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Luteoforol, a flavan 4-ol, is induced in pome fruits by prohexadione-calcium and shows phytoalexin-like properties against *Erwinia amylovora* and other plant pathogens

Francesco Spinelli[1], John-Bryan Speakman[2], Wilhelm Rademacher[2], Heidi Halbwirth[3], Karl Stich[3] and Guglielmo Costa[1]
[1]*Department of Arboriculture, University of Bologna, viale Fanin 46, 40127Bologna, Italy; Phone: +39-51-2096447; Fax: +39-51-2096401; E-mail: spinelli@agrsci.unibo.it);* [2]*BASF Agricultural Center, Global Research Crop Protection PR/HB, Li 470 67114 Limburgerhof, Germany;* [3]*Institute of Technical BioScience, Technical University of Vienna, Getreidemarkt 9, 1060Vienna, Austria*

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Abstract

Treatments with prohexadione-calcium led to lowered incidence of fire blight, scab and other diseases in pome fruit trees and other crop plants. In addition to acting as a growth regulator, prohexadione-calcium interferes with flavonoid metabolism and induces the accumulation of the 3-deoxycatechin luteoliflavan in shoots of pome fruit trees. Luteoliflavan does not possess any remarkable antimicrobial activity. Therefore luteoforol, its unstable and highly reactive precursor, has been tested *in vitro* for its bactericidal and fungicidal activities. Luteoforol was found to be highly active against different strains of *Erwinia amylovora*, the causal agent of fire blight, and all other bacterial and fungal organisms tested. Phytotoxic effects were also observed in pear plantlets. The results obtained indicate that prohexadione-calcium induces luteoforol as an active principle with non-specific biocidal properties. It is proposed that luteoforol is released upon pathogen attack from its cellular compartment and inhibits further disease development by destroying pathogen cells as well as by inducing a hypersensitive-like reaction in the host plant tissue. This mechanism would be closely analogous to the one known for structurally related phytoalexins in sorghum.

Abbreviations: FHT – flavanone 3-hydroxylase; ProCa – prohexadione-calcium; TrixE – trinexapac-ethyl.

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora*, is the most devastating bacterial disease of apples (*Malus domestica*) and pears (*Pyrus communis*) (Vanneste, 2000). The bacterium penetrates the plant through the nectaries of flowers, but it may also enter leaves via wounds and natural openings in the cuticle. *Erwinia amylovora* can invade all parts of a tree solely by internal progression through the host tissues. Thus, a single infection can potentially kill a whole tree (Vanneste, 2000). Economic damages caused by fire blight are substantial and a severe outbreak can disrupt fruit production of an orchard for several years. Most of the economically interesting apple and pear varieties are very susceptible to fire blight. As a result, the disease significantly limits the area for

successful pome fruit production. It is likely that the negative economical impact of fire blight will increase since the disease is still spreading geographically into new apple- and pear-growing areas (Vanneste, 2000; Jock et al., 2002; van der Zwet, 2002). In addition, modern high-density orchards, trained to continuous hedgerow-systems, facilitate the disease diffusion (Longstroth, 2001). Furthermore, there are no products currently known, with the exception of streptomycin and some other antibiotics, which can effectively control fire blight (Momol et al., 1999; Psallidas and Tsiantos, 2000). On the other hand, the use of streptomycin becomes increasingly limited, due to the presence of streptomycin-resistant strains of *E. amylovora* and due to concerns about development of antibiotic resistance in human pathogens (McManus et al., 2002). For instance, a restricted use of streptomycin against fire blight is presently allowed in the European Union (as of April 2004) only in Germany, Greece, and The Netherlands. From the foregoing, it is obvious that new and reliable strategies to control fire blight are urgently needed.

An alternative and promising approach to control fire blight is the use of growth-regulating acylcyclohexanediones, such as prohexadione-calcium (calcium 3-oxido-4-propionyl-5-oxo-cyclohexene carboxylate; ProCa) and trinexapac-ethyl [4-(1′-cyclopropyl{1′-hydroxy}methylene)-3,5-dioxocyclohexane-1-carboxylate; TrixE].

These compounds reduce plant susceptibility to both bacterial and fungal diseases: it has been demonstrated that pre-treated pome fruit trees were less infected by *E. amylovora* (Winkler, 1997; Fernando and Jones, 1999; Momol et al., 1999; Yoder et al., 1999; Costa et al., 2001a; Aldwinckle et al., 2002; Bubán et al., 2002; Deckers and Schoofs, 2002; Maxson and Jones, 2002; Bazzi et al., 2003; Norelli et al., 2003) and *Venturia inaequalis* (Costa et al., 2001b; Spinelli et al., 2002). Whereas fire blight shoot infections can be controlled by ProCa at a relatively high degree of activity, its effect against flower infections requires further optimization (Bazzi et al., 2003). ProCa and trinexapac-ethyl exhibit a structural similarity to 2-oxoglutaric acid, thereby blocking 2-oxoglutarate-dependent dioxygenases, which are involved in the formation of growth-active gibberellins and in flavonoid metabolism (Rademacher, 2000). Work by Römmelt et al. (1999, 2000, 2002, 2003) and Halbwirth et al. (2002) with ProCa has demonstrated particularly that the inhibition of flavanone 3-hydroxylase (FHT) leads to an alternative pathway in phenylpropanoid metabolism and causes considerable changes in the spectrum of flavonoids and related compounds present in apple and pear shoot tissues (Figure 1). Most notably, luteoliflavan (3-deoxycatechin) is formed, which does not normally occur in apple and pear tissues. In young apple leaves, luteoliflavan starts to accumulate approximately 8 days after treatment, which would coincide with the onset of fire blight resistance. However, neither luteoliflavan nor several other induced phenolic compounds showed sufficiently inhibitory effects on *E. amylovora in vitro* to provide a clear explanation for the enhanced fire blight resistance after treatment with ProCa (Römmelt et al., 1999).

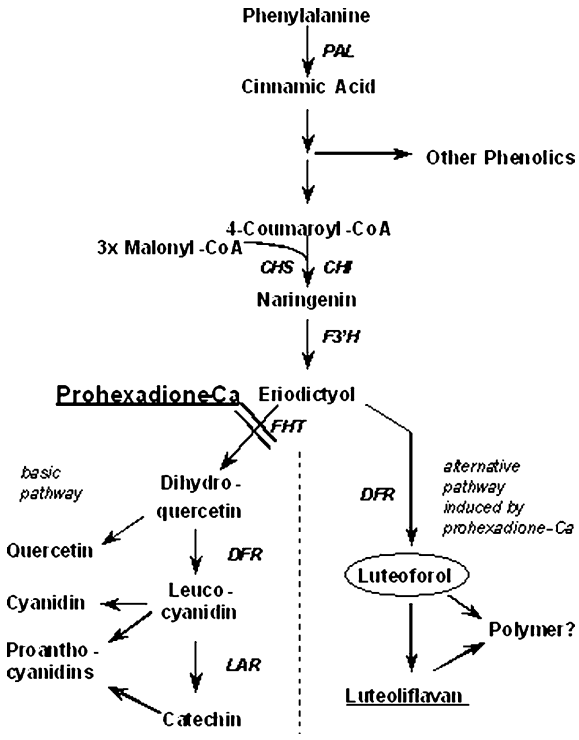

Figure 1. Effect of ProCa on flavonoid metabolism (after Halbwirth et al., 2002). CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3′H, flavonoid 3′-hydroxylase; FHT, flavanone 3-hydroxylase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia lyase.

The 3-deoxyanthocyanidin apigeninidin, luteolinidin and derivatives thereof, act as phytoalexins in *Sorghum bicolor* (Nicholson et al., 1988). Moreover, extracts of the *Euphorbiaceae Bridelia crenulata*, supposed to contain luteoforol, have been reported to inhibit the growth of human pathogenic bacteria (Ramesh et al., 2001), although the experimental evidence given in this contribution is scarce. Since 3-deoxyanthocyanidins appear to be of special interest as antimicrobials, special attention was paid to luteoforol, the unstable precursor of luteoliflavan. Its high reactivity, which results in rapid formation of polymeric structures, impedes direct detection and extraction from apple leaves (Halbwirth et al., 2002). However, the formation of luteoforol and related compounds by apple leaves after prohexadione-Ca application has, indirectly and directly, been shown in numerous publications (Römmelt et al., 1999, 2000, 2002, 2003; Halbwirth et al., 2002; Fischer et al., 2003)

This contribution describes the effect of luteoforol on a range of different bacteria and fungi, with and without relevance as plant pathogens. The bacteria *Pantoea agglomerans* and *Pseudomonas fluorescens* were specifically included in the investigation, since they could be of value as fire blight antagonists (Ishimaru et al., 1988; Johnson et al., 1993; Lindow et al., 1996; Vanneste et al., 1996). Growth-inhibitory effects of luteoforol on these microorganisms might influence their biological performance when co-applied with ProCa.

Materials and methods

Chemicals

Luteoforol was chemically synthesized following methods published by Bate-Smith (1969) and Stich and Forkmann (1988). Quantification was performed as described by Bate-Smith and Rasper (1969) using a calibration curve obtained by adding a defined quantity of (^{14}C)-labelled eriodictyol to a luteoforol synthesis. The activities of luteoforol and the phytoalexins luteolinidin and apigeninidin (both as their chloride salts from Extrasynthese, Lyon Nord, France) were tested on several bacterial (Table 1) and fungal (Table 2) species and strains. Ethyl acetate and methanol, in the amounts used as solvents of luteoforol, apigeninidin and luteolinidin, respectively, were also included in the assays. All chemicals tested and their respective concentrations are listed in Table 3.

Evaluation of bactericidal effect

The antibacterial activity of the different samples was tested on solid and liquid media. For the inhibition test on solid medium, plates with M9 minimal medium (Sambrook et al., 1989) were prepared and overlaid with a thin layer (2–3 mm) of minimal medium containing 10^7 cfu ml^{-1} of the different bacteria. Minimal medium M9 was chosen to mimic the lack of nutrients in the plant apoplast (Eastgate et al., 1997). Sterile disks of filter paper (5 mm diam.), each imbibed with 10 μl

Table 1. Bacterial organisms used for testing

Bacterial strains	Organisms	Type	Isolation
Ea 1/79	*Erwinia amylovora*	Plant pathogenic	K. Geider[a]
Ea 286	*Erwinia amylovora*	Plant pathogenic	K. Geider[a]
Ea 1540	*Erwinia amylovora*	Plant pathogenic	D.V. Dye[b]
Ea 8865	*Erwinia amylovora*	Plant pathogenic	S. V. Thomson[b]
Ea DCA289/01	*Erwinia amylovora*	Plant pathogenic	F. Spinelli
Eh 252	*Pantoea agglomerans*	Epiphytic	J. L. Vanneste[b]
Eh C9-1	*Pantoea agglomerans*	Epiphytic	C.A. Ishimaru[a]
Eh DCA269/01	*Pantoea agglomerans*	Epiphytic	F. Spinelli
Pf A506	*Pseudomonas fluorescens*	Epiphytic	S.E. Lindow[b]
Microbacterium sp.	*Microbacterium* sp.	Epiphytic	F. Spinelli
S. mutans 20742	*Streptococcus sobrinus*	Human pathogenic	DSMZ[c]

[a]Strains obtained from Prof. K. Geider, Max Planck Institut, Heidelberg, Germany.
[b]Strains obtained from Dr. Joel Vanneste, HortResearch, Hamilton, New Zealand.
[c]Strain obtained by DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Table 2. Fungal organisms used for testing

Organism	Spore concentration (spores ml^{-1})
Alternaria solani	2×10^4
Alternaria brassicicola	2×10^4
Botrytis cinerea	2×10^3
Colletotrichum graminicola	2×10^4
Colletotrichum lagenarium	2×10^4
Fusarium culmorum	2×10^3
Phytophthora infestans	2×10^4
Pyricularia oryzae	2×10^4
Venturia inaequalis	2×10^4

The differences among the spore concentrations used are due to the diversities in the growth and germination rate of the different organisms. All isolates were obtained from Dr. J.B. Speakman, BASF.

of 10 mM luteolinidin, 10 mM apigeninidin, 1 mM luteoforol, methanol or ethyl acetate were placed on the agar surface. Additionally, aqueous streptomycin sulphate (200 ppm) and sterile water were used in the same way as positive and negative controls, respectively. After a 24–48 h incubation at 27 °C, the presence of inhibition haloes was assessed. To quantify the effect of different concentrations of luteoforol, luteolinidin and apigeninidin on bacterial growth, the growth curves of the various bacterial species and strains cultured in liquid M9 medium were determined. Bacteria were grown in 96-well microtiter plates, incubated at 27 °C. Since the final incubation volume in each well was only 150 μl, no agitation was needed. After 0, 4, 24, 48, 72, and 96 h of incubation, the bacterial population densities were determined by plating 10-fold dilutions.

Evaluation of fungicidal effect

The effects of luteoforol, luteolinidin and apigeninidin were tested against several plant pathogenic fungi. Fungal spore suspensions of distinct concentrations (Table 2) were incubated in 96-well microtiter plates, with each well filled with 150 μl of *Aspergillus nidulans* minimal medium (DSMZ©) containing the test compound (Table 3). The plates were wrapped in aluminum foil and incubated at room temperature. Spore germination and mycelial growth were assessed daily with the aid of a stereomicroscope. *Venturia inaequalis* and *C. graminicola* were especially included in this investigation, since the first is an important pathogen for apple, whereas the second is a pathogen for sorghum where it induces the biosynthesis of apigeninidin and luteolinidin (Nicholson et al., 1988).

Fire blight control on immature pear fruit cores (IPF test)

An immature pear fruit (IPF) test was performed, according to Vanneste et al. (1996), to determine the efficacy of luteoforol to control fire blight symptom development. Immature pear fruits of cv. Conference were used for the test. The fruits were

Table 3. Samples tested for their ability to inhibit bacterial growth and fungal spore germination

Chemical	Concentration
Luteoforol	1 mM
	0.1 mM
	0.01 mM
Luteolinidin	10 mM
	1 mM
	0.1 mM
Apigeninidin	10 mM
Apigeninidin	1 mM
Apigeninidin	0.1 mM
Ethyl acetate 2.6%	Volume used to reach 1 mM luteoforol in the incubation
Ethyl acetate 0.26%	Volume used to reach 0.1 mM luteoforol in the incubation
Ethyl acetate 0.026%	Volume used to reach 0.01 mM luteoforol in the incubation
Methanol 3%	Volume used to reach 10 mM luteolinidin, apigeninidin in the incubation
Methanol 0.3%	Volume used to reach 1 mM luteolinidin, apigeninidin in the incubation
Methanol 0.03%	Volume used to reach 0.1 mM luteolinidin, apigeninidin in the incubation
Water	Used as negative control

surfaced-sterilized for 5 min in a bleach solution, which contained 3% active chlorine, followed by several rinses with sterile water. Twenty fruit cores were used for each test sample. Each core was inoculated with a 10 μl drop of a 4.2×10^7 cfu ml^{-1} culture of Ea 1/79. Immediately after inoculation, 1 mM luteoforol and streptomycin and water as controls were added as a 10 μl drop. The cores were incubated at 27 °C and high relative humidity in a growth chamber for 1 week. Symptom development was assessed daily.

Fire blight control on micropropagated plants

Finally, the effects of luteoforol, luteolinidin and their respective solvents were tested *in vitro* against fire blight infection on rootless micropropagated cv. Williams pear plantlets. Two or three microcuttings were transferred to sterile vials, which contained 2 ml of an aqueous solution of luteoforol (1 mM), luteolinidin (10 mM) or ethyl acetate, methanol and water as controls. The same volume of ethyl acetate and methanol was added as for that used to apply luteoforol and luteolinidin, respectively. The vials were closed with cotton wool to allow transpiration and uptake of the aqueous solution. The plants were maintained at 24 °C for 96 h and successively inoculated by cutting the leaf tips with scissors dipped into a bacterial suspension of Ea 1/79 (4.2×10^7 cfu ml^{-1}).

Results

The inhibition test on M9 minimal medium plates was used to screen the efficacy of luteoforol and the phytoalexins luteolinidin and apigeninidin on the growth of different strains of *E. amylovora* and other bacteria. In all cases, luteoforol gave a faint, but distinct, inhibition halo. The most significant effect was observed in the plates inoculated with Ea 1540 (Figure 2). This observation suggests that different strains of *E. amylovora* possess different degrees of sensitivity to luteoforol. Both luteolinidin and apigeninidin were not effective in this kind of test, although applied at a 10-fold higher dosage. Low diffusion in the agar medium or chemical interactions with the filter paper or with constituents of the medium could be possible

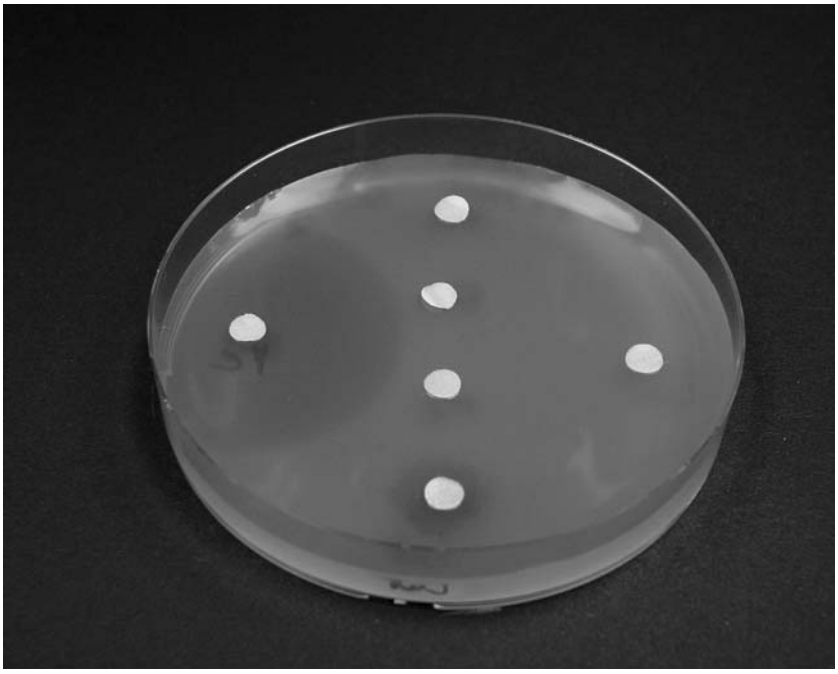

Figure 2. Inhibition test on minimal medium. On the left: the positive control (streptomycin 0.14 mM); on the bottom: luteoforol (1 mM); on the right: luteolinidin (10 mM); on the upper end: the negative control (water). The other disks contained methanol and ethyl acetate. The plate had been inoculated with *E. amylovora* 1540.

explanations. Neither methanol nor ethyl acetate had an effect on bacterial growth.

In liquid cultures, luteoforol inhibited the growth in all bacterial organisms tested. Its activity was more than 10-times higher than that of luteolinidin (Figures 3). Apigeninidin showed an effect similar to luteolinidin (data not shown). The effect of luteoforol is concentration-dependent and even at the lowest concentration tested (0.01 mM) activity could be observed. On *Ea*1/79, *Ea*8865, *Pf*A506, *Microbacterium* sp., luteoforol reduced the bacterial populations under the detectable threshold. Methanol and ethyl acetate were practically without activity in the different incubations.

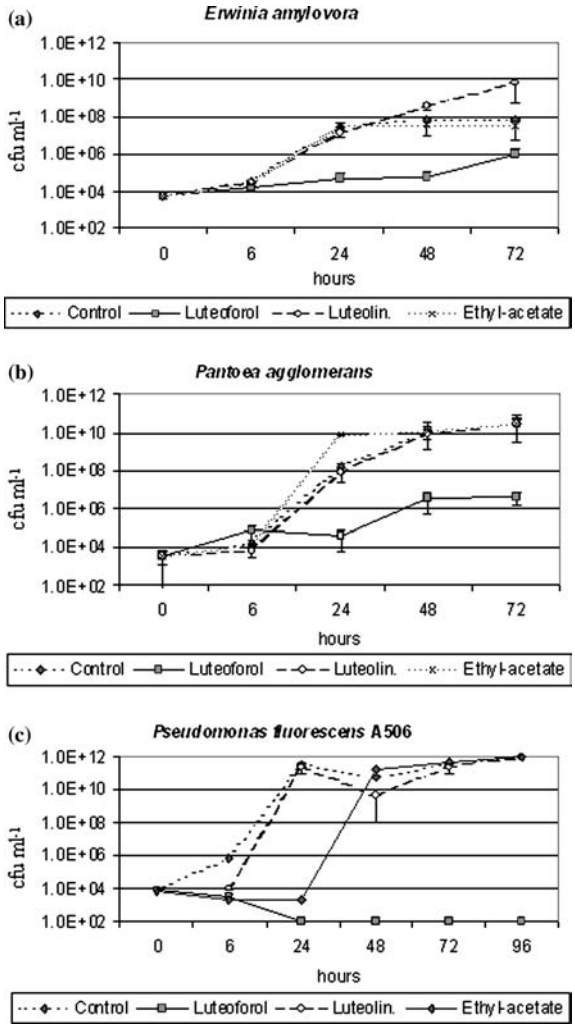

Figure 3. Bactericidal effect of luteoforol 1 mM against *Erwinia amylovora* Ea DCA289/01 (a), *Pantoea agglomerans* Eh DCA269/01 (b) and *Pseudomonas fluorescens Pf*A506 (c). Among each bacterial species, all the strains considered in the experiments reacted in a comparable way to the tested compounds. Standard error is shown.

As with bacteria, the effects of luteoforol and the standard phytoalexins, luteolinidin and apigeninidin, were also tested on several phytopathogenic fungi. When applied at its highest dosage (1 mM), luteoforol completely inhibited spore germination of all fungi examined. Again, the efficacy of luteoforol was concentration-dependent and its activity clearly surpassed that of luteolinidin. However, both luteolinidin and apigeninidin formed crystals in the 1 mM solution, and this crystallization was even more pronounced at 10 mM, which made direct observation of the conidial germination very difficult. Both phytoalexins delayed spore germination at 10 mM at similar degrees of activity. This delay in germination was apparent for approximately 36 h; mycelial growth was profuse in all wells after 72 h. Methanol did not affect spore germination, whereas a slight inhibition was observed with ethyl acetate at the highest concentration. The results obtained are shown in Figures 4 and 5.

The efficacy of luteoforol to control fire blight symptom development was assessed in the IPF test. Luteoforol application reduced fire blight symptom development but did not reach the activity of streptomycin (Figure 6). Luteoforol induced phytotoxicity when applied to micropropagated pear plantlets. The treated plantlets died after 12 h. Therefore, this treatment did not allow *in planta* investigations on the effect of luteoforol on fire blight infection. None of the other compounds tested gave a similar effect.

Discussion

Chemical analyses of apple shoot tissues treated with ProCa revealed the occurrence of flavonoids, which are not commonly formed in apple. In particular, luteoliflavan (3-deoxycatechin) accumulated, which has not been detected in rosaceous species so far (Römmelt et al., 1999, 2002). The experiments reported here provide evidence that luteoforol, which is the immediate precursor of luteoliflavan, could be the active compound responsible for enhanced pathogen resistance after ProCa treatment.

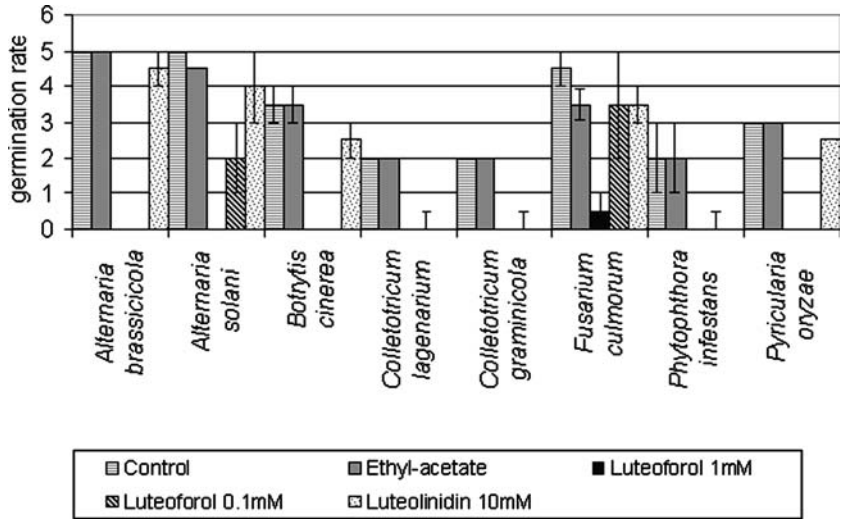

Figure 4. Effects of luteoforol, luteolinidin and their solvents on spore germination of different phytopathogenic fungi. The data shown were recorded after 6 days of incubation. In case of *A. brassicicola, B. cinerea*, *C. lageniarium*, *C. graminicola, P. infestans*, *P. orizae* and *V. inaequalis* the germination rate was zero with both luteoforol concentrations tested. Standard error is shown.

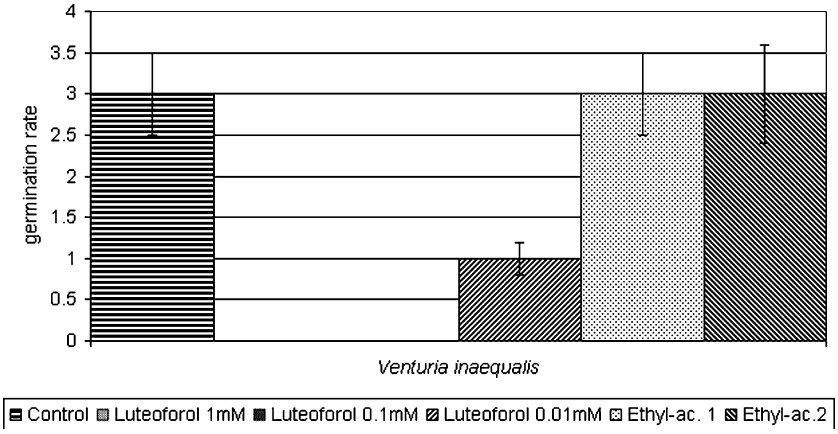

Figure 5. Fungicidal effect of luteoforol against *V. inaequalis*. At the highest dosage, luteoforol completely inhibited the spore germination of this phytopathogenic fungus. At concentrations of 1 and 0.1 mM, the germination rate was zero. Standard error is shown.

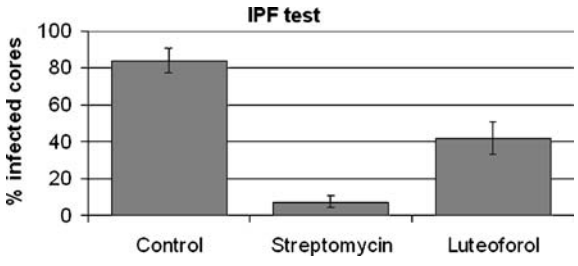

Figure 6. Effects of streptomycin and luteoforol on reducing symptoms development in IPF test. Standard error is shown.

The antimicrobial activity of luteoforol is more than 10 times higher than that of the structurally related phytoalexin luteolinidin. Furthermore, relatively high concentrations of luteoliflavan, which is formed from luteoforol, are detected in treated shoots of pome fruits (Römmelt et al., 2000). Therefore, without having specific information yet on luteoforol pool sizes and localization inside cells, it appears likely that its induction by ProCa represents the main cause of the reduced pathogen incidence observed. Luteoforol is active

against both bacteria and fungi. This broad and obviously unspecific biocidal effect is an important feature since it would make resistance formation in pathogens highly unlikely. Luteoforol is also active against *P. agglomerans* and *P. fluorescens*, which represent valuable biological control agents against fire blight. Nevertheless, the combined use of ProCa and biological control is not necessarily impeded, since bacterial antagonists do not usually need to penetrate into plant tissues to become effective (Vanneste, 2000).

Even at the lowest dosage tested (1 mM), luteoforol caused phytotoxicity in micropropagated pear plantlets. Since luteoforol was taken up passively by transpiration, it is likely that its concentration in the plant apoplast was even much lower than 1 mM. Therefore it can be hypothesized that endogenously formed luteoforol is normally compartmentalized inside plant cell organelles, such as the vacuole or specific vesicles, minimizing its toxic effect. During infection, *E. amylovora* or other pathogens destroy the integrity of cell membranes (Sjulin and Beer 1978; Youle and Cooper, 1987), which would lead to the release of luteoforol. In the apoplast next to the site of infection, luteoforol will then act both against pathogens and plant cells, thus restricting pathogen spread to the immediate neighbouring plant cells. This mechanism would be closely related to the one found for the structurally related phytoalexins apigeninidin and luteolinidin in sorghum (Nicholson et al., 1987).

The increased resistance observed in pome fruits after application of ProCa can also be partially due to other effects of ProCa on plant metabolism. For example, ProCa acts as a growth retardant and affects morphological and histological parameters in leaves and shoots. Nevertheless, the application of other growth retardants, such as chlormequat chloride and paclobutrazol, which leads to comparable effects on plant morphology and histology, does not result in resistance induction (Costa et al., 2001a, b). Therefore, it appears likely that the morphological and histological changes, such as shoot growth reduction, lignification, modification of shoot orientation and canopy density are *per se* not sufficient to explain the reduced disease incidence.

Products containing ProCa are promising tools to control vegetative growth in apple and pear (Rademacher, 2000). In addition, they are effective in decreasing host susceptibility in a number of host-pathogen systems (Bazzi et al., 2003). Therefore in contrast to acibenzolar-*S*-methyl and other inducers of systemic acquired resistance, ProCa can simultaneously be used for two different purposes. The compound has very favourable toxicological and eco-toxicological features, a low propensity for crop residues and no health risk for user or consumer is indicated (Winkler, 1997). It should also be noted in this context that fruits from trees treated with ProCa showed a regular flavonoid composition at harvest (D. Treutter, Technical University of Munich, Germany, pers. comm.), due to the relative rapid disintegration of the compound and its type of translocation (Rademacher and Kober, 2003). Finally, on account of the fact that ProCa induces plant resistance, the occurrence of resistance to this compound in phytopathogenic fungi and bacteria is very unlikely. Therefore, when integrated into spray programme, the compound may also be of value for reducing resistance formation against modern fungicides and antibiotics. In conclusion, it can be stated that ProCa allows a new, promising and safe method for resistance induction in pome fruit trees against fire blight and other bacterial and fungal diseases.

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