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Investigations on the Possible Use of Abiotic and Biotic Elicitors in Defence-Related Responses in Plants

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Summary

Experiments were conducted to determine the protective effect triggered by the plant growth retardant prohexadione-Ca (Regalis®), bacterial lipopolysaccharides (LPS) and oligosaccharides (OLS) in different host-pathogen systems. These molecules did not show any direct antibacterial activity *in vitro*. On tobacco leaves, LPS infiltration (100 μg ml^{-1}) prevented hypersensitive reaction (HR), whereas OLS at different concentrations did not give constant and reliable responses. On micropropagated pear plantlets in vials, only Regalis® and LPS (100 μg ml^{-1}) gave a significant defence-related response against fire blight (*Erwinia amylovora*): in particular, plant-derived antimicrobial compounds were related to flavan-3-ols and appeared to be newly synthesized after LPS application. Regalis®, applied at different concentrations, significantly lowered progression of bacterial wilt (*Ralstonia solanacearum*) and reduced leaf lesions of bacterial speck (*Pseudomonas syringae* pv. *tomato*) and spot (*Xanthomonas vesicatoria*) on tomato plants in the greenhouse. OLS application (100 μg ml^{-1}) significantly protected tomato leaves from bacterial speck, but not from bacterial spot. Two treatments with Regalis®, at a twelve-day interval and at different concentrations (75+50 ppm), gave a valuable protection of grapevine leaves to downy mildew (*Plasmopara viticola*) in the greenhouse. When Regalis® was sprayed at different concentrations and at different times on potted plum trees kept outdoors it was apparently ineffective against bacterial spot/canker (*Xanthomonas arboricola* pv. *pruni*). These findings further support the potential of prohexadione-Ca and of other biotic molecules as tools for plant defence responses against different pathogens, in the perspective of integrated, eco-friendly and sustainable control programmes.

Zusammenfassung

Untersuchungen über eine mögliche Anwendung von biotischen und abiotischen Elizitoren von Schutzmechanismen in Pflanzen. Die Untersuchungen wurden durchgeführt, um die Schutzwirkung des Wuchshemmstoffs Prohexadion-Ca (Regalis®) und von bakteriellen Lipopolysacchariden (LPS) sowie Oligosacchariden (OLS) in unterschiedlichen Wirt-Pathogen-Beziehungen zu ermitteln. *In vitro* weisen diese Moleküle keinerlei direkte antibakterielle Wirkung auf. Auf Tabakblättern verhindert die LPS-Infiltration (100 μg ml^{-1}) Hypersensitivitätsreaktionen (HR), während OLS in unterschiedlichen Konzentrationen keine konstante und zuverlässige Reaktion hervorruft. Mikrovermehrte, getopfte Birnenpflänzchen zeigten nur bei Regalis® und LPS (100 μg ml^{-1}) signifikante Abwehrreaktionen gegen Feuerbrand (*Erwinia amylovora*): die von den Pflanzen gebildeten antimikrobiellen Verbindungen waren insbesondere mit Flavan-3-ol verwandt und wurden offenbar nach der LPS-Gabe neu synthetisiert. Regalis®, das in unterschiedlichen Konzentrationen angewendet wurde, verlangsamte bei Gewächshaustomaten die Entwicklung der bakteriellen Welkekrankheit (*Ralstonia solanacearum*) signifikant und reduzierte Blattläsionen, die durch Blattflecken- (*Pseudomonas syringae* pv. *tomato*) und Ringfleckenkrankheit (*Xanthomonas vesicatoria*) verursacht wurden. Die OLS-Gabe (100 μg ml^{-1}) schützte Tomatenblätter mit signifikantem Erfolg vor Blattflecken jedoch nicht vor Ringflecken. Zwei Behandlungen mit Regalis®, im Abstand von zwölf Tagen und in unterschiedlichen Konzentrationen (75+50 ppm), führten bei Reben unter Gewächshausbedingungen zu einem Schutz gegen Falschem Mehltau (*Plasmopara viticola*). Regalis®, das in unterschiedlichen Konzentrationen und Zeitpunkten auf getopften Pflaumenbäumen im Freiland ausgebracht wurde, war gegen eckige Fleckenkrankheit/Krebs (*Xanthomonas arboricola* pv. *pruni*) wirkungslos.

Die beobachteten Ergebnisse ermöglichen die Anwendung als Pflanzenschutzmittel sowohl von Prohexadion-Ca als auch von anderen biotischen Signalmolekülen bakterieller Herkunft, welche bestimmte Abwehrreaktionen der Pflanze hervorrufen können. Das ist besonders wichtig im Hinblick auf integrierte, umweltfreundliche und nachhaltige Kontrollprogramme.

Key words. Induced protection – phytopathogenic bacteria – *Plasmopara viticola* – prohexadione-Ca – lipopolysaccharides – oligosaccharides

Introduction

In the last few years, new strategies have been developed to control important diseases of cultivated plants (KUC et al. 1999): these are based on the induction of a transient and/or permanent modification of biochemical pathways of the plant cellular metabolism using particular molecules called elicitors. These complex molecules might be of biotic or abiotic origin, and *in planta* they trigger the intercellular production of secondary metabolites, for example by modifying the phenylpropanoid pathway, thus making the host plant more resistant towards its pathogens (BENHAMOU and PICARD 1999; LUCAS 1999; RADEMACHER 2000; TREUTTER 2000). Bacterial proteins called harpins might be effective against different diseases (WANG and LIU 1999) and other molecular complexes of bacterial origin, such as pr-LPS, showed their efficacy in reducing the amount of wildfire in tobacco leaves (BIZARRI et al. 1996). Non-biotic elicitors of plant defence responses are also known: for example, salicylic acid, acybenzolar-S-methyl (Bion®), phosethyl-Al, trinexapac-ethyl (TrixE) and prohexadione-Ca (Regalis®): the latter two, in particular, are known to be competitive inhibitors of dioxygenases involved in the phenylpropanoid pathway *in planta*.

Prohexadione-Ca is a synthetic plant growth regulator and several trials showed its use to reduce shoot growth and recent studies outlined both its effect against apple scab (ROEMMELT et al. 2002) and its possible use in the control of fire blight by modifying the flavonoid metabolism in plants and transiently inhibiting flavanone-3-hydroxylase (FHT) activity (STICH et al. 2002). Thus several studies provided evidence that molecules of bacterial origin may act as signal molecules or part of the signal transduction pathways in the plant-pathogen relationship: this might be due to the activation of avirulence genes (*avr*) involving the induction of systemic resistance. In this study the possible use of both abiotic and biotic elicitors to evoke plant defence responses was investigated in different plant-pathogen combinations, in order to give further evidence for possible, alternative and/or complementary control strategies based on the induction of resistance mechanisms *in planta*.

Materials and Methods

Dioxygenase inhibitor

Regalis®, a water-dispersible granular formulation containing 10 % of prohexadione-Ca (BAS 125 10 WG) as active ingredient, was used at different concentrations in all the *in vitro* and *in planta* experiments.

Specific molecules of bacterial origin

The Gram-negative strain IPV-BO G19 of *Pseudomonas putida* (IPV-BO = Istituto di Patologia Vegetale, University of Bologna, Italy) was used for the extraction of cell-associated lipopolysaccharides (LPS "endotoxin"). The bacterium was grown in low osmolarity YM medium (MILLER et al. 1990) and. LPS were obtained by the phenol-water extraction (PW) (WESTPHAL and JAHN 1965, modified). LPS dispersions (2 mg ml^{-1}) were characterised by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 15 %) and detected in the gels by silver staining (HITCHCOCK and BROWN 1983). LPS (smooth type) from *Salmonella friedenau* were used as a control (gift from Dr. O. Holst).

The bacterial strains IPV-BO 1285 d (*Bacillus subtilis*), IPV-BO G19 (*Pseudomonas putida*) – chosen for their evident antagonistic effect in inhibiting *Erwinia amylovora* growth *in vitro* and *in planta* – , IPV-BO 1326 (*Pseudomonas syringae* pv. *syringae*) and NCPPB 2664 (*P. syringae* pv. *aptata*) were used to extract periplasmic oligosaccharides (OLS). The use of the *B. subtilis* strain (Gram-positive) was taken into account as a potential source of similar signal molecules active in the induction of plant resistance responses. The bacteria were grown overnight in low osmolarity YM medium and pellets were extracted using 1 % trichloroacetic acid (TCA) (MILLER et al. 1986). Aliquots of 2 ml were fractionated onto the size exclusion column Sephadex G-50 and 5 ml fractions were collected. Fractions were analysed for the presence of OLS using the phenol-H_2SO_4 method (HANSON and PHILLIPS 1981); the presence of proteins was checked by reading each fraction spectrophotometrically at 280 nm. Fractions corresponding to the first OLS peak (Fig. 2 A and B) were pooled and freeze-dried.

In vitro tests

The activity of purified compounds against *E. amylovora* was tested on KB-agar plates. Assay discs (Schleicher & Schuell, 9 mm) treated with 50 µl of different concentrations of LPS (100, 250 µg ml^{-1}), OLS (100, 500, 1000 µg ml^{-1}) and Regalis® (50, 100, 250, 500, 1000 ppm) were aseptically placed on the surface of KB-agar plates previously spread with 100 µl of an aqueous suspension of *E. amylovora* strain OMP-BO 1077.7/94 (OMP-BO = Osservatorio per le Malattie delle Piante, Regional Phytosanitary Service, Bologna, Italy). SDW and streptomycin sulphate (100 ppm) were used as controls. The plates were kept at 4 °C overnight and then incubated at 27 °C. The presence of inhibition haloes was assessed after 24 h.

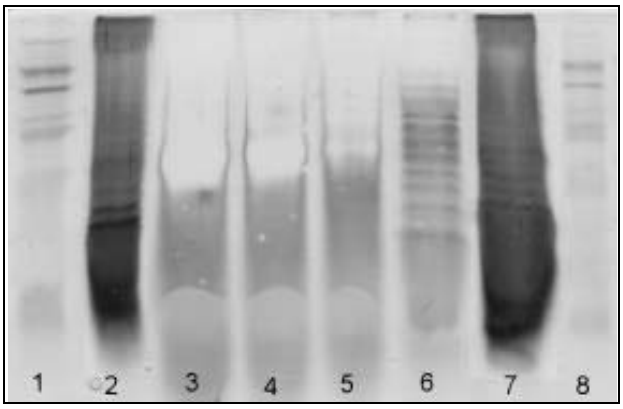


Fig. 1 Gel electrophoresis of LPS. The well samples are as follows: 1 and 8: 10 µg of MW marker; 2 and 7: 5 µg of *S. friedenau* S-LPS (control) 3, 4 and 5: 10 µg of supernatant from ultracentrifugation; 6: 10 µg of S-LPS of strain IPV-BO G 19 (sample from freeze-dried preparation).

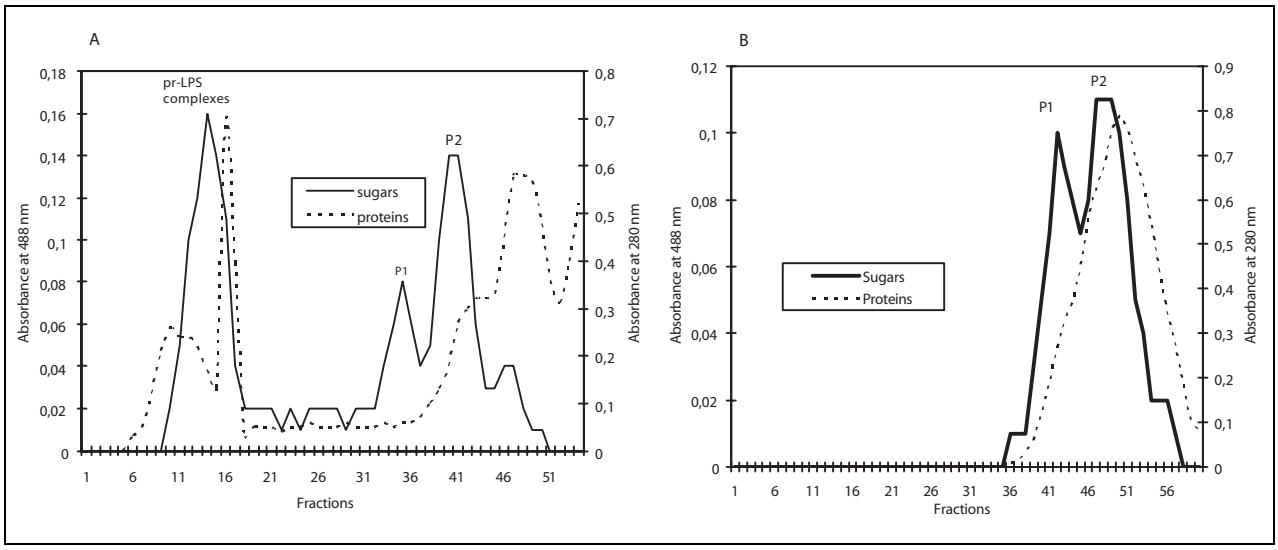


Fig. 2 A and B Chromatograms obtained after fractioning the TCA extract from *P. putida* (A) and *B. subtilis* (B) onto a Sephadex G-50 column. The continuous line corresponds to the galacturonic acid equivalents of extract (readings at λ=488 nm, after phenol-H_2SO_4 treatment); the dotted line corresponds to the protein content of extract (readings at λ=280 nm). As expected, the pr-LPS peak is absent in the chromatogram of the Gram-positive bacterium.

Bioassays on tobacco leaves

Tobacco leaf panels cv. 'White Burley' were infiltrated with LPS (100μg ml^{-1}) and OLS (100–1000 μg ml^{-1}); SDW was used as a control. Twenty-four hours later the same leaf panels were infiltrated with the challenge *E. amylovora* strain (7x10^8 cfu ml^{-1}). Tobacco plants were kept in a growth chamber at 25 °C with 70–90 % RH and under a 14 h photoperiod (9000 lx; 6 a.m. to 8 p.m.). The injected leaves were observed up to 48 h to monitor the development of hypersensitive reaction (HR). Experiments were repeated twice.

Bioassays on micropropagated pear plantlets

Bioassays were carried out at different timings using micropropagated pear plantlets cv. 'Abbé Fétel' grown in vials containing 40 ml of the QUOIRIN and LEPOIVRE (1977) substrate without hormones; the vials were kept in the growth chamber. Experiments were conducted on 4 treatments (OLS from *P. putida* and from *B. subtilis*; LPS; SDW as a control) distributed in 4 randomised replications, each consisting of 20 plantlets. Volumes of 1 ml of LPS dispersions and OLS solutions were dispensed in each vial (100 μg ml^{-1}). In other experiments, 1 ml of an aqueous dispersion of Regalis® (100 ppm) was aseptically dispensed in each vial of a group of 80 micropropagated pear plantlets; a second group of 80 plantlets was used as a control by dispensing 1 ml of SDW.

After 48 h, all the plantlets were inoculated with *E. amylovora* by cutting the apical portion of the two youngest leaves with scissors contaminated in an aqueous suspension containing 7x10^7 cfu ml^{-1}. Blight symptom progression was daily monitored and disease incidence was assessed 12 days after inoculation. Five samples of each treatment (youngest apical leaves) were collected just before challenge inoculation, frozen in liquid nitrogen and lyophilised for chemical analysis (HPLC).

Chemical characterisation of phenolic compounds of plant origin

The apical three youngest and unfolded leaves from micropropagated pear plantlets were individually sampled 48 h after application of elicitors (LPS, and OLS from *P. putida* and *B. subtilis*) and SDW (controls); the samples were then lyophilized, ground in a mortar and the phenolic compounds were extracted with pure methanol using flavon (0.1 mg ml^{-1}) as a standard. The extract was used for HPLC-analysis. Phenolic acids and flavonols were detected at 280 nm, whereas the flavan-3-ols were estimated at 640 nm after post-column derivatization with p-dimethyl-aminocinnamaldehyde (DMSA) (TREUTTER 1989).

Assays on different crop plants

Experiments were carried out under greenhouse and outdoor conditions to evaluate the effect of prohexadione-Ca and molecules of bacterial origin (LPS, OLS) in eliciting protection against *Ralstonia solanacearum* (tomato bacterial wilt), *Pseudomonas syringae* pv. *tomato* (tomato bacterial speck), *Xanthomonas vesicatoria* (tomato bacterial spot), *X. arboricola* pv. *pruni* (plum bacterial spot/canker) and *Plasmopara viticola* (grapevine downy mildew).

Greenhouse experiments with tomato and *R. solanacearum*. Experiments were done on 30 tomato plants cv. 'Moneymaker' grown in pots. At the fifth-sixth true leaf stage, 10 plants were sprayed with 125 ppm of Regalis® and 10 plants with LPS (250 μg ml^{-1}) from *P. putida*; the remaining 10 plants were used as a positive control. High relative humidity (90–100 %) was constantly guaranteed for 24 h after application of the elicitors. Challenge inoculation with the virulent *R. solanacearum* strain PD 2762 (PD = Plantenziektenkundige Dienst, Wageningen, The Netherlands) (about

10^8 cfu ml^{-1}) was performed 7 days after application of Regalis® and 2 days after application of LPS and SDW by cutting the most distal part of its root apparatus and dipping into the bacterial suspension.. The development of typical symptoms in different treatments was daily determined for one month by a disease index: scores 0–5 were based on the progression of leaf wilt (0 = no symptoms; 1 = 1–2 wilted leaflets; 2 = ≥ 3 wilted leaflets; 3 = one wilted leaf; 4 = ≥ 2 wilted leaves; 5 = wilted plant). Individual scores were analysed using Cruskall-Wallis non-parametric test. For the plants of all treatments, the height and the number of leaves were determined.

Greenhouse experiments with tomato and *P. syringae* pv. *tomato* and *X. vesicatoria*. Four experiments were carried out at different times on 240 tomato plants cv. 'Moneymaker' grown in pots. In each of the first two experiments, Regalis® (100 ppm), OLS from *P. syringae* pv. *aptata* and SDW (control) were thoroughly sprayed on 20 tomato plants per treatment. In the other two experiments, OLS from *P. syringae* pv. *syringae* (100 μg ml^{-1}) were used. Inoculation with the challenge bacteria *P. syringae* pv. *tomato* (10+10+10 plants) and *X. vesicatoria* (10+10+10 plants) (about 10^8 cfu ml^{-1}) was performed ten days and two days after application of prohexadione-Ca and both OLS and SDW, respectively.

The development of typical symptoms (necrotic leaf lesions with chlorotic haloes) was periodically monitored for up to four weeks and disease severity was determined on leaf samples collected from different treatments. For each sample, consisting of 30 leaves per treatment (3 youngest leaves from 10 plants), lesions were counted.

Outdoor experiments with plum trees and *X. arboricola* pv. *Pruni*. An experiment was carried out on 30 one-year-old plum trees (*Prunus salicina*) cv. 'Angeleno' grafted on myrobalan (*P. myrobalanum*) kept in pots to evaluate the efficacy of Regalis® application in inducing a resistance response to *X. arboricola* pv. *pruni* infections and its effect on shoot growth. The trees were divided into three groups of 10 trees each: the dioxygenase inhibitor was applied three times on two groups at different concentrations (Table 3); one group was used as a control. Challenge inoculation with *X. arboricola* pv. *pruni* strain IPV-BO 1196 (about 10^8 cfu ml^{-1}) was performed 10–14 days after each application of Regalis®. Leaf symptom progression was periodically monitored for up to two weeks and disease severity was determined by counting spots on leaves collected from different treatments.

Greenhouse experiment with grapevine and *P. viticola*. An experiment was carried out on 120 grafted vines (60 of the cv. 'Pergolone' and 60 of the cv. 'Trebbiano') in pots to determine the efficacy of prohexadione-Ca application against downy mildew caused by *P. viticola*. On 30 vines of each cultivar, Regalis® (125 ppm) was sprayed two times at 12-day interval and at different concentration (75+50 ppm); deionized water was sprayed on the other vines (controls). Each treatment consisted of 5 replications of 6 vines. After application of the chemical, high relative humidity (90–100 %) was maintained for 24 h at 23 °C. Ten days after the last application of Regalis®, the vines (and in particular, the lower leaf surface) were sprayed with an aqueous suspension of *P. viticola* sporangia (about $5x10^4$ ml^{-1}) obtained by washing infected leaves collected from grapevines in the vineyard. A film of water on the plant tissues and high relative humidity were maintained after experimental inoculation for 24 h. Leaf symptoms were monitored and, after 10 days, the vines were enclosed in polythene bags overnight in order to support the development of a downy growth of the sporangiophores of the fungus on the lower surface of leaves, directly under the spots. In the two treatments, the infected leaf area (%) was determined on the 4–5 central leaves of 1–3 canes per vine.

Results

The PW-extraction method combined with specific purification steps, was suitable to obtain LPS preparations ready to be dispersed in water. These molecules were referred to the smooth type (S-LPS) when compared to the SDS-PAGE pattern of the S-LPS from *S. friedenau* used as a control (Fig. 1).

Size exclusion chromatography of phenol-H_2SO_4 extracts from bacterial strains, including the Gram-positive *B. subtilis*, resulted in the separation of three molecular complexes. The first peak in the chromatogram corresponded to the pr-LPS fraction of the Gram-negative bacterium: a similar peak was absent in the fractionation of the extracts obtained from the Gram-positive one; the two following peaks (P1, P2) corresponded to two different OLS complexes. The most interesting peak for the planned experiments appeared to be the first one (Fig. 2 A and B).

In vitro tests

Regalis®, purified LPS and OLS preparations used at different concentration, did not show any direct inhibition activity against *E. amylovora* growth.

Tobacco bioassays

In mature tobacco leaf panels, locally injected LPS completely prevented the development of confluent hypersensitive necrosis (HR) after *E. amylovora* infiltration, whereas the control interveinal areas (SDW) developed typical hypersensitivity. Infiltration of OLS did not give constant responses: experiments on different tobacco plants with different concentrations gave protection ranging from 0–100 %. However, using an OLS concentration ≥250 μg ml^{-1} protection was more evident. Control leaf tissue (SDW) always showed confluent hypersensitive necrosis.

Bioassays on micropropagated pear plantlets

Regalis®, LPS and OLS (from the antagonist strains *B. subtilis* and *P. putida*) were tested on micropropagated pear plantlets cv. 'Abbé Fétel' in vials as elicitors of resistance against fire blight. As regards Regalis® (100 ppm), there was a trend toward a reduction of disease incidence: in particular, statistical analysis of data showed a significant defence-related response 11 days

Table 1. Effect of prohexadione-Ca application on tomato plant growth. Values followed by different letters are significantly different (Duncan's test, $p<0.05$).

Treatments	Leaves (No.)	Plant Height (cm)
H_2O	5.7 a	7.8 a
LPS	5.4 a	9.7 a
Regalis®	5.0 b	4.2 b

Chemical characterisation of phenolic compounds of plant origin

Chemical analyses (HPLC), performed on phenolic extracts from tissue of micropropagated pear plantlets, showed the presence of newly synthesised products after application of bacterial LPS. These compounds seemed to belong to the flavan-3-ols category; furthermore, it was observed a concomitant strong reduction of hydroxycinnamic acids levels (Fig. 4)

Table 2. Defence response of tomato plants to bacterial spot and bacterial speck induced by Regalis® and OLS. Values refer to the mean number of lesions per leaf. Values followed by different letters are significantly different (Duncan's test, $p<0.05$). n. d. = not determined.

Treatments	*X. vesicatoria* – No. of lesions			
	Exp. I	Exp. II	Exp. III	Exp. IV
H_2O	109.57 a	94.30 a	34.57 a	n. d.
OLS_{2664}	65.60 a	91.00 a	32.70 a	n. d.
Regalis®	24.40 b	14.20 b	3.30 b	n. d.
	P. syringae pv. *tomato* – No. of lesions			
	Exp. I	Exp. II	Exp. III	Exp. IV
H_2O	19.10 a	76.60 a	29.30 a	127.20 a
OLS_{1386}	7.80 b	42.60 b	17.90 b	91.83 b
Regalis®	3.70 b	4.90 c	4.83 c	19.33 c

Table 3. Effect of Regalis® on the growth of plum trees and on the progression of the bacterial spot caused by *X. arboricola* pv. *pruni*. Data show the differences among the treatments at different times. Values followed by different letters are significantly different (Duncan's test, $p<0.05$).

Time	Treatments	Shoots (no.)	Shoot length (cm)	Diseased leaves (no.)	Spots (no.)
1	H_2O	9.4 a	259.3 a	1.7 a	76.2 a
	Regalis® (50+50+50 ppm)	8.7 a	218.3 a	1.8 a	22.9 a
	Regalis® (100+50+50 ppm)	6.8 a	81.6 b	1.5 a	35.7 a
2	H_2O	9.9 a	295.1 a	3.6 a	36.9 a
	Regalis® (50+50+50 ppm)	9.0 a	264.3 a	6.6 a	41.6 a
	Regalis® (100+50+50 ppm)	7.9 a	144.3 b	6.3 a	46.9 a
3	H_2O	11.1 a	300.0 a	5.9 a	45.3 a
	Regalis® (50+50+50 ppm)	11.4 a	282.2 a	7.5 a	56.9 a
	Regalis® (100+50+500 ppm)	9.3 a	154.1 b	6.5 a	65. a

after challenge inoculation with *E. amylovora* (Table 1). As regards LPS and OLS, both at 100µg ml^{-1}, only the former significantly reduced disease incidence during the whole monitoring period; moreover, there was a trend towards a slower disease symptom progression as compared with the other treatments. In particular, the highest protective effect was observed between the third and the fourth day after challenge inoculation (relative disease incidence: 50 % and 62 %, respectively) (Fig. 3).

Assays on different crop plants

Greenhouse experiments with tomato and *Ralstonia solanacearum*. Treatment of tomato plantlets with Regalis® (125 ppm) resulted in a progression of bacterial wilt lower than the untreated controls: the protection was significant starting from the sixth day after *R. solanacearum* inoculation. In particular, values of disease index scores reached 1.9 in the untreated control, whereas in the Regalis® treated plants the disease index

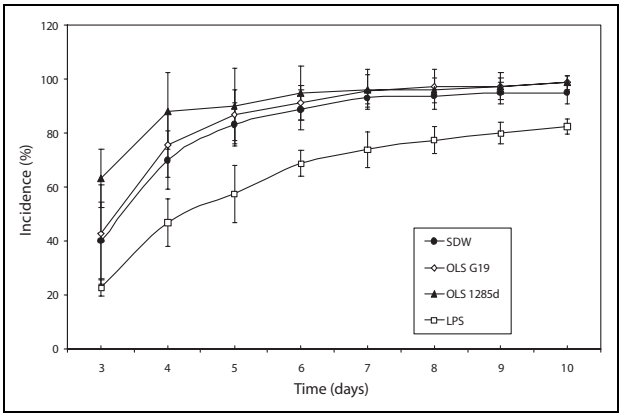


Fig. 3 Effect of LPS and OLS on fire blight progression in micropropagated pear plantlets 'Abbé Fétel' in vials.

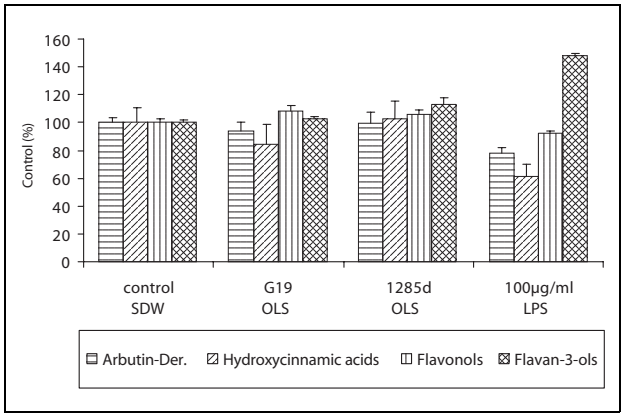


Fig. 4 Effect of bacterial OLS and LPS on phenol biosynthesis in micropropagated pear plantlets 'Abbé Fétel' in vials.

scores did not exceed 1.5 (Cruska-Wallis test, p 0.01). No protection was observed in the treatment with LPS (250 µg ml^{-1}). The effect of Regalis® application on tomato plant growth resulted in a significant reduction of both plant height and number of leaves (Table 1). No phytotoxicity was observed on tomato plantlets.

Greenhouse experiments with tomato and *P. syringae* pv. *tomato* and *X. vesicatoria*. Regalis® application (100 ppm), ten days before challenge inoculation, resulted in a significant reduction of leaf lesions caused by both pathogens. Also OLS application (100 µg ml^{-1}) significantly protected tomato leaves from bacterial speck (*P. syringae* pv. *tomato*), but it was always less effective than Regalis®; in all cases, OLS did not show any protective effect against bacterial spot (*X. vesicatoria*) (Table 2).

Outdoor experiments with plum trees and *X. arboricola* pv. *Pruni*. Regalis® application to plum trees, at different concentrations and at different times, did not give any protection to infection triggered by *X. arboricola* pv. *pruni*. Table 3 shows the same amount of disease among the three treatments, indicated as number of spots and number of diseased leaves, for each time considered. The only significant difference reported in this table is related to the shoot length: the treatment with 100+50+50 ppm of Regalis® caused phytotoxic effects on leaves and inhibited shoot growth for several weeks. As a result of this, the mean value of shoot length of this treatment was approx. half of the one obtained with the two other treatments.

Greenhouse experiments with grapevine and *P. viticola*. The activity of Regalis® against downy mildew (*P. viticola*) was determined on the grapevine cvs. 'Trebbiano' and 'Pergolone'. The chemical, applied two times at a twelve-day interval and at different concentrations (75+50 ppm), gave a good level of protection of the leaves to infection. The mean infected leaf area (%) of the treated grapevines was significantly lower than that of the control and the protection level tended to be higher on cv. 'Trebbiano' than on cv. 'Pergolone' (Table 4). No phytotoxicity was observed on foliage of both cultivars.

Table 4. Response of grapevine leaves to downy mildew after application of Regalis®. Values followed by different letters are significantly different (Duncan's test, $p<0.05$).

Treatments	'Trebbiano' Infected leaf area (%)	'Pergolone' Infected leaf area (%)
H_2O	13.12 b	14.01 b
Regalis® (75+50 ppm)	2.94 a	4.80 a

Discussion

These experiments, carried out in the frame of the multidisciplinary project "*Erwinia*" financed by the European Commission, give further evidence on the potential use of molecules from different sources (prohexadione-Ca, bacterial OLS and LPS) to activate plants in their defence responses against phytopathogens. In other words, these "information-rich molecules", acting as chemical messengers or signals with no direct antimicrobial activity, have the possibility to modulate plant susceptibility/resistance in various host-pathogen systems, through activation of a cascade of complex mechanisms and physiological events.

In particular, the plant growth retardant Regalis® (prohexadione-Ca) acts as an inhibitor of 2-oxoglutarate-dependent dioxygenases in gibberellin biosynthesis and triggers defence reactions against fire blight (*E. amylovora*) and scab (*Venturia inaequalis*) in apple and pear by qualitatively and quantitatively changing their spectrum of flavonoids and other phenylpropanoids (Fernando and Jones 1999; Yoder et al. 1999; Rademacher 2000; Kampan et al. 2000; Bazzi et al. 2000; Costa et al. 2001; Aldwinckle et al. 2002; Deckers and Schoofs 2002; Roemmelt et al. 2002; Stich et al. 2002). Regalis® application at different concentrations and at different times on micropropagated pear plantlets in vials, tomato plants and grapevines resulted in a significant protection from infections caused by challenge inoculation of *E. amylovora*, *P. syringae* pv. *tomato*/*X. vesicatoria* and *P. viticola*, respectively. These first findings, together with previous experimental evidences on the protection from apple and

peach powdery mildew and grape grey mould obtained by other researchers (see ROEMMELT et al. 2002), open the doors to a potential practical use of this dioxygenase inhibitor as an elicitor of defence reactions against economically important diseases on different crops other than apple and pear. In contrast, Regalis® does not seem effective in controlling bacterial spot/canker (*X. arboricola* pv. *pruni*) on plum trees, but it must be pointed out that further experiments are necessary to confirm these preliminary data. Such a result can be explained considering that recent studies (GOSCH et al, this issue) showed that FNR activity in plum leaves is lower than in other crop plants and thus a less efficacy in controlling disease can be observed in these plants. A lack of effectiveness was also observed in other systems such as pepper/grey mold, wheat/powdery mildew and tomato/late blight (ROEMMELT et al. 2002).

Cell associated S-type LPS, from a fluorescent pseudomonad suitable as a possible biocontrol agent against fire blight (GALASSO et al. 2002) purified and locally injected in tobacco interveinal panels, completely prevented induction of hypersensitive response (HR) after challenge infiltration of a virulent *E. amylovora* strain. In general, prevention of HR is regarded as an increased tolerance (MAZZUCCHI et al. 1979) and/or as a manifestation of plant resistance towards pathogens, known as "localized induced resistance" (Lir, *sensu* SEQUEIRA 1983) or "localized induced response" (NEWMAN et al. 2001). LPS were also reported to induce systemic resistance (ISR) in plants against pathogens (VAN PEER and SCHIPPERS 1992; LEEMAN et al. 1995). When absorbed by micropropagated pear plantlets in vials, these bio-active LPS contributed to an enhanced defensive capacity against fire blight. This protective effect was reported in several plant-microbe interactions (ALEXANDROVA et al. 2000; COVENTRY and DUBERY 2001; NEWMAN et al. 2001). Further studies are needed to support the hypothesis that this activity might be in relation with phenol biosynthesis in plant tissue. With this respect, LPS can be said to have sensitised pear plant tissue inducing the expression of some defence-related genes. In the assays on tomato plants, application of these molecules did not cause a marked change in the level of bacterial wilt progression with respect to the control, following challenge inoculation with *R. solanacearum*. This results might be related to conditions associated with infection, the specificity of the structural motifs of the O-antigen polysaccharide in the bacterial LPS used and to the ability of phytopatogenic bacteria to evolve mechanisms, which remain still unclear, to suppress the effects of such molecules on plant cells (NEWMAN et al. 2000; LEROUGE and VANDERLEYDEN 2001).

OLS are molecules with several and different roles in many biological systems: in the plant-microbe interaction they may act as signal molecules (ALBERSHEIM et al. 1983; RYAN 1987). Several bacterial species of clinical, environmental and agricultural relevance may produce OLS, both membrane-derived and periplasmic and their role is crucial in the adaptation of the bacterium to its different environments, in our case to its interaction with the host plant. Bacterial OLS used in our experiments *in planta* are probably a mixture of homo- and heteropolymers with a degree of polymerisation lower than 20: then we may call them oligosaccharins and their role as signal molecules or elicitors is still poorly understood (STEFANI et al. 1994). Their mode of action appears to be more specific than that of LPS or pr-LPS, since nanomolar concentration of OLS may prevent the susceptible reaction but not the HR, leading to the conclusion that a particular bacterial OLS may inhibit the disease caused by the same bacterium, but not other diseases (TREBBI 1996). Our results confirm this statement: the use of OLS in control strategies should be purpose-built, after further and deeper studies on the structure-function relationship. This might explain both the variability of the results in some experiments and the failure in controlling a disease in other assays.

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