Journal of Phytopathology

ORIGINAL ARTICLE

The Lactoperoxidase System: a Natural Biochemical Biocontrol Agent for Pre- and Postharvest Applications

Françoise Bafort[1], Olivier Parisi[1], Jean-Paul Perraudin[2] and Mohammed Haïssam Jijakli[1]

1 Gembloux Agro-Bio Tech, Laboratory of Plant Pathology, University of Liège, Passage des Déportés 2, B-5030 Gembloux, Belgium
2 Taradon Laboratory, Avenue Léon Champagne 2, B-1480 Tubize, Belgium

Keywords
Botrytis cinerea, lactoperoxidase, *Penicillium digitatum*, *Penicillium expansum*, *Penicillium italicum*, *Phytophthora infestans*

Correspondence
F. Bafort, University of Liège, Gembloux Agro-Bio Tech, Laboratory of Plant Pathology, Gembloux, Belgium.
E-mail: francoise.bafort@ulg.ac.be

Received: June 30, 2016; accepted: September 28, 2016.

doi: 10.1111/jph.12532

Abstract

Controlling pests in pre- and postharvest crops using natural and low-impact products is a major challenge. The lactoperoxidase system is an enzymatic system that exists in all external secretions in mammals and is part of the non-immune system. We tested its efficacy in *in vitro* microplates on *Phytophthora infestans, Penicillium digitatum, Penicillium italicum, Penicillium expansum* and *Botrytis cinerea* to determine the most suitable concentrations for use. Then, we verified its efficacy *in planta* under controlled conditions. Solutions prepared with 5.4 mM iodide and 1.2 mM thiocyanate and diluted threefold inhibited pathogen growth *in vitro* by 63–100%. Twofold-diluted solutions protected potato plants against *P. infestans* by 60–74% under controlled conditions. Undiluted solution inhibited orange's and apple's postharvest pathogens in curative application with efficacy levels ranging between 84 and 95% in orange and between 63 and 74% in apple. 1.5-fold concentrated solutions inhibited postharvest pathogens of apple in curative application with efficacy levels ranging between 84 and 92%. Our results also show that the oxidative stress response of fruit following wounding could interfere with ion efficiency. Our tests demonstrate for the first time that this biochemical method is as efficient as a conventional synthetic chemical method under controlled conditions.

Introduction

For some years now, consumers and subsequently legislators and growers have been concerned by environmental issues, leading to the emergence of organic farming. Then, the industry followed with the creation of different certifications imposing a more rational and integrated use of pesticides. On a legal perspective, the European Union regulates pesticide use, and this has resulted in many active substances being withdrawn from the market because of their excessive toxicity or deleterious effects on the environment. The authorization process has been hardened: products can be simply removed from the market on the basis of certain criteria called qualifiers. As a result, the number of authorized active substances has decreased even more. Some crops eventually end up without any licensed product available, mainly in the sector of minor crops such as horticultural crops. The decrease in the number of authorized synthetic plant protection products coupled with societal demands for a more oriented organic farming involves searching for biopesticides to protect crops together with other alternatives such as innovative orchard management, crops rotation and diversity of plant species.

Potato late blight is caused by the fungus-like oomycete *Phytophthora infestans* (Mont.) de Bary. Until 1980, clonal reproduction of the pathogen was observed everywhere except in Mexico (Cooke et al. 2011). Then, sexual reproduction spread across northern Europe, leading to higher genetic diversity (Cooke et al. 2011; Yuen and Andersson 2013; Wiik 2014). This also increased the capacity of the pathogen to

infect soils for years, and favoured its adaptability and aggressiveness (Cooke et al. 2011; Yuen and Andersson 2013). Under favourable weather conditions, that is temperatures in the 15–20°C range combined with a high level of humidity and a dense foliage, leaf lesions occur after 3–5 days, and foliage destruction within 10–15 days (Schöber 1992; Sedláková et al. 2011). Disease management is mainly focused on crop rotation, use of resistant potato varieties, control of primary inoculum sources, use of chemical fungicides and use of decision support systems (DSSs) (Cooke et al. 2011; Wiik 2014). Resistant seed potatoes are not widely used in northern Europe because their agronomic characteristics do not meet consumer expectations and the resistance genes are regularly overcome (Cooke et al. 2011; Akino et al. 2013). Decision support systems are based on several criteria such as weather conditions, potato variety or pathogen life cycle. They provide farmers with information that helps them manage late blight disease. They reduced fungicide inputs between 8 and 62% as compared to routine treatments in trials across Europe in 2001 (Cooke et al. 2011). Nevertheless, potato late blight is still mainly controlled through the intensive use of fungicides despite growing consumer and legislation demand to reduce their use.

Postharvest pests are still a major issue in fruit storage. Even with modern storage facilities, losses of apples stored in cold chambers can still reach 5–25%. They are caused by physiological diseases and fungi such as *Botrytis cinerea*, *Penicillium expansum and Gloeosporides* (Jijakli and Lepoivre 2004). Postharvest pests are primarily controlled through handling practices and applications of synthetic fungicides such as imazalil and thiabendazole. In citrus fruit, losses are linked to fruit senescence, physiological disorders and pests and represent approximately 25% in developing countries and approximately 5–10% in developed countries (Ladaniya 2010). The pathogens *Penicillium digitatum*, *Penicillium italicum* and *Geotrichum citri-aurantii* are the causal agents of green mould, blue mould and sour rot, respectively. They are the main causes of losses during storage (Ismail and Zhan 2004). Refrigeration, sanitation of packing houses, fungicide application and drenching, hot water treatments, degreening treatments and fruit cleaning are the main postharvest control practices.

In mammals, the lactoperoxidase system is a natural protection system present in several secretory glands: salivary glands, lacrimal glands, airway and intestinal mucus, vaginal secretions and milk (Kussendrager and van Hooijdonk 2000; Hawkins 2009). It consists of an enzyme, namely lactoperoxidase, and its substrates, namely hydrogen peroxide and a halogen (chloride, bromide or iodide) or pseudo-halogen (thiocyanate). In the presence of the enzyme, hydrogen peroxide is transformed into water and the halogen or pseudo-halogen is oxidized into an ion, an hypo (pseudo) halide, that has antimicrobial and antiviral properties (Kussendrager and van Hooijdonk 2000; Välimaa et al. 2002; Moskwa et al. 2007; Welk et al. 2009). Combination of iodide and thiocyanate in the lactoperoxidase system increased fungicidal and bactericidal effects (Bosch et al. 2000). The system has been widely tested against human and animal pathogens (de Wit and van Hooydonk 1996; Kussendrager and van Hooijdonk 2000), but has been less investigated against plant pathogens. This technique is already successfully applied in dental hygiene, preservation of milk, food and cosmetics, as well as health care of calves, piglets and fish (de Wit and van Hooydonk 1996; Kussendrager and van Hooijdonk 2000; Hawkins 2009). The objective of this study was to evaluate the *in vitro* and *in vivo* efficacy of the ions produced by the lactoperoxidase system and applied separately from the enzyme on pests in pre- and postharvest crops.

Materials and Methods

Phytophthora infestans culture

Phytophthora infestans (Montagne de Bary) from CRA Libramont was used for all trials. Cultures were grown on modified V8 juice agar (200 ml of V8 juice, 18 g of agar, 3 g of calcium carbonate and distilled water to 1 l) at 15°C in a dark culture chamber. After 3 weeks of incubation, sporangia were collected by adding 5 ml of sterile 0.05% Tween water on the plate. Sporangia were scraped off with a sterile inoculating loop and filtered through a sterilized double-layer fine cloth. The concentration of sporangia was adjusted using a Bürker counting chamber.

Penicillium expansum, *Penicillium digitatum*, *Penicillium italicum* and *Botrytis cinerea* cultures

Penicillium expansum (CBS 484.75), *P. digitatum* (MUCL 39704), *P. italicum* (MUCL 15608) and *B. cinerea* strain V (isolated from decayed fruit in our laboratory) were grown on potato dextrose agar at 25°C in a culture chamber. After 10 days (for the *Penicillium* strains) and 3 weeks (for *B. cinerea*) of incubation, conidia were collected by adding 9 ml of sterile 0.05% Tween water on the plate. Conidia were scraped off with a sterile inoculating loop and filtered through a sterilized double-layer fine cloth. The

concentration of conidia was adjusted using a Bürker counting chamber.

Production of an ions solution using the lactoperoxidase system

Lactoperoxidase (Taradon Laboratory, Tubize, Belgium), 50 U/ml, was mixed with a thickening agent (bentonite clay, Bentonil®, Clariant Functional Minerals SE) and then added to 1 l of tap water and mixed thoroughly for 1 min. Freshly prepared solutions of potassium iodide (KI) (VWR International Europe BVBA, Leuven, Belgium), potassium thiocyanate (KSCN) (VWR International Europe BVBA) and H_2O_2 35% (VWR International Europe BVBA) at concentrations mentioned in '*In vitro* efficacy tests' below were added under gentle shaking. The solution was kept under shaking for 1 min, and then, 150 μl of coagulant (Pac Sachtoklar from Brenntag N.V.) was added. One more minute later, the shaking was stopped, the enzyme and thickening agent decanted, and the upper part of the solution was filtered through a paper filter (VWR International Europe BVBA).

In vitro growth tests

The growth tests were performed on microplates following the protocol of (Kouassi et al. 2012), using three pathogen concentrations (10^4, 10^5 and 10^6 spores/ml) and three medium concentrations (potato dextrose broth diluted threefold, 30-fold and 300-fold) for 5 days at 22°C in the dark, that is a total of nine growth experiments. The optimal growth conditions were selected to perform the *in vitro* efficacy tests.

In vitro efficacy tests

The pathogens at a final concentration of 3×10^5 spores/ml or 1×10^6 spores/ml were cultured for 5 days in threefold-diluted sterile potato dextrose broth in the presence or absence (control) of an inhibiting solution, in the dark at 22°C in ELISA plate wells following the protocol of (Kouassi et al. 2012). The controls were tested in four replicates. The inhibiting ions solutions were performed in eight replicates. Optical densities (OD) at 650 nm were measured at incubation time of 48 h and 120 h, and mean OD at 120 h were calculated to perform data analysis. Four substrate ratios (5.4 mM KI/1.2 mM KSCN/6.6 mM H_2O_2; 3.6 mM KI/0.8 mM KSCN/4.4 mM H_2O_2; 2.7 mM KI/0.6 mM KSCN/3.3 mM H_2O_2; and 0.78 mM KI/0.34 mM KSCN/1.12 mM H_2O_2) were prepared. Each solution was diluted threefold, fivefold and 10-fold. All tests were performed in two independent experiments. The percentage of inhibition of a pathogen was calculated using the following formula (Kouassi et al. 2012):

$\%\ \text{inhibition} = ((OD_{\text{control}} - OD_{\text{inhibiting solution}})/(OD_{\text{control}})) * 100$, where OD_{control} is the optical density of the pathogen culture after subtracting the optical density of the medium, and $OD_{\text{inhibiting solution}}$ is the optical density of the inhibiting solution-treated pathogen culture after subtracting the optical density of the medium and of the inhibiting solution.

Control solutions without enzyme with (5.4 mM KI + 1.2 mM KSCN) from one part and 6.6 mM H_2O_2 for another part were tested against each pathogen for evaluating their inhibiting activities.

Dosages

Ion concentrations were measured from the oxidation of the NH_2-group of 3.3′,5.5′-tetramethylbenzidine (TMB), as described in study by Bafort et al. 2014.

Thiocyanate was measured with a colorimetric method based on the red complex formed by thiocyanate ion and iron (III) ion measured at 447 nm as described in study by Lahti et al. 2000.

Iodide was measured with an iodine combination electrode (Hanna HI 4111) and a Hanna Instruments Ltd bench pH/ISE meter (HI 4222).The meter was calibrated with Hanna iodide calibrators. Samples were prepared as described by Hanna.

Peroxide hydrogen was measured with VWR International MQuant™ with 0.5-2-5-10-25 mg/l H_2O_2 range.

The quantity of substrate consumed during enzymatic reaction was calculated with the following formula:

$\%\ \text{substrate used} = (\text{initial concentration of substrate} - \text{final concentration of substrate})/\text{initial concentration of substrate} * 100$, where initial concentration of substrate is the concentration of substrate before enzymatic reaction and final substrate concentration is the concentration of substrate measured after enzymatic reaction.

Catalase test

Catalase was purchased from Sigma-Aldrich NV/SA (Overijse, Belgium) and added to the reaction mixture at 200 U/ml or at 5000 U/ml.

Phytotoxicity tests on detached leaves

Solanum tuberosum sprouted tubers were cultivated in plastic pots (one tuber/pot) containing compost in

glasshouse conditions. Healthy 4-week-old leaflets were taken and then left at room temperature overnight to favour evapotranspiration. One leaflet was placed in each Petri dish (9 cm diameter, 64 cm^2) filled with water agar. Each leaflet was laid over the agar surface with its adaxial face upward. Treatments were performed using a manual atomizer (under air pressure) to apply 5 $\mu l/cm^2$ of leaf and 320 μl/dish. Three different concentrations (5.4 mM KI/1.2 mM KSCN/6.6 mM H_2O_2; 3.6 mM KI/0.8 mM KSCN/4.4 mM H_2O_2; and 2.7 mM KI/0.6 mM KSCN/3.3 mM H_2O_2) were applied undiluted, or diluted fivefold or 10-fold. Eight replicates were prepared per dilution and per concentration. Eight replicates of a negative control (sprayed with water) were used as a reference. After treatment, the Petri dishes were maintained at 25°C under a 16-h light/8-h dark photoperiod. Leaflets were observed 6 and 14 days after the treatments were applied to determine phytotoxicity (expressed by symptoms such as necrosis spots, burns, brown spots or premature senescence).

Phytotoxicity tests on fruit

Fruits were disinfected with a 1% hypochloride solution for 2 min and rinsed twice with water. Then, they were dipped into one of the ion solutions (prepared as described previously) or into water for 1 min (nine replicates/treatment). They were checked after 24 days and compared to the control for burnings, spots and stains.

In vivo efficacy assays on potato plants

Trials were designed in random blocks. *Solanum tuberosum* tubers were cultivated in pots (one tuber/pot) at 22°C under the glasshouse for 15 days before the tests. Spores of *P. infestans* were applied at a concentration of 5×10^4 spores/ml until saturation of the two leaf faces, using a manual atomizer. Treatments were applied 4 h after the pathogen was inoculated, following the same methodology. After the treatments, the plants were kept in the glasshouse at 22–25°C and high relative humidity (>90%) under a 16-h light/8-h dark photoperiod for 1 month. The conditions (control, fungicide, ion doses) were applied in trials 1 and 2 with five replicates per condition. The third trial was designed with nine replicates per condition to confirm the results. The control treatment was made of copper oxychloride (50% CU) (Cupravit Forte from Bayer SA-NV, Diegem, Belgium) at a concentration of 5 kg/ha. The negative control was sprayed with water. Ions were prepared from a stock solution prepared as described in 'Production of an ions solution using the lactoperoxidase system' with 5.4 mM KI/1.2 mM KSCN/6.6 mM H_2O_2. Other levels were prepared by diluting the stock solution with water.

Late blight symptoms on leaves were estimated between 10 and 24 days after inoculation using a visual six-level scale used in the CRA Station of Libramont (Belgium). It indicates the proportions of leaf surfaces covered by late blight symptoms, where 0 corresponds to 0%, 1 to 1–19%, 2 to 20–49%, 3 to 50–79%, 4 to 80–95% and 5 to 96–100%. The leaves were located between 30 cm and 70–80 cm aboveground.

In vivo tests on fruits

Fruits were disinfected with a 1% hypochloride solution for 2 min and then rinsed twice with water. Three injuries of 4 mm in diameter and 3 mm depth were made per fruit. In the preventive test, the fruits were first treated by dipping 1 min in the treatment solutions and then inoculated 24 h later with 40 μl of the pathogen at 10^6 (*B. cinerea*), 10^5 (*P. expansum*) or 10^4 (*P. italicum* and *P. digitatum*) spores/ml. In the curative test, the fruits were first inoculated, and then treated 24 h later. The negative control was made of water, and the positive control was a mixture of imazalil and pyrimethanil at 1.25 ml/l (Philabuster 400 SC from Janssen Pharmaceutica N.V.). After drying, the fruits were placed individually in a closed plastic container containing a filter paper and 3 ml of water. They were incubated at 25°C and checked after 5 or 6 days. Each treatment was replicated 10 times. Infection was assessed by measuring lesion diameter to determine the level of infection.

Data analysis

Data were analysed using MINITAB Ltd version 17 (Coventry, UK). Normality, equal variance and one-way parametric analysis of variance (ANOVA) were performed on optical density mean values (*in vitro* test) or on infection mean values (*in vivo* test). When the analysis was statistically significant, the means were separated using Fisher's LSD tests. Differences at $P < 0.01$ (*in vitro* tests and *in vivo* fruit tests) or at $P < 0.05$ (*in vivo* potato plant tests) were considered as significant.

Results

In vitro results

Growth test

The growth curves giving optimal discrimination between the exponential phase and the stationary

phase of pathogens growth are shown in Fig. 1. The lowest concentration and a too diluted broth failed to ensure good fungal growth. Based on these results, we chose a concentration of 10^6 spores/ml in PDB diluted threefold for our *in vitro* efficacy tests.

Dosages

For each mixture of substrates, concentrations of ions produced, thiocyanate, iodide and peroxide hydrogen were measured after enzymatic reaction. Results are shown in Table 1.

In all mixtures, hydrogen peroxide is consumed almost entirely. Thiocyanate and iodide are consumed in identical proportion, from 42 to 32%, in mixture with a ratio of 1:4.5 between thiocyanate and iodide. The ratio of 1:2.29 promotes thiocyanate consumption at the expense of iodide. Higher substrate amount gives higher ion concentrations.

In vitro efficacy

The mixtures resulting in the action of lactoperoxidase on the substrates (thus containing the produced ions) were tested at different concentrations and dilutions against the pathogen (Table 2). The substrates alone demonstrate their absence of efficacy on the pathogens (Table 2).

A substrate dose-effect can be noted: pathogens were more inhibited as substrate concentrations increased. The first and most concentrated solution diluted threefold inhibited all strains although the least concentrated solution diluted threefold inhibited one fungal strain. We noted maximum inhibition for *P. infestans* and *B. cinerea*, which were inhibited by more than 61% by 9 solutions of 10. *Penicillium expansum* and *P. italicum* were inhibited relatively efficiently, with, respectively, 4 and 6 treatments of 10 giving inhibition results above 56%. Finally, *P. digitatum* was weakly inhibited: only one treatment of 10 induced 100% inhibition. The effect of catalase, produced by *P. digitatum* (Macarisin et al. 2007), on ions concentration has been tested, and results are shown in Fig. 2. Catalase decreased ion concentrations by 16–68% depending on the catalase concentration.

Phytotoxicity

The ratios giving the best *in vitro* efficacy were sprayed on potato leaflets at three levels: undiluted, diluted fivefold and diluted 10-fold. After 6 days, no phytotoxicity was observed. After 14 days, one leaflet of 8 (12.5%) presented burns with the undiluted first ratio (5.4 mM KI/1.2 mM KSCN/6.6 mM H_2O_2).

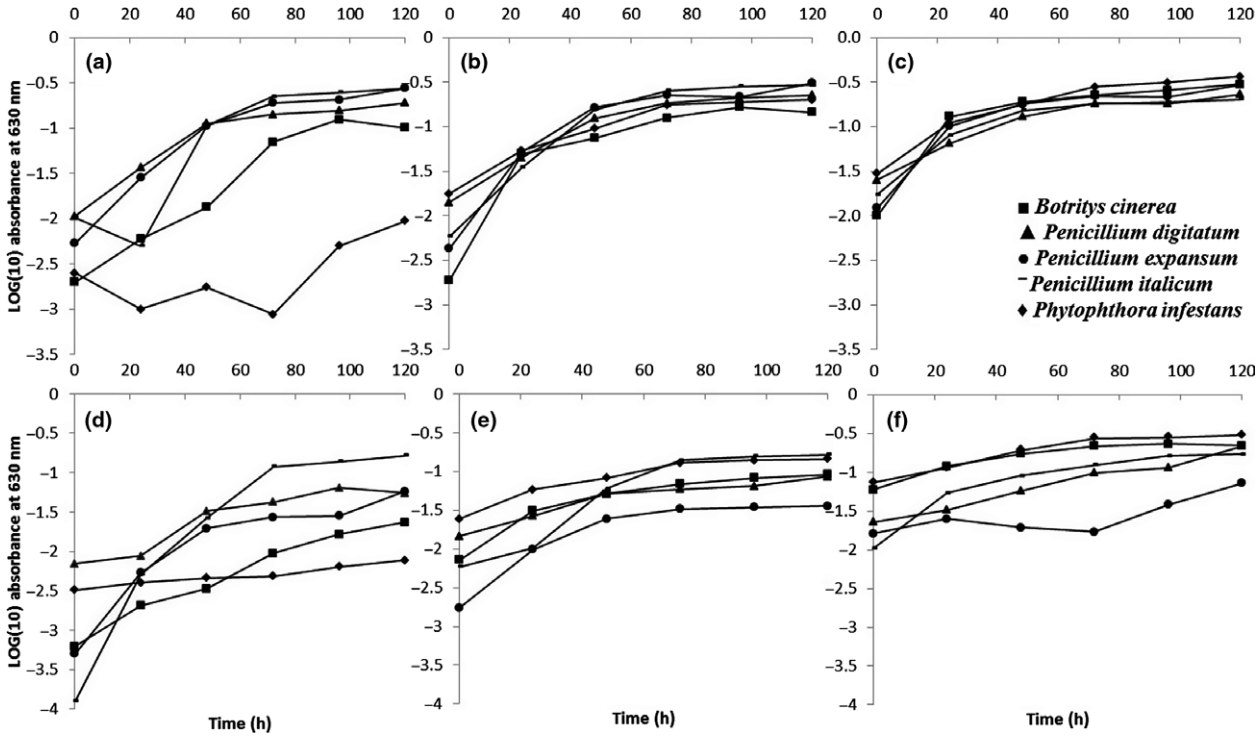

Fig. 1 Growth curves of *Botrytis cinerea*, *Penicilium digitatum*, *Penicilium expansum*, *Penicilium italicum* and *Phytophthora infestans* at 10^4 (a or d), 10^5 (b or e) and 10^6 (c or f) spores/ml in potato dextrose broth diluted threefold (a, b or c) or 30-fold (d, e or f). Optical density is expressed in Log10.

Table 1 Substrate concentration and ratio between iodide and thiocyanate before enzymatic concentration, substrate and ion concentrations after enzymatic reaction and percentage of substrates consumed. Mean of five independent experiences with standard deviations. KI, potassium iodide; KSCN, potassium thiocyanate; H_2O_2, hydrogen peroxide; SCN^-, thiocyanate ion; I^-, iodide ion

Before enzymatic reaction		After enzymatic reaction						
KI/KSCN/H_2O_2 (mM/l)	Ratio KI/KSCN	Ions (μM/l)	SCN^- (mM/l)	% used	H_2O_2 (mM/l)	% used	I^- (mM/l)	% used
5.4/1.2/6.6	4.5	400 ± 32	0.69 ± 0.2	42%	0.05 ± 0.01	99%	3.2 ± 0.5	40%
3.6/0.8/4.4	4.5	310 ± 26	0.52 ± 0.1	34%	0.01 ± 0	99%	2.5 ± 0.4	32%
2.7/0.6/3.3	4.5	234 ± 24	0.40 ± 0.03	34%	0.01 ± 0	99%	1.8 ± 0.2	35%
0.78/0.34/1.1	2.29	110 ± 11	0.18 ± 0.01	46%	0 ± 0	100%	0.5 ± 0.1	27%

Table 2 Lactoperoxidase concentration, substrate concentration before enzymatic reaction, dilutions applied and percentages of *in vitro* inhibition of *Botrytis cinerea*, *Penicilium expansum*, *Phytophthora infestans*, *Penicilium digitatum and Penicilium italicum* by the lactoperoxidase system produced ions after 120-h incubation. Inhibition percentages are the means of two independent assays. Fisher's comparison of means was applied at 99% confidence; means that do not share a letter are significantly different. ns, not significantly different from the control

LPO U/ml	KI/KSCN/H_2O_2 (mM/l)	Dilution	*Botrytis cinerea* % inhibition at 120 h	*Penicillium expansum* % inhibition at 120 h	*Phytophthora infestans* % inhibition at 120 h	*Penicillium digitatum* % inhibition at 120 h	*Penicillium italicum* % inhibition at 120 h
50	5.4/1.2/6.6	1/3	88^a	87^a	63^a	100^a	100^a
		1/5	84^a	86^a	66^a	38^{ns}	100^a
		1/10	83^a	62^b	72^a	22^{ns}	97^a
50	3.6/0.8/4.4	1/3	88^a	10^{ns}	69^a	0^{ns}	100^a
		1/5	88^a	0^{ns}	61^a	3^{ns}	77^a
		1/10	70^a	0^{ns}	69^a	7^{ns}	48^b
50	2.7/0.6/3.3	1/3	84^a	56^b	63^a	0^{ns}	81^a
		1/5	81^a	0^{ns}	72^a	0^{ns}	13^{ns}
		1/10	24^b	0^{ns}	67^a	0^{ns}	13^{ns}
50	0.78/0.34/1.1	1/3	90^a	0^{ns}	35^{ns}	0^{ns}	19^{ns}
Effect of substrates							
0	0/0/6.6	1/3	0^{ns}	0^{ns}	14^{ns}	6^{ns}	7^{ns}
0	5.4/1.2/0	1/3	0^{ns}	0^{ns}	11^{ns}	0^{ns}	0^{ns}

In apple and citrus fruit, no phytotoxicity was observed after the 24 days of incubation at the highest concentration and the same ion ratio.

In vivo results

Efficacy against Phytophthora infestans

Based on the previous results, the ratio 1:4.5 between thiocyanate and iodide with the highest concentration of substrates was selected and diluted twofold, fourfold or eightfold before being tested in controlled conditions. Three independent trials were carried out. Results are described in Fig. 3. They show that diluted ions reduced late blight symptoms and offered similar or better curative protection than the copper fungicide. Whatever the ion dilution, no phytotoxicity was recorded.

Statistical test results are presented in Table 3. All treatments significantly reduced the development of symptoms on treated leaves. Although dilution 1/8 reduced late blight symptoms as compared to the control, it was less efficient, with low protection rates in two trials of three. Protection rates at the ¼ dilution varied from 36 to 65%. Finally, the lowest dilution (1/2) provided 60–74% protection and offered comparable protection to the copper fungicide.

Efficacy against postharvest pathogens

The ions were also tested against postharvest pathogens of apple and citrus fruit. Results against *B. cinerea and P. expansum* on Golden Delicious apples are shown in Tables 4 and 5, respectively. Results against *P. digitatum* and *P. italicum* on organically-grown Valencia orange are shown in Tables 6 and 7, respectively.

Against *B. cinerea*, preventive ion applications did not protect apples whatever the concentration level of ions. In curative applications, fungal development was limited, following a substrate dose-effect: the

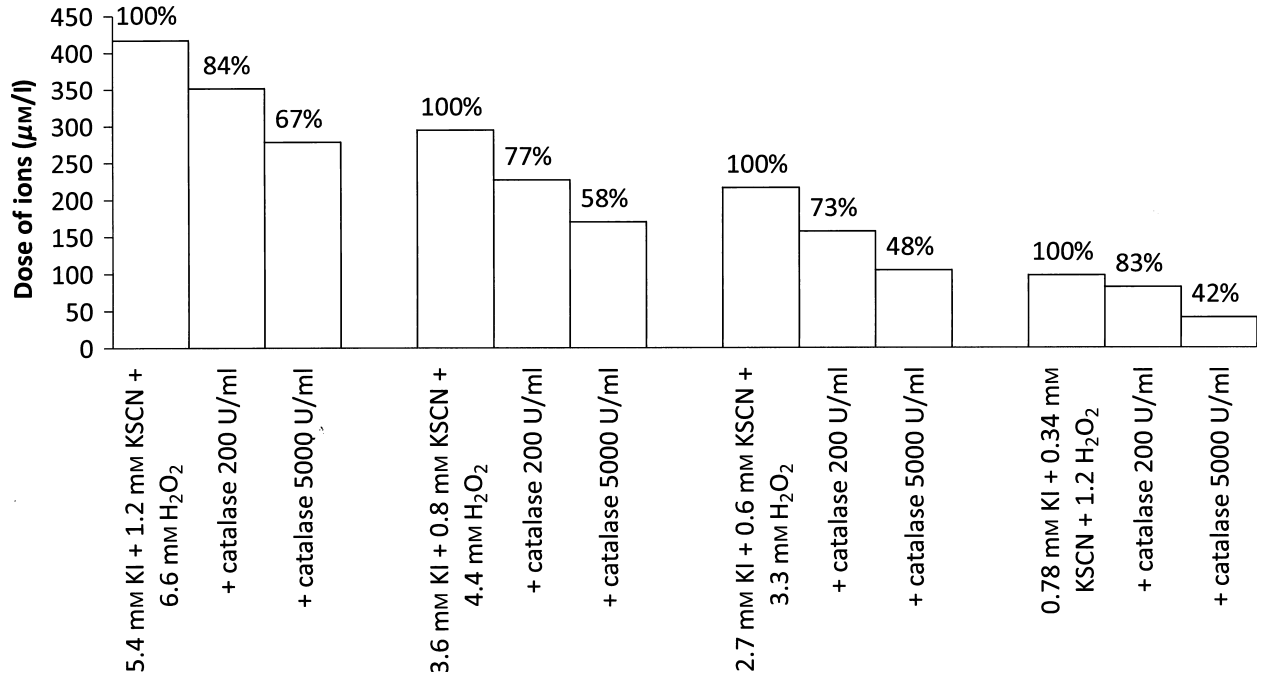

Fig. 2 Decrease in ion concentrations (µM/l) after incubation with catalase, expressed as % of the residual concentrations measured as compared to the catalase-free solution. Dosages were performed without catalase added and after 5-min incubation with 200 U or 5000 U of catalase/ml. Results are the means of two independent assays.

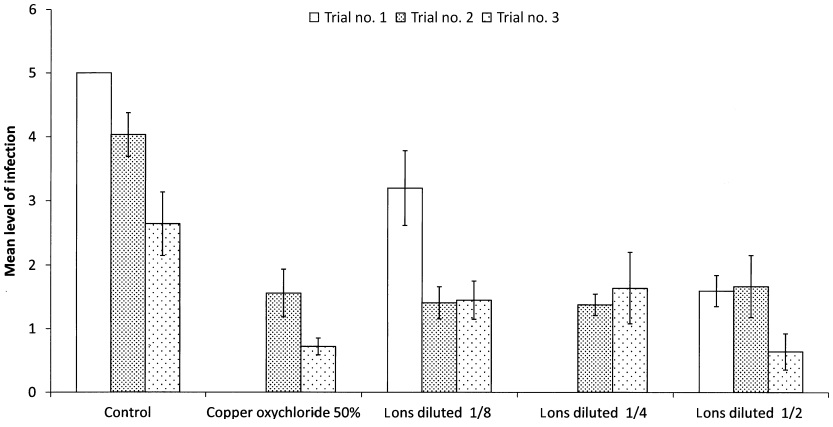

Fig. 3 Mean levels of *Solanum tuberosum* infection by *Phytophthora infestans* on day 24 in three trials. Trials 1 and 2: five replicates/treatment; trial 3: nine replicates/treatment. Control: water treatment; fungicide: copper oxychloride 50%; lactoperoxidase-produced ions prepared with [5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2] and diluted at three concentration levels (dilution 1/8; dilution 1/4; and dilution 1/2). Bars: standard errors.

Table 3 Mean levels of infection, protection rates (%) and Fisher's comparisons (95%) of means of *Phytophthora infestans* lesions on *Solanum tuberosum* in three trials. Protection rate (%) = [(Control - Treatment)/Control]*100. Ratio 1: lactoperoxidase oxidoreduction of (5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2); diluted 1/8, ¼ or ½

	Trial no 1			Trial no 2			Trial no 3		
Treatment	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate
Control	5	a		4	a		2.6	a	
Ratio 1 dil. 1/8	3.2	b	36%	1.4	b	65%	1.5	a, b	45%
Ratio 1 dil. 1/4	–		–	1.4	b	65%	1.6	a, b	38%
Ratio 1 dil. 1/2	1.6	c	68%	1.7	b	60%	0.6	b	74%
Copper oxychloride	–		–	1.6	b	61%	0.7	b	73%

Table 4 Mean levels of infection (mm) and Fisher's comparisons (99%) of means of *Botrytis cinerea* infection on Golden Delicious apples in two trials. Treatments were applied in preventive (24 h before inoculation) or curative (24 h after inoculation) application. Ratio 1: lactoperoxidase oxidoreduction of (5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2), diluted 1/5 or undiluted; ratio 2: ratio 1 with 1.5× more concentrated substrates

	Curative application						Preventive application					
	Trial no 1			Trial no 2			Trial no 1			Trial no 2		
Treatment	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate
Control	27	a		28	a		20	a		17	a	
Ratio 1 dil. 1/5	24	a	11%	–	–	–	19	a	6%	–	–	–
Ratio 1	13	b	51%	7	b	76%	20	a	0%	17	a	1%
Ratio 2	–	–	–	2	b	92%	–	–	–	13	b	26%
Imazalil pyrimethanil	6	c	79%	6	b	79%	0	b	100%	0	c	100%

Table 5 Mean levels of infection (mm) and Fisher's comparisons (99%) of means of *Penicillium expansum* infection on Golden Delicious apples in two trials. Treatments were applied in preventive (24 h before inoculation) or curative (24 h after inoculation) application. Ratio 1: lactoperoxidase oxidoreduction of (5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2), diluted 1/5 or undiluted; ratio 2: ratio 1 with 1.5× more concentrated substrates

	Curative application						Preventive application					
	Trial no 1			Trial no 2			Trial no 1			Trial no 2		
Treatment	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate
Control	30	a		16	a		18	a		13	a	
Ratio 1 dil. 1/5	26	a	12%	–	–	–	12	a	34%	–	–	–
Ratio 1	12	b	61%	2	b	88%	2	b	91%	4	b	73%
Ratio 2	–	–	–	3	b	84%	–	–	–	1	c	95%
Imazalil pyrimethanil	6	c	81%	3	b	82%	0	b	98%	0	c	100%

Table 6 Mean levels of infection (mm) and Fisher's comparisons (99%) of means of *Penicillium digitatum* infection on citrus fruit in two trials. Treatments were applied in preventive (24 h before inoculation) or curative (24 h after inoculation) application. Ratio 1: lactoperoxidase oxidoreduction of (5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2), diluted 1/5 or undiluted; ratio 2: ratio 1 with 1.5× more concentrated substrates

	Curative application						Preventive application					
	Trial no 1			Trial no 2			Trial no 1			Trial no 2		
Treatment	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate
Control	21	a		39	a		32	a		33	a	
Ratio 1 dil. 1/5	4	b	81%	–	–	–	25	a	23%	–	–	–
Ratio 1	1	b	95%	2	b, c	95%	13	b	60%	9	c	73%
Ratio 2	–	–	–	9	b	76%	–	–	–	20	b	39%
Imazalil pyrimethanil	0	b	100%	0	c	100%	0	c	99%	0	c	100%

highest ratio clustered with the synthetic fungicides imazalil and pyrimethanil ($P < 0.001$). Against *P. expansum,* both preventive and curative applications inhibited the fungus, with efficacy levels depending on the ion ratio. Again higher ratios clustered with imazalil and pyrimethanil ($P < 0.001$).

Table 7 Mean levels of infection (mm) and Fisher's comparisons (99%) of means of *Penicillium italicum* infection on citrus fruit in two trials. Treatments were applied in preventive (24 h before inoculation) or curative (24 h after inoculation) application. Ratio 1: lactoperoxidase oxidoreduction of (5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2), diluted 1/5 or undiluted; ratio 2: ratio 1 with 1.5× more concentrated substrates

	Curative application						Preventive application					
	Trial no 1			Trial no 2			Trial no 1			Trial no 2		
Treatment	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate	Infection (mm)	Fisher test	Protection rate
Control	11	a		44	a		17	a		43	a	
Ratio 1 dil. 1/5	8	b	33%	–	–	–	16	a	4%	–	–	–
Ratio 1	2	c	84%	7	b	84%	15	a	12%	35	b	19%
Ratio 2	–	–	–	8	b	81%	–	–	–	26	c	39%
Imazalil pyrimethanil	0	c	100%	0	c	100%	0	b	98%	0	d	100%

In citrus fruit, curative application against *P. digitatum* resulted in good inhibition of fungal growth and clustered with chemical fungicide ($P < 0.001$). The highest ratio was less efficient than the medium. Preventive application was less efficient than curative application. Against *P. italicum*, preventive application did not prevent infection. Curative application resulted in good inhibition of fungal growth for the highest ratios ($P < 0.001$).

Discussion

In vitro results

Enzymatic reaction

Lactoperoxidase is an oxidoreductase enzyme of the family of the mammalian haeme peroxidases characterized by identical reaction mechanisms (O'Brien 2000). The first reaction is the activation of the native form of lactoperoxidase through the reaction with hydrogen peroxide which is reduced into water. The enzyme, which has undergone a two-electron oxidation, is called Compound I. Compound I is reduced back to its native form through a two-electron transfer from a halogen or a pseudo-halogen (Pruitt and Tenovuo 1985). The two-step reaction is described as follows:

Peroxidase (native form) + H_2O_2 →
Compound I + H_2O

Compound I + X^- (halogen or pseudo-halogen) →
Peroxidase (native form) + OX^- (hypo(pseudo) halides)

All hypo(pseudo)halides (OX^-) are in an acid–base equilibrium association with their corresponding acid hypo(pseudo)halide (HOX). The acid form has a higher oxidation potential and is more soluble in non-polar media so that it passes through hydrophobic barriers such as cell membranes more easily but is less stable than the basic form (OX^-) (Pruitt and Tenovuo 1985; Ashby 2012). Thiocyanate and iodide are oxidized, respectively, in $OSCN^-$ and OI^- (de Wit and van Hooydonk 1996; Bosch et al. 2000). Hypoiodide, with a pKa of 10,6, is predominant as HOI in basic media, whereas hypothiocyanite with a pKa of 5.3 is predominant in the acid form in acid media (Thomas 1981; Ashby 2012). Both molecules react with the thiol moiety of enzymes and proteins, an essential moiety for the activity of numerous enzymes and proteins (Aune and Thomas 1978; Thomas and Aune 1978). OI^- can also react with thioethers, amine groups and NAD(P)H (Prütz et al. 2000).

If two substrates are present for the second step of reaction, there is a competition between both substrates to fix the enzyme. Thiocyanate is the favourite electron donor for lactoperoxidase which explains that when reducing the amount of iodide, the consumption of thiocyanate is favoured. Due to the high value of rate constants for the reaction, the reaction proceeds in milliseconds with, in our experimental conditions, 99–100% consumption of hydrogen peroxide, approximately 30–40% consumption of thiocyanate and iodide. H_2O_2 level after oxidation ranged from 66 to 0 μM/l. Thiocyanate levels used in the study (1.2–0.34 mM) are similar to physiological concentration found in saliva (0.5–6 mM) (Tenovuo and Makinen 1976). The concentration of oxidizing molecules is correlated with the concentration of substrates; higher levels of active molecules are obtained with higher concentration substrates (Table 1) and reveal that the enzymatic reaction has been correctly carried out *id est* hydrogen peroxide, KI and KSCN being consumed. The level of oxidant molecules in ion solutions was controlled at each production and

has to be identical with those described in Table 1 to be used in *in vitro* and *in vivo* experiments.

In vitro inhibition tests

H_2O_2 level after enzymatic oxidation does not exceed 66 μM/l (Table 1), and we have checked the role of peroxide hydrogen in the inhibition process by adding a treatment containing 6.6 mM H_2O_2, without lactoperoxidase nor iodide or thiocyanate. H_2O_2 is a relatively stable molecule with slow reactivity towards biological molecules. It has already been showed that *in vitro* 2 mM H_2O_2 treatment against *P. digitatum*, *P. expansum* and *P. italicum* did not affect conidia germination or fungal growth (Kim and Chan 2014; Buron-Moles et al. 2015). In our study, we show that 120-h incubation with 2.2 mM H_2O_2 (final concentration, see Materials and Methods) has no effect on strain growth (Table 2). Also we measured the inhibition capacity of iodide and thiocyanate with a treatment containing 5.4 mM KI and 1.2 mM KSCN, without H_2O_2 nor lactoperoxidase, and no inhibition was measured on strains growth (Table 2). As the substrates alone did not inhibit the growth of pathogens, we can afford that the inhibition results observed in Table 2 are only due to the produced ions.

Our results show that better inhibition is obtained with higher ion concentration and that all fungi are not equally susceptible to the lactoperoxidase system, even within a same genus. This could indicate that some pathogens are able to buffer the ions or to recover if the concentration is not sufficiently high.

We have shown that in the presence of catalase, the ion concentrations diminish. It has been showed that OCl^-, an ion produced by myeloperoxidase in the presence of chloride and H_2O_2, rapidly inactivated catalase by binding the haeme site of the enzyme (Mashino and Fridovich 1988). The ions $OSCN^-$ and OI^- are weaker oxidants as compared to OCL^-, but a reaction with the haeme site or with a cysteine of the enzyme could be possible.

When pathogens face a stress, reactive oxygen species (ROS) and antioxidant enzymes such as catalase and superoxide dismutase are produced. The differences in susceptibility to ions of the tested pathogens could be related with variability in ROS defence mechanisms between species. *In vitro* and in fruits, studies have shown that *P. digitatum* is more resistant to H_2O_2 than other *Penicillium* species and that *P. infestans* and *B. cinerea* are sensitive to H_2O_2. It was hypothesized that *P. digitatum* production of catalase suppressed citrus wound-induced H_2O_2 production and is less sensitive to H_2O_2 than *P. expansum* in *in vitro* experiments (Macarisin et al. 2007; Buron-Moles et al. 2015). *In vitro* tests showed that *P. italicum* had similar susceptibility to H_2O_2 as *P. expansum* (Kim and Chan 2014). In apples artificially inoculated with *B. cinerea*, the authors demonstrated that the reduction in wound-induced defence in late-harvested apples was due to the decrease in wound-induced H_2O_2 production that goes along with ripening (Su et al. 2011). Transgenic potato plants that generate high levels of H_2O_2 in leaf and tuber tissues were resistant to *P. infestans,* but resistance was counteracted by exogenously added catalase (Wu et al. 1995).

Resistance to ions could also be linked to the production of glutathione, a small peptide containing a thiol moiety. Glutathione is an altruistic metabolite present in yeast and filamentous fungi (Pócsi et al. 2004). Glutathione is an important antioxidant molecule and reacts among others with OCL^-, $OSCN^-$ or OI^-, which can buffer their antimicrobial action (Kussendrager and van Hooijdonk 2000; Prütz et al. 2000). An increased production of catalase or glutathione production could account for partial *P. digitatum* resistance observed in *in vitro* tests.

In vivo efficacy tests

Potato plant pathogen

A protective effect against *P. infestans* was noted, particularly following application of the twofold-diluted first ratio, and to a lesser extent of the other dilutions of that first ratio. Protection rates were statistically equivalent to protection by copper oxychloride. A single spray did not allow the ions to completely destroy the pathogen, as copper, which is a residual fungicide, does. The pH of the ion solution is neutral which means that hypothiocyanite is present in the anionic form although hypoiodide is present in the acidic form. Anionic molecules are not believed to pass through hydrophobic barriers such as biological membranes, but the small size of $OSCN^-$ could offset this hurdle. HOI could pass more easily through hydrophobic cellulose pathogen membranes and oxidize any molecules containing amine, thioether or thiol groups, resulting in non-specific denaturation of the respiration chain and glycolysis enzymes. Our results are encouraging, and it will be interesting to evaluate ion efficacy in field conditions. The ions were in aqueous solution without any additives. Consequently, it would be worthwhile to work on formulation to ensure better resilience whatever the weather conditions. In field conditions, applying fungicides up

to the end of haulm desiccation is necessary with 7-day intervals between applications in many cases, and even less under high disease pressure (Cooke et al. 2011). A spreading and a fixing agent would increase the chances for the ions to be maintained on the leaves for several days. It would also be interesting to test the ions in combination with a translaminar fungicide.

Increasing concentration of potassium ions through the xylem of plants is known to increase flow rates in the vessels and to contribute to the regulation of water flow through the plants (Zwieniecki et al. 2001). The impact of K^+ derived from the lactoperoxidase system could be a possible cofactor beneficial for the reduction in infection but the possible penetration through the cuticle of K^+ in the xylem has to be studied. A different way of distribution of ions, for example through roots, could also be relevant. Differences in size and density of vessels between olive and kiwifruit species are part of hydraulic adaptations of *Olea europaea* (L.) to Mediterranean ecosystems and underlined the importance of water flow through the stem (Dichio et al. 2013). Interaction between plant species-specific structures, functions of xylem and ions derived from the lactoperoxidase system could be of interest to study, especially for some xylem-limited pest such as *Xylella fastidiosa*.

Postharvest pathogen

Apple blue and grey moulds are pests that cause postharvest decay via airborne and waterborne spores. Infections by the blue mould *P. expansum* originate from wounds, stem-end invasions, corerots or lenticels. They can produce patulin, a mycotoxin, known to be a virulence factor (Sanzani et al. 2012). Blue mould is the most widespread wound pathogen, even if its incidence is now lower thanks to modern controlled atmosphere storage, sanitization, soft handling methods and postharvest fungicides. A wound is also necessary for infection by *B. cinerea,* which causes grey mould on fruit. Wounds can be caused by biotic factors such as harvest, transport, packing-house handling or insects and abiotic factors such as rain, wind and hail (Vilanova et al. 2013). The postharvest disease starts from a decayed fruit next to healthy fruit. High-dose ions can efficiently control grey mould in curative applications, but not in preventive applications. *Botrytis cinerea* seems to be more resistant to ions in preventive treatment than *P. expansum,* suggesting a different way of action or a different defence mechanism from the pathogen when there is a contact between the pathogen and a natural matrix.

In citrus fruit, *P. digitatum* and *P. italicum* are the causal agents of green and blue moulds and develop through injuries. Both pathogens are ubiquitous organisms, commonly found in soil, water, air and fresh and processed foods. Those characteristics contribute to high inoculum during all steps of harvesting, handling, storage, packing-house and transport chain (Louw and Korsten 2015). The green mould in citrus fruit can account for 90% of total losses during the postharvest chain (Louw and Korsten 2015). In orange, curative application against *P. digitatum* resulted in good inhibition of fungal growth. The highest ratio was less efficient than the medium ratio perhaps due to non-phenotypic phytotoxicity. Preventive application was less efficient than curative application. Against *P. italicum*, preventive application did not prevent infection. Curative application resulted in good inhibition of fungal growth for the highest ratios.

These results show that the pathogen and the ions do not react in the same way as *in vitro* when they are in contact with fruit. Preventive application did not inhibit decay, except in the case of *P. expansum* infection. As the ions proved efficient in one preventive model, the lack of efficiency in the other preventive models cannot be explained by short shelf life or ion instability. In preventive application, the fruits were wounded and treated with water or ions, and the wounds were infected by one pathogen 24 h later. This allows the fruit to initiate the oxidative stress response with production of reactive oxygen species (ROS) such as H_2O_2 (Torres 2006; Torres et al. 2011). In parallel, different antioxidant enzymes are produced, such as superoxide dismutase and catalase (Torres et al. 2011). Catalase activity increases from 4–5 h to 15–24 h after wounding and then decreases (Torres et al. 2003, 2011).This increased activity could be responsible for the loss of efficacy in preventive application, as shown in our *in vitro* results. Moreover, $OSCN^-$ reacts with H_2O_2, so that this could increase ion consumption before inoculation (Pruitt and Tenovuo 1985). Fruit maturity is also an important factor in ROS production because H_2O_2 production decreases during the ripening process. Moreover, catalase activity differs between oranges wounded immediately after commercial harvest (catalase activity increases 4 h up to 72 h after wounding) and oranges harvested at the same date but stored 2 months at 4°C before being wounded (catalase activity only increases 72 h after the injury) (Torres et al. 2011). Differences in maturity and/or storage conditions between the fruit batches used for *P. expansum* tests could explain the different results. They

could also be explained by a lower capacity of the fungus to produce catalase or to buffer oxidant molecules such as ions.

The production of the active solution has been kept as simple as possible with a minimum of ingredients to fit agricultural practices. To that end, we used tap water. Nevertheless as enzyme works better with an enzyme-specific buffer such as phosphate or citrate buffer, it would be interesting to check the efficacy of the active solution produced with an optimized buffer instead of tap water.

Human saliva contains levels of SCN^- up to 6 mM in smokers and up to 3 mM in non-smokers, which is higher than the concentration of 1.2 mM used in our first ratio (Schultz et al. 1996). Mouth levels of $OSCN^-$ range between 20 and 60 μM, but the ion is rapidly degraded (Tenovuo et al. 1982; Nagy et al. 2009). Commercial toothpaste using the lactoperoxidase system contains a similar HOSCN concentration to the ratio 5.4 mM I^-/1.2 mM SCN^- (Lenander-Lumikari et al. 1993). HOSCN ions are lowly or non-toxic in human saliva at levels that are used in the present study. These observations lead us to consider these ions as a soft biochemical method for controlling plant pathogens. We demonstrate for the first time that this biochemical method is as efficient as conventional chemical pesticides under controlled conditions. Our results also show that the oxidative stress response of fruit after wounding could interfere with ion efficiency. Pest protection and repeated applications in both preventive and curative applications will have to be tested in glasshouse and field conditions for potato crops and under packing-house conditions for harvested fruits.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

Akino S, Takemoto D, Hosaka K. (2013) *Phytophthora infestans*: a review of past and current studies on potato late blight. J Gen Plant Pathol 80:24–37.

Ashby MT. (2012) Hypothiocyanite. Adv Inorg Chem 64:263–303.

Aune TM, Thomas EL. (1978) Oxidation of protein sulfhydryls by products of peroxidase-catalyzed oxidation of thiocyanate ion. Biochemistry 17:1005–1010.

Bafort F, Barthelemy J-P, Parisi O, Perraudin J-P, Jijakli H. (2014) Development of a colorimetric method for the dosage of OI^- anions and I_2 in aqueous media. Commun Agric Appl Biol Sci 79:155–160.

Bosch EH, van Doorne H, de Vries S. (2000) The lactoperoxidase system: the influence of iodide and the chemical and antimicrobial stability over the period of about 18 months. J Appl Microbiol 89:215–224.

Buron-Moles G, Torres R, Teixidó N, Usall J, Vilanova L, Viñas I. (2015) Characterisation of H_2O_2 production to study compatible and non-host pathogen interactions in orange and apple fruit at different maturity stages. Postharvest Biol Technol 99:27–36.

Cooke LR, Schepers HTAM, Hermansen A et al. (2011) Epidemiology and Integrated Control of Potato Late Blight in Europe. Potato Res 54:183–222.

Dichio B, Montanaro G, Sofo A, Xiloyannis C. (2013) Stem and whole-plant hydraulics in olive (*Olea europea*) and kiwifruit (*Actinidia deliciosa*). Trees 27:183–191.

Hawkins CL. (2009) The role of hypothiocyanous acid (HOSCN) in biological systems. Free Radic Res 43:1147–1158.

Ismail M, Zhan J. (2004) Post-harvest citrus diseases and their control. Outlooks Pest Manag 15:29–35.

Jijakli MH, Lepoivre P. (2004) State of the art and challenges of post-harvest disease management in apples. In: Mukerij KG. (ed) Fruit and Vegetable Diseases. New York, Springer Netherlands, pp 59–94.

Kim JH, Chan KL. (2014) Augmenting the antifungal activity of an oxidizing agent with kojic Acid: control of *penicillium* strains infecting crops. Molecules 19:18448–18464.

Kouassi HS, Bajji M, Brostaux Y, Zhiri A, Samb A, Lepoivre P, Jijakli MH. (2012) Développement et application d'une méthode de microplaques pour évaluer l'efficacité d'huiles essentielles contre *Penicillium italicum Wehmer*, *Penicillium digitatum Sacc.* et *Colletotrichum musea* (Berk. & M.A. Curtis) Arx, trois champignons pathogènes de post-récolte sur fruits. Biotechnol Agron Soc Environ 16:325–336.

Kussendrager KD, van Hooijdonk AC. (2000) Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. Br J Nutr 84(Suppl 1): S19–S25.

Ladaniya M. (2010) Citrus Fruit: Biology, Technology And Evaluation. San Diego CA, USA, Academic Press.

Lahti M, Vilpo J, Hovinen J. (2000) Spectrophotometric determination of thiocyanate in human saliva. J Chem Educ 76:1281–1282.

Lenander-Lumikari M, Tenovuo J, Mikola H. (1993) Effects of a lactoperoxidase system-containing toothpaste on levels of hypothiocyanite and bacteria in saliva. Caries Res 27:285–291.

Louw JP, Korsten L. (2015) Pathogenicity and host susceptibility of *Penicillium* spp. on Citrus. Plant Dis 99:21–30.

Macarisin D, Cohen L, Eick A, Rafael G, Belausov E, Wisniewski M, Droby S. (2007) *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of Citrus fruit. Phytopathology 97:1491–1500.

Mashino T, Fridovich I. (1988) Reactions of hypochlorite with catalase. Biochim Biophys Acta - Protein Struct Mol Enzymol 956:63–69.

Moskwa P, Lorentzen D, Excoffon KJDA, Zabner J, McCray PB Jr, Nauseef WM, Dupuy C, Bánfi B. (2007) A novel host defense system of airways is defective in cystic fibrosis. Am J Respir Crit Care Med 175:174–183.

Nagy P, Jameson GN, Winterbourn CC. (2009) Kinetics and mechanisms of the reaction of hypothiocyanous acid with 5-thio-2-nitrobenzoic acid and reduced glutathione. Chem Res Toxicol 22:1833–1840.

O'Brien PJ. (2000) Peroxidases. Chem Biol Interact 129:113–139.

Pócsi I, Prade RA, Penninckx MJ. (2004) Glutathione, altruistic metabolite in fungi. Adv Microb Physiol 49:1–76.

Pruitt KM, Tenovuo JO. (1985) The lactoperoxidase system: chemistry and biological significance. In: Pruitt KM, Tenovuo JO. (eds) Immunology Series. New York, CRC Press, pp 15–177.

Prütz WA, Kissner R, Koppenol WH, Rüegger H. (2000) On the irreversible destruction of reduced nicotinamide nucleotides by hypohalous acids. Arch Biochem Biophys 380:181–191.

Sanzani SM, Reverberi M, Punelli M, Ippolito A, Fanelli C. (2012) Study on the role of patulin on pathogenicity and virulence of *Penicillium expansum*. Int J Food Microbiol 153:323–331.

Schöber B. (1992) *Phytophthora infestans*, the cause of late blight of potato. Potato Res 35:78.

Schultz CP, Ahmed MK, Dawes C, Mantsch HH. (1996) Thiocyanate levels in human saliva: quantitation by Fourier transform infrared spectroscopy. Anal Biochem 240:7–12.

Sedláková V, Dejmalová J, Hausvater E, Sedlák P, Doležal P, Mazáková J. (2011) Effect of *Phytophthora infestans* on potato yield in dependence on variety characteristics and fungicide control. Plant, Soil Environ 57:486–491.

Su J, Tu K, Cheng L, Tu S, Wang M, Xu H, Zhan G. (2011) Wound-induced H_2O_2 and resistance to *Botrytis cinerea* decline with the ripening of apple fruit. Postharvest Biol Technol 62:64–70.

Tenovuo J, Makinen KK. (1976) Concentration of thiocyanate and ionizable iodine in saliva of smokers and nonsmokers. J Dent Res 55:661–663.

Tenovuo J, Pruitt KM, Thomas EL. (1982) Peroxidase antimicrobial system of human saliva: hypothiocyanite levels in resting and stimulated saliva. J Dent Res 61:982–985.

Thomas EL. (1981) Lactoperoxidase-catalyzed oxidation of thiocyanate: equilibria between oxidized forms of thiocyanate. Biochemistry 20:3273–3280.

Thomas EL, Aune TM. (1978) Lactoperoxidase, peroxide, thiocyanate antimicrobial system: correlation of sulfhydryl oxidation with antimicrobial action. Infect Immun 20:456–463.

Torres MA. (2006) Reactive Oxygen Species Signaling in Response to Pathogens. Plant Physiol 141:373–378.

Torres R, Valentines MC, Usall J, Viñas I, Larrigaudiere C. (2003) Possible involvement of hydrogen peroxide in the development of resistance mechanisms in "Golden Delicious" apple fruit. Postharvest Biol Technol 27:235–242.

Torres R, Teixidó N, Usall J, Abadias M, Mir N, Larrigaudiere C, Viñas I. (2011) Anti-oxidant activity of oranges after infection with the pathogen *Penicillium digitatum* or treatment with the biocontrol agent *Pantoea agglomerans* CPA-2. Biol Control 57:103–109.

Välimaa H, Waris M, Hukkanen V, Blankenvoorde MFJ, Nieuw Amerongen AV, Tenovuo J. (2002) Salivary defense factors in herpes simplex virus infection. J Dent Res 81:416–421.

Vilanova L, Torres R, Viñas I, González-Candelas L, Usall J, Fiori S, Solsona C, Teixidó N. (2013) Wound response in orange as a resistance mechanism against *Penicillium digitatum* (pathogen) and *P. expansum* (non-host pathogen). Postharvest Biol Technol 78:113–122.

Welk A, Meller C, Schubert R, Schwahn C, Kramer A, Below H. (2009) Effect of lactoperoxidase on the antimicrobial effectiveness of the thiocyanate hydrogen peroxide combination in a quantitative suspension test. BMC Microbiol 9:134.

Wiik L. (2014) Potato late blight and tuber yield: results from 30 Years of field trials. Potato Res 57:77–98.

de Wit JN, van Hooydonk ACM. (1996) Structure, functions and applications of lactoperoxidase in natural antimicrobial systems. Ned melk en Zuiveltijdschr 50:227–244.

Wu G, Shortt BJ, Lawrence EB, Levine EB, Fitzsimmons KC, Shah DM. (1995) Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357–1368.

Yuen JE, Andersson B. (2013) What is the evidence for sexual reproduction of *Phytophthora infestans* in Europe? Plant Pathol 62:485–491.

Zwieniecki MA, Melcher PJ, Holbrook NM. (2001) Hydrogel control of xylem hydraulic resistance in plants. Science 291:1059–1062.