The effect of Regalis® (prohexadione calcium) on the reduction of fire blight *(Erwinia amylovora)* severity in apple trees

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Summary: Due to the lack of effective and non-phytotoxic materials for control of the blossom and shoot blight phase of fire blight in pome fruit trees, two novel control strategies have emerged: shoot growth retardation by bioregulators and applying resistance inducer compounds. Prohexadione calcium (ProCa) is the active ingredient of the bioregulator Regalis® registered in several European countries. The reduction of shoot growth elongation is the most obvious effect of ProCa. Furthermore, it causes significant changes in the spectrum of flavonoids and their phenolic precursors, leading to the considerable reduction of susceptibility to fire blight.

In Poland, potted one-year-old apple trees of cvs. Gala Must grafted on M.26 and Sampion on M.9 (in 2001) as well as Gala Must on P.60 (in 2002) were treated with Regalis® at a range of concentration of 250, 150 or 150 + 100 ppm, respectively. The inoculation of shoots was made with the strain No.691 of *E. amylovora* (10^7 cfu/ml), on the 7th and 21st day after treatments with Regalis. In Hungary, during the years of 2002 and 2003 one-year-old container grown apple trees of the cvs. Idared/M.9 and Freedom/M.9 were treated with the prohexadione-Ca, the active ingredient of Regalis® 100, 150 or 200 ppm, two weeks before inoculation with the Ea1 strain of *E. amylovora* (10^7 cfu/ml).

In Poland, the suppression of fire blight in shoots reached up to 80%, depending on concentration and application time of Regalis®. In Hungary, the effect of prohexadione-Ca treatments, determined by the length of necrotic lesion developed, proved to be better than that of streptomycin used for comparison.

Key words: fire blight, *Erwinia amylovora*, Regalis®, prohexadione-Ca, resistance inducers, apple, preventive plant protection

Introduction

Fire blight is one of the most serious diseases affecting apple and pear trees as well as many other *Rosaceae* plants (*van der Zwet & Keil* 1979; *van der Zwet & Beer,* 1992; *Sobiczewski et al.* 1997; *Bonn & van der Zwet,* 2000). It has been known for over 200 years; the first foci of the disease were found on the East coast of USA. Presently it has occurred in over 40 countries of the world (*van der Zwet,* 2002). Poland was one of the first countries of continental Europe where fire blight was discovered in 1966. Now, it is detected in most apple and pear production regions of this country. Fire blight was identified (*Hevesi,* 1996) 30 years later in Hungary as the 36th country in chronological order regarding the fire blight history all over the world. It causes economical losses in many fruit production regions mainly located East of Danube river (*Németh,* 2003).

Fire blight attacks all organs of the aboveground part of the host plants often leading to their death. In some years plants covering hundreds of hectares were destroyed. The severity of the disease is a matter of its destructive character, ability to systemic distribution in the plants and rapid dissemination in nature as well as the lack of effective control methods. At present its control is directed to the integration of various activities including sanitation, chemical treatments, selection of resistant cultivars and soil management (*Steiner,* 2000). The most important chemicals recommended against fire blight are copper compounds and antibiotics (*Psallidas and Tsiantos,* 2000). However, their use has some limitations: copper usually causes phytotoxicity, especially on apple fruits, and streptomycin is not allowed for plant protection in many countries. In some countries (like USA, New Zealand, Israel) its extensive use has led development the resistance of *Erwinia amylovora* to this antibiotic (*McManus et al.,* 2002).

Recently, new control possibilities of fire blight based on resistance induction to the disease have been developed. Some newly registered materials, like: fosetyl-Al (Aliette), harpin proteine (Messenger®), benzothiadiazole (Bion® or Actigard®) and prohexadione calcium (Regalis® or Apogee®) are presumed to trigger plant defence mechanisms (*Momol et al.,* 1999; *Steiner,* 2000; *Aldwinckle et al.,* 2002, *McManus et al.,* 2002; *Maxson and Jones,* 2002). The best results were noticed with ProCa. It gives an efficacious

control of shoot growth elongation by inhibiting formation of highly active gibberellins from inactive precursors (*Evans et al.* 1999, *Rademacher* 2000). The 2-oxoglutaric acid-dependent dioxygenases, as in the biosynthesis of gibberellins, are involved in the flavonoid metabolism too and cause considerable changes in the formation of flavonoids and their phenolic precursors (*Roemmelt et al.*, 2002). It is suggested, that induction of physiological resistance against fire blight, or apple scab on shoots of apple should be based on these qualitative and quantitative changes in flavonoids or other phenylpropanoids (*Rademacher*, 2000).

ProCa treated apple trees were significantly less infected by *Erwinia amylovora* (reviewed by *Bubán et al.*, 2003b) and a reduced incidence of fire blight was also found in pears (*Costa et al.* 2001, *Bubán et al.* 2002a, *Deckers and Schoofs* 2002).

From the environmental saving points of view, ProCa represents reduced risk to human health and environment, i.e. prohexadione-Ca is a candidate for "reduced risk" registration by the U.S. Environmental Protection Agency; EPA (*Winkler*, 1997). Its acute oral toxicity is LD_{50}> 5000 mg/kg, and no carcinogenic, mutagenic or teratogenic effects were observed. Animal and plant metabolism studies show that the active ingredient is bioconverted to naturally occurring products.

Materials and methods

A) Poland

The studies were conducted in 2001–2002 in the experimental greenhouse of the Research Institute of Pomology and Floriculture in Skierniewice (RIPF) on potted one-year-old apple trees. In 2001 trees of cvs. Gala Must/ M.26 and Sampion/ M.9 and in 2002 trees of cv. Gala Must/ P.60 were used. During entire period of growth the trees were fertilized once a week with universal liquid fertilizer Florovit in dose of 5 ml/l. Protection against diseases and pests was performed according to present recommendations for orchards.

Treatment of trees with Regalis®:

The trees were sprayed once or twice with water suspension of Regalis® (Regalis 10WG with a.i. 10% of prohexadione calcium) using following concentrations: 0.25, 0.15 or 0.15 and 0.1%. The first spraying was done when young green shoots reached the length of 9–18 cm for Gala Must/M.26, 7–14 cm for Sampion/M.9 and 9–22 cm for Gala Must/ P.60.

Preparation of *E. amylovora* suspension and inoculation of trees:

Strain No. 691 of *E. amylovora* from collection of Laboratory of Bacteriology of RIPF was used for shoot inoculation. This strain was originally isolated in 1988 from apple shoots and stored in glycerol at temperature of –70 °C. For inoculum preparation bacteria had been passaged twice on NAS medium (2.3% Difco Nutrient Agar with 5% sucrose). The concentration of bacteria in water was adjusted to 10^7 cfu/ml on spectrocolorymeter (Specol; length of wave 630 nm) and serial dilution plating method on NAS.

The inoculations of apple shoots were made on 7th or 21st day after treatment with Regalis® by cutting-off the shoot tips under the first undeveloped leaf using scissors previously immersed in inoculum. After inoculation the shoot tips were covered with plastic bags for 48 hours to protect desiccation of inoculum.

Evaluation of shoot infestation with fire blight:

The measurements of total length of shoots and the length of their necrotized parts were made after 2, 4 and 6 weeks from the inoculation. The diseased part of given shoot was expressed as the percentage of its total length.

The results were subjected to an analysis of variance. Each combination consisted of 16 trees (4 per replication). For separation of means the Duncan's multiple t-test at 5% significance was used.

B) Hungary

One-year-old container-grown trees of two apple cultivars Freedom/M.9 and Idared/M.9 known to be tolerant and susceptible to fire blight, respectively, were used in the trials on shoot infection. The plants were cultivated in a shaded glasshouse from June to September in 2002 as well as in 2003. Water and nutrients were supplied when necessary. Vigorously growing shoots were selected for the experiments to assure a strong challenge.

Inoculation of the youngest 2–3 leaflets around the shoot tip was done by cutting through them with scissors immersed in the inoculum suspension (10^7 cfu/ml) of the Ea1 strain of *E. amylovora*. Inoculated shoots, one to three in each tree, were covered with transparent plastic bags for 24 hours. To simulate natural conditions of the nurseries, the treatments were started when the trees have reached their height of 65–70 cm. Therefore, while evaluating treatment effects the necrotic lesion length starting at the shoot tips on inoculated shoots was not expressed as the percentage of their total length. There were two separate series of experiments, in July and September of 2002, repeated in July of 2003, including 10 trees/cultivar for each treatment and date.

Regalis® (BASF) and streptomycin as Streptomycin 20WP (Summit-Agro Hungaria) were applied at a timing appropriate to their mode of action and in doses corresponding to the usual rates when sprayed them under field conditions (details are given in *Tables 6 to 9*).

All data were elaborated on an analysis of variance using One-Way ANOVA with SPSS 9, and the means were compared using Tukey's multiple range test at P=0.01.

Results

A) Poland

Treatments of apple trees with Regalis® significantly suppressed development of fire blight on their vegetative

shoots. In experiment performed in 2001 on Gala Must, the intensity of disease determined 2 weeks after shoots inoculation with *E. amylovora* was reduced in about 50%, independent of inoculation time after Regalis® treatment *(Table 1)*. However, 4 weeks after inoculation such evaluation showed that fire blight was reduced in 34–37% depending on time of inoculation. It should be pointed out that the length of necrotized part of shoots was much smaller, both on treated and non treated trees, when inoculations were performed 21 days after treatment with Regalis®.

The suppression of fire blight on Sampion shoots inoculated with *E. amylovora* 7 days after Regalis® treatment reached about 40 and 50% when determined 2 and 4 weeks after inoculation, respectively *(Table 2)*. However, the efficiency was much higher (i.e. 76 and 67%, respectively, when the shoot inoculation was done 21 days after the ProCa treatment. Similarly to Gala Must/M.26, and both on treated and non treated trees of the cv. Sampion, the diseased part of shoots was much shorter when they were inoculated after longer period of time at treatment with Regalis®.

In the experiment performed in 2002 non significant differences in suppression of fire blight have been found between treatments with 0.15 and 0.25% of Regalis® when inoculation of shoots with *E. amylovora* was done 7 days after those treatments *(Table 3)*. However, when inoculation was made 21 days after treatments with both concentrations of this preparation, there were obvious differences in disease intensity *(Table 4)*. Evaluation performed 2 weeks after inoculation showed that reduction of necrotized part of shoots ranged from 91 to 71% depending on applied concentration of Regalis®. These trends were also observed 6 weeks after inoculation, although, suppression of fire blight was about 60% in case of lower concentration of Regalis®.

Two applications of Regalis® at concentrations of 0.15% and 0.1% with an interval of 7 days, followed by inoculation of shoots with *E. amylovora*, reduced disease severity to about 80% *(Table 5)*. This rate of reduction was stable during entire period of experiment, as compared to control. However, it gradually decreased, from 87 to 66%, on shoots inoculated 21 days after last treatment with prohexadione-Ca.

B) Hungary

During the first year of investigation the length of strikes, recorded on the 7th and 14th day after inoculation in Regalis® pre-treated shoots of the cv. Idared was not related to its doses applied *(Table 6)*. It was found that length of lesions on Regalis® treated shoots measured on 7th day after inoculation constituted only 19–25% of those on shoots treated with streptomycin and on the 14th day they were longer constituting 38–42%. As for the cv. Freedom, the length of strikes (compared to those of 'Idared') was slightly shorter after 7th day (16–18%) and slightly longer on the 14th day after inoculation (42–53%). While comparing the necrotic lesion length in Regalis® treated shoots to ones in non-treated (only inoculated) shoots, these differences were much higher.

The efficacy of treatments with ProCa in the second experiment *(Table 7)* was more pronounced than in the first one *(Table 6)*. For example, in trees of the cultivar Idared treated with the higher dose of Regalis®, after 1 and 3 weeks

Table 1 The effect of Regalis® on severity of fire blight in shoots of apple cv. Gala Must/M.26 Skierniewice, 2001

Treatment	Percentage of necrotized part of shoots inoculated with *Erwinia amylovora* after			
	2 weeks		4 weeks	
	A*	B*	A*	B*
Check (inoculation only)	82.1 b	45.5 b	98.8 b	59.1 b
Regalis® 0.25%	41.6 a	22.6 a	71.8 a	38.7 a

*Shoots were inoculated 7 days (A) or 21 days (B) after treatment with Regalis®;
statistical analysis were made separately for each time of evaluation and shoots inoculation.

Table 2 The effect of Regalis® on severity of fire blight in shoots of apple cv. Sampion/M.9 Skierniewice, 2001

Treatment	Percentage of necrotized part of shoots inoculated* with *Erwinia amylovora* after			
	2 weeks		4 weeks	
	A*	B*	A*	B*
Check (inoculation only)	39.0 b	14.2 b	66.7 b	45.9 b
Regalis® 0.25%	23.7 a	3.4 a	34.6 a	15.3a

*see Table 1

Table 3. Severity of fire blight in shoots of apple cv. Gala Must/P.60 treated with Regalis® 7 days before inoculation with *Erwinia amylovora* Skierniewice, 2002

Treatment	Percentage of necrotized part of shoots inoculated* with *Erwinia amylovora* after		
	2 weeks	4 weeks	6 weeks
Check (inoculation only)	72.8 b	92.2 b	93.6 b
Regalis® 0.25%	21.8 a	22.6 a	23.4 a
Regalis® 0.15%	19.6 a	21.3 a	20.3 a

*Statistical analysis was made separately for each time of shoot inoculation and evaluation

Table 4. Severity of fire blight in shoots of apple cv. Gala Must/P.60 treated with Regalis® 21 days before inoculation with *Erwinia amylovora* Skierniewice, 2002

Treatment	Percentage of necrotized part of shoots inoculated* with *Erwinia amylovora* after		
	2 weeks	4 weeks	6 weeks
Check (inoculation only)	56.4 c	72.9 c	75.6 c
Regalis® 0.25%	4.9 a	5.3 a	10,1 a
Regalis® 0.15%	16.3 b	25.6 b	31.1 b

*see Table 3

Table 5. Severity of fire blight in shoots of apple cv. Gala Must/P.60 pretreated with Regalis® at two concentrations Skierniewice, 2002

Treatment	Percentage of necrotized part of shoots inoculated with *Erwinia amylovora* after					
	2 weeks		4 weeks		6 weeks	
	A*	B*	A*	B*	A*	B*
Check (inoculated)	64.6 b	49.6 b	79.6 b	54.0 b	82.9 b	56.2
Regalis® 0.15% +0.1%⊙	14.1 a	6.5 a	17.3 a	17.7 a	17.1 a	19.2 a

*see table 1;
⊙Regalis® was applied at 2 concentrations with the interval of 7 days

Table 6. Length of cortical lesions caused by *Erwinia amylovora*[1] in shoots of Regalis® pre-treated apple trees Újfehértó, 2002 July (Bubán et al., 2003b)

Cultivars and treatments	Length (mm) of cortical lesion on the	
	7th	14th
	day after inoculation	
'Idared'/ M.9		
Check (inoculation only)[1]	140 c	174 c
Regalis® [2] 100 g/100 L	27 a	54 a
Regalis® [2] 200 g/100 L	20 a	61 a
Streptomycin 20 WP[3], 0.5 kg/ha	106 b	144 bc
'Freedom'/M.9		
Check (inoculation only)[1]	233 c	277 c
Regalis® [2] 100 g/100 L	28 a	70 a
Regalis® [2] 200 g/100 L	24 a	88 a
Streptomycin 20 WP[3], 0.5 kg/ha	154 b	166 b

[1]Inoculation with Ea1 strain of *Erwinia amylovora*, 10^7 bacteria/ml
[2]Treatment with Dash® HC 13 days before inoculation,
[3]Treatment on the same day several hours before inoculation

Table 7 Length of cortical lesions caused by *Erwinia amylovora*[1] in shoots of Regalis® pre-treated apple trees Újfehértó, 2002, September (Bubán et al., 2003b)

Cultivars and treatments	Length (mm) of cortical lesion on the		
	9th	16th	23th
	day after inoculation		
'Idared'/ M.9			
Check (inoculation only)[1]	68 b	100 b	115 b
Regalis® [2] 100 g/100 L	10 a	38 a	44 a
Regalis® [2] 200 g/100 L	3 a	18 a	21 a
Streptomycin 20 WP[3], 0.5 kg/ha	57 b	81 b	104 b
'Freedom'/M.9			
Check (inoculation only)[1]	68 b	133 b	141 b
Regalis® [2] 100 g/100 L	6 a	19 a	25 a
Regalis® [2] 200 g/100 L	3 a	17 a	18 a
Streptomycin 20 WP[3], 0.5 kg/ha	56 b	83 b	99 ab

*see table 6

Table 8 Length of cortical lesions caused by of *Erwinia amylovora*[1] in shoots of Regalis® pre-treated apple trees Újfehértó, 2003, July

Cultivars and treatments	Length (mm) of cortical lesion on the		
	7th	14th	21th
	day after inoculation		
'Idared'/ M.9			
Check (inoculation only)[1]	93 c	201 b	242 b
Regalis® [2] 150 g/100 L	4 a	84 a	105 a
Streptomycin 20 WP[3], 0.5 kg/ha	56 b	175 b	203 b
'Freedom'/M.9			
Check (inoculation only)[1]	77 b	148 bc	198 b
Regalis® [2] 150 g/100 L	14 a	69 a	109 a
Streptomycin 20 WP[3], 0.5 kg/ha	83 b	188 b	229 b

* see table 6

following inoculation the length of cortical lesion was only 5 and 20% respectively of those on trees treated with streptomycin.

At the time of the experiment (conducted in September) the trees still had actively growing shoot tips, but tissues of leaves and shoot axes were more mature than they had been in July (at the period of the first experiment). Due to the coincidence of the natural hardening of those tissues with the ProCa-induced alterations in the metabolic pathway of phenolics, the shoots had become less susceptible to fire blight. This assumption is supported by the fact, that in shoots, which were non-treated (only inoculated), necrotic lesions were much longer in the first experiment (July, *Table 6*) than in the second one (September, *Table 7*).

Results of the experiments carried out in 2003 present the convincing efficiency of ProCa for both apple cultivars investigated *(Table 8)*.

It is known, that the length of cortical lesion is not the only parameter to estimate how the disease evolved gradually within the shoot. While cutting the shoots alongside, the length of the brown discoloration of vascular bundles was consequently longer than that of the cortical lesions outside *(Table 9)*. The length of inner symptoms was distinctly shorter in shoots pre-treated with ProCa than in inoculated and otherwise untreated shoots.

Discussion

Our experiments showed that application of Regalis® on one-year-old trees of various apple cultivars caused significant reduction of fire blight severity in shoots. Depending on cultivar, year and place of study as well as time of evaluation, the limitation of disease ranged from over 30 to over 90% as compared to control. These results confirmed our experiences from the field trials (*Bubán et al.*, 2002b, 2003a). Also other authors obtained satisfactory and encouraging results on apples and pears growing both, in greenhouse and orchards (*Winkler*, 1997; *Yoder et al.*, 1999; *Costa et al.*, 2001; *Glozer et al.*, 2002; *Aldwinckle*, et al., 2002; *Maxson & Jones*, 2002). It should be pointed out that Regalis® appeared to be valuable tool for controlling secondary fire blight in pears as reported by *Deckers and*

Table 9 Length of cortical lesions[1] and inner symptoms of vascular bundles[2] caused by *Erwinia amylovora* in shoots of Regalis® pre-treated apple trees Újfehértó, on 4th of August, 2003

Cultivars and treatments	Length (mm) of	
	Cortical lesions	Inner symptoms
'Idared'/ M.9		
Check (inoculation only)[3]	275 b	283 b
Regalis®[4] 150 g/100 L	114 a	160 a
'Freedom'/M.9		
Check (inoculation only)[3]	217 b	226 ab
Regalis®[4] 150 g/100 L	128 a	157 a
Streptomycin 20 WP[5], 0.5 kg/ha	272 b	280 b

[1] visible outside
[2] brownish discoloration is visible after cutting the shoot axes lengthwise
[3] Inoculation with Ea1 strain of *Erwinia amylovora*, 10^7 cfu/ml
[4] Treatment with Dash® HC on June 25,
[5] Treatment on the same day but several hours before inoculation (July 9),

Schoofs (2002) and by *Bubán et al.* (2002a). Application of this compound at the end of apple bloom period caused, besides of disease reduction, a significant decrease of *E. amylovora* survival ability on leaves (*Fernando and Jones,* 1999). It also reduced rat-tail bloom in apple trees and subsequently late season fire blight, but did not decline early season infection (*Holtz et al.,* 2002). It was documented that movement of endophytic populations of this bacterium in Regalis® treated and inoculated pear shoots was shorter than in the control (*Roemmelt et al.* 2002). However, the post-infection treatment with ProCa is of no practical value in reducing fire blight symptoms in apple trees (*Schupp et al.,* 2002).

Results of our studies showed that spraying of trees with water suspension of Regalis® before inoculation with *E. amylovora* was more efficient when the interval between treatment and inoculation was longer. In earlier experiments (*Sobiczewski et al.,* 2001), the inculation of Idared/M.26 trees 7 days after Regalis® treatment caused higher disease intensity than when inoculation was made 21 days after such a treatment. It was also influenced by the natural limitation of shoots' growth observed under greenhouse conditions.The time as an important factor in developing the resistance to fire blight was pointed out by *Bazzi et al.* (2003).

Two applications of Regalis® on Gala Must/P.60 apple trees at the distance of 7 days followed 7 or 21 days after inoculation decreased the disease severity by 79 to 66% respectively (evaluated after 6 weeks). *Momol et al.* (1999) obtained after two application of Apogee (27% of ProCa), two and three weeks before inoculation, the reduction of fire blight by 86% on 20-year-old trees cv. Idared. These results appeared to be much better than after use of streptomycin (decreasing of disease by 39%). In our experiments efficacy of streptomycin was much lower. However, *Bazzi et al.* (2003) reported that incidence and severity of fire blight on potted pear trees cv. 'Abbe Fetel' did not show differences between treatments with Regalis® and streptomycin.

It is well known that susceptibility of apple scion cultivars to fire blight is influenced by rootstocks. Presumably such dependence occurs also in case of relation between ProCa treatment and severity of the disease. *Cline and Hunter* (2002) found that susceptibility to fire blight in plants of six apple rootstocks inoculated two weeks after spraying with ProCa proved to be different, because there was a significant rootstock influence on disease development. In our studies Regalis® applied on trees of Gala Must grafted on M.26 reduced occurrence of the disease about 30% (evaluation 4 weeks after inoculation) but on trees of the same cultivar grafted on P.60 (highly resistant) such reduction ranged from over 70 to 90%.

The presented studies indicated some new aspects related to application of Regalis® in apple and pear orchards. Useful advisement of Rademacher and *Kober* (2003) must be emphasized, i.e. in order to apply ProCa in the most efficient way strictly prerequisites have to be regarded. Also, their recommendations for preparing of spray solution, timing the spray application, choosing best conditions for treating trees and selecting rates depending on the fruit species and cultivars in the orchards are to be treated.

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