonoid routes induced by the dioxygenase inhibitor prohexadione-Ca in apple (*Malus* x *domestica*). Phytochemistry **64**, 709–716.

Roemmelt S., Fischer T. C., Halbwirth H., Peterek S., Schlangen K., Speakman J.-B., Treutter D., Forkmann G., and Stich K. (2003b), Effect of dioxygenase inhibitors on the resistance-related flavonoid metabolism of apple and pears: chemical, biochemical and molecular biological aspects. Eur. J. Hortic. Sci. **68**, 129–136.

Ruehmann S. and Treutter D. (2003), Effect of N-nutrition in apple on the response of its secondary metabolism to prohexadione-Ca treatment. Eur. J. Hortic. Sci. **68**, 152–159.

Schlangen K., Gosch C., Roemmelt S., Knott J., Fischer T. C., Treutter D., Forkmann G., Stich K., and Halbwirth H. (2003), Can prohexadione-Ca induce antimicrobial flavonoids in rose? Eur. J. Hortic. Sci. **68**, 137–143.

Snyder B. A. and Nicholson R. L. (1990), Synthesis of phytoalexins in *Sorghum* as a site-specific response to fungal ingress. Science **248**, 1637–1639.

Stich K. and Forkmann G. (1988), Biosynthesis of 3-deoxyanthocyanins with flower extracts from *Sinningia cardinalis*. Phytochemistry **27**, 785–789.

Styles E. D. and Ceska O. (1975), Genetic control of 3-hydroxy- and 3-deoxyflavonoids in *Zea mays*. Phytochemistry **14**, 413–415.

Viswanathan R., Mohanraj D., Padmanaban P., and Alexander K. C. (1996), Accumulation of 3-deoxyanthocyanidin, phytoalexins, luteolinidin and apigeninidin in sugarcane in relation to red rot disease. Indian Phytopathol. **49**, 174–175.

Yoder K. S., Miller S. S., and Byers R. E. (1999), Suppression of fire blight in apple shoots by prohexadione-calcium following experimental and natural inoculation. HortScience **34**, 1202–1204.