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Iodus 40, salicylic acid, heptanoyl salicylic acid and trehalose exhibit different efficacies and defence targets during a wheat/powdery mildew interaction

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Abstract

Prophylactic efficacies of Iodus 40 and salicylic acid (SA) against wheat powdery mildew caused by *Blumeria graminis* f. sp. *tritici* have been shown and compared with those of heptanoyl salicylic acid (HSA) and trehalose. Plantlets treated once exhibited 55%, 50%, 95%, and 38% protection levels, respectively. Two sprayings increased these levels up to 60%, 65%, 100%, and 60%, respectively. Biological effects of these resistance inducers on reactive oxygen species (ROS) metabolism and lipid peroxidation were also investigated. We found clear differences in the extent and the type of induced responses, with HSA exhibiting both the most numerous and the highest effects. HSA and SA induced a 5.5-fold increase of whole cell DAB staining due to hydrogen peroxide accumulation, whereas Iodus 40 and trehalose increased staining intensity at the penetration sites only. However, these effects were not correlated with any modification of catalase (CAT), oxalate oxidase (OXO) or lipoxygenase (LOX) activities, except for HSA which decreased CAT in non-inoculated conditions and increased LOX in infectious conditions. HSA also induced an increase in the rate of lipid peroxidation, whereas Iodus 40 induced a decrease. The effects of the inducers on germinating conidia and wheat epidermal cells responding to fungal penetration were also investigated. Papilla-linked autofluorescence was affected by SA and Iodus 40 whereas germination was slightly altered by Iodus 40. The newly described protective efficacies and the partial, distinct and non-overlapping activities of these inducers on the wheat/powdery mildew interaction are discussed.

Keywords: *Blumeria graminis*; Resistance inducer; *Triticum aestivum*

1. Introduction

The obligate parasite *Blumeria graminis* (DC.) E.O. Speer f. sp. *tritici* is responsible for powdery mildew, one of the most damaging foliar diseases on wheat (*Triticum aestivum* L.). If an extensive use of fungicides was not undergone, this disease would be responsible for worldwide heavy losses. New control strategies based on the use of resistance inducers offer an opportunity to overcome the negative impact of conventional fungicides on the environment, but also on human health (Walters et al., 2005). Commercial inducers that target at wheat powdery mildew have been released in the past few years. The first of them was benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), synonymous of acibenzolar-*S*-methyl (ASM), a structurally-related functional analogue of salicylic acid (SA) (Görlach et al., 1996). Numerous works that showed its wide effectiveness have been performed within the last 6–7 years (Walters et al., 2005). In the last fifteen

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years, SA itself has been described as playing a key role in the activation of defence systems against pathogens in plants. Despite several reports (Spletzer and Enyedi, 1999; Saikia et al., 2003; Sparla et al., 2004; Galis et al., 2004), works focusing on SA as a resistance inducer are far from being as extensive as those concerning BTH and they do not involve wheat. However, heptanoyl salicylic acid (HSA), which has been synthesized by esterification of 2-OH benzoic acid with heptanoic acid, was recently shown to induce protection on wheat powdery mildew (Muchembled et al., 2006). Iodus 40 is another commercial product that has been released in France several years ago. It is used to decrease wheat powdery mildew damages in the field and to reduce the applications of conventional fungicides. Its active ingredient is a polysaccharide, which consists of β-1,3-D-glucan, a laminarin extracted from the brown algae *Laminaria digitata*. It has been shown to induce a protection of grapevine against *Botrytis cinerea* and *Plasmopara viticola* (Aziz et al., 2003). However, no data are currently available about the efficacy or any biological activities of Iodus 40 on wheat, which is its target crop. Trehalose, a nonreducing disaccharide found in a wide variety of organisms, induces resistance on wheat against powdery mildew (Reignault et al., 2001; Muchembled et al., 2006). Reignault et al. (2001) showed that trehalose activates wheat defences such as papilla deposition, and phenylalanine ammonia lyase (PAL) peroxydase (PO) activities.

We recently showed the efficacy of Milsana, an ethanolic extract from leaves of the giant knotweed *Reynoutria sachalinensis*, on wheat powdery mildew, altogether with its partial defence-inducing activity and its direct fungistatic effect (Randoux et al., 2006). This product was initially considered as an elicitor of plant defences but exhibited a weak defence-inducing activity and a direct fungistatic activity. There is therefore an urgent need in understanding the actual extent of resistance inducers biological activities. For these reasons, we examined a range of potential defence targets for HSA, SA, Iodus 40 and trehalose. The aims of the present work were as follows: (i) to test and to measure the efficacies of SA and Iodus 40 and to compare them with those of trehalose and HSA on the wheat/powdery mildew pathosystem in controlled laboratory conditions, (ii) to evaluate the ability of the four inducers to modulate wheat defence responses linked to reactive oxygen species (ROS) metabolism and lipid peroxidation, (iii) to test for any effect on fungal penetration and conidia germination. To our knowledge, this work is the first one to show and measure the efficacies of SA and Iodus 40 and to provide first evidences for the biological effects of Iodus 40 on this interaction. It is also the first comparison of four distinct inducers using a single experimental procedure at the laboratory scale. Distinct physiological targets of these inducers on wheat plantlets have been found out. We also show here how effective is the molecular modification of SA that led to HSA in terms of biological activities.

2. Results

2.1. Iodus 40, salicylic acid, heptanoyl salicylic acid and trehalose exhibit different efficacies

Plantlets were sprayed with HSA, SA, Iodus 40 and trehalose solutions or with the corresponding controls. After one treatment, partial resistance was obtained with Iodus 40, SA and trehalose with protection levels of 55%, 50% and 38%, respectively (Fig. 1a and b). However, a high, almost total (95%) protection was obtained with HSA (Fig. 1b). The effects of two treatments were also investigated. Partial resistances were obtained again with Iodus 40, SA and trehalose, but with a higher efficacy and within a smaller range of values: both Iodus 40 and trehalose led to a 60% protection whereas SA showed a 65% efficacy. After two sprayings instead of one, HSA treatment resulted in a total protection against wheat powdery mildew.

2.2. Iodus 40, SA, HSA and trehalose induce different modifications in H_2O_2 accumulation, CAT and LOX activities, and lipid peroxidation

We used DAB staining and microscopy to analyze the accumulation of H_2O_2 associated with fungal penetration in epidermal cells. We performed syringe infiltration as a treatment instead of spraying. This allowed us to ensure that each penetration event evaluated for the DAB staining intensity did occur in a cell that was subjected to the tested inducer. The water-treated control infiltrated leaves exhibited the class 0 (no staining, 19.5%) class 1 (weak staining, 56.2%) and class 2 (average staining, 22%) staining intensities. For the Iodus 40 and the trehalose-infiltrated samples, class 1 dropped to 28% and 29.5%, respectively, whereas class 2 became more frequent (50% and 46.1%, respectively). We therefore observed variations in the size of the staining intensity class population that were induced by these treatments: the highest intensity class increased, whereas the lowest intensity class decreased (Fig. 2a). Such a shift towards higher DAB staining intensities was not observed neither for the SA treatments compared to the water control, nor the HSA treatments compared to the 30% ethanol control (data not shown). However, while examining these samples under light microscopy, the DAB staining revealed a higher frequency of entirely stained cells. For the water and 30% ethanol control samples, 3% and 6% of entirely stained cells were observed. These proportions rose to 16% and 33% in the SA and the HSA-treated samples, respectively. SA and HSA therefore both induced a 5.5-fold increase of the number of cell responding to fungal penetration with a global DAB staining (Fig. 2b).

In order to further investigate the effect of the treatments on the ROS metabolism, we assessed oxalate oxidase (OXO) and catalase (CAT) activities (Bolwell and Wojtaszek, 1997), as well as lipoxygenase (LOX) activity which, besides ROS metabolism, is involved in lipid peroxidation

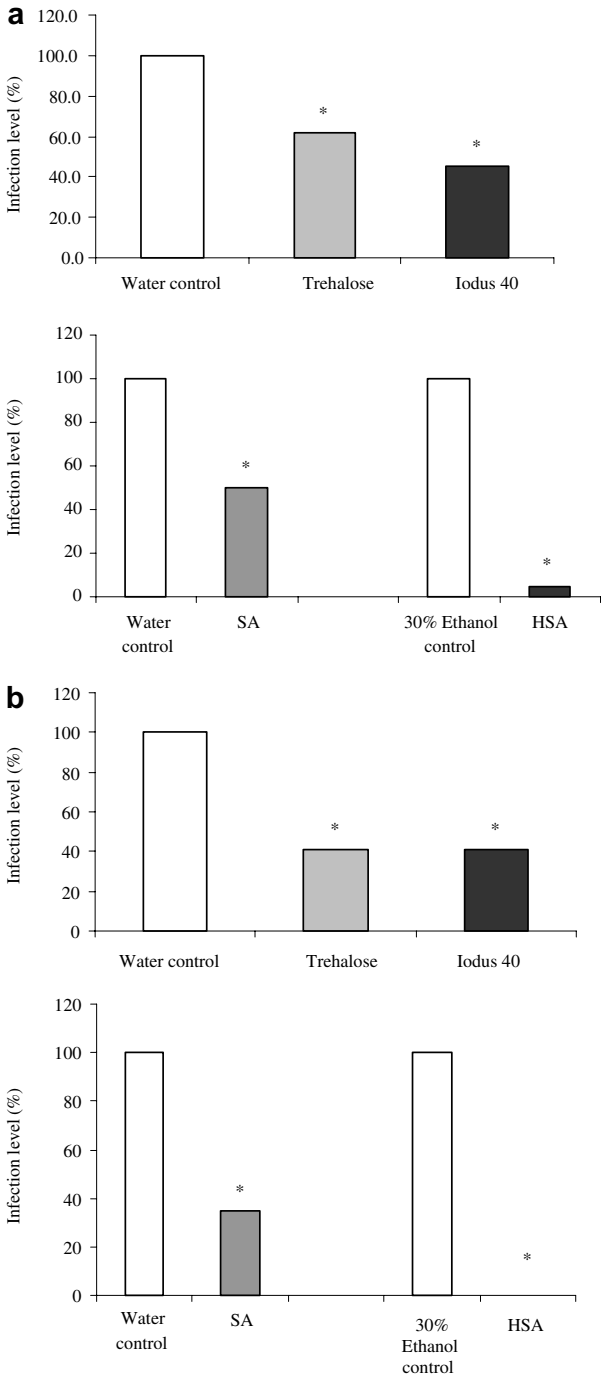

Fig. 1. Efficiency of preventive Iodus 40, SA and trehalose treatments on wheat plantlets compared to the distilled water control and of HSA compared to the 30% ethanol control. The efficiencies of Iodus 40 and trehalose have been compared to each other using the same control whereas efficiencies of SA and HSA were compared using their own controls. All data represent means of three independent series of experiments. In each experiment, 10 sprayed leaves from controls and treated plants were randomly collected. Depending on the experiment, the mean number of sporulating colonies on the control leaves ranged between 70 and 140. The number of sporulating powdery mildew colonies were therefore used to determine the percentage of infection following each treatment compared to the corresponding control. A 100% infection level was assigned to each control. Bars with an asterisk are significantly different from the corresponding control. a. Efficiencies of one single spraying. b. Efficiencies of two distinct sprayings.

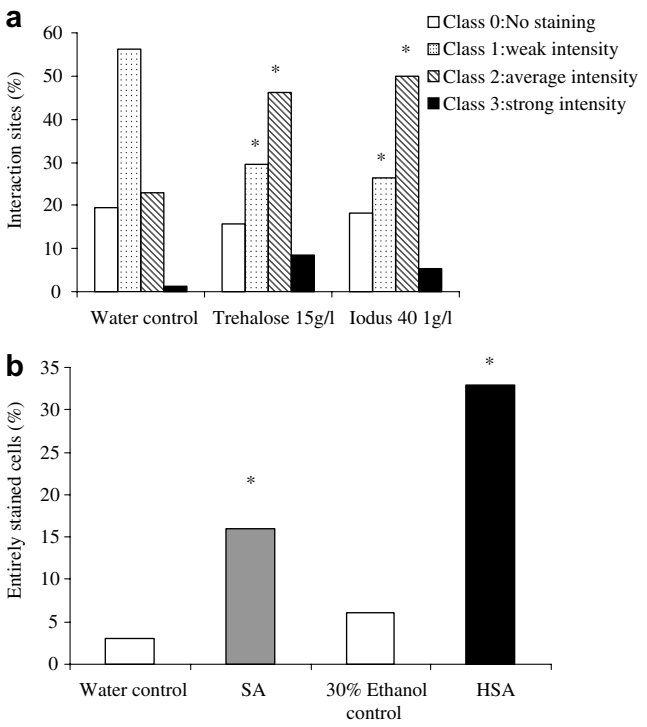

Fig. 2. (a) Effect of Iodus 40 and trehalose treatments on H_2O_2 accumulation at the fungal penetration site. Data represent means of three independent series of experiments. For each intensity class, the treated samples were compared to the corresponding infiltrated water control. Bars with an asterisk are significantly different from control. (b) Effect of SA and HSA treatments on whole cell DAB staining compared to the corresponding infiltrated control, i.e. distilled water and 30% ethanol, respectively. The number of entirely stained cells are expressed from assessment of 100 penetration events for each condition. Data represent means of three independent series of experiments. Bars with an asterisk are significantly different from control.

(Bolwell and Wojtaszek, 1997; Shah, 2005). Leaves were sprayed and examined every day over a 4 days period for these activities. Treated plants were also compared to the corresponding controls in both infectious and non-infectious contexts. With only one exception, no significant difference was observed between the controls and any of the treated leaves for all the considered activities over the whole period of the experiment (data not shown). Only HSA, 4 days after treatment and therefore 2 days after inoculation, induced significant modifications. It decreased the CAT activity, in both inoculated (15%) and non-inoculated (24%) conditions (Table 1). HSA also induced in non-inoculated conditions a 49% increase of LOX activity, whereas a 217% increase occurred in inoculated conditions (Table 1). The lipid peroxidation level was assessed using the same experimental conditions for the four tested inducers but over a 7 days period, except on day 6. During the Iodus 40/trehalose experiment, a first type of effect was observed from day 1, i.e. one day after treatment, since Iodus 40 induced a partial but significant 27% decrease. On day 3, a slighter but still significant effect was also observed with Iodus 40: the non-inoculated (ni) condition led to a 20% decrease, whereas the inoculation performed

Table 1
Specific catalase (CAT) and lipoxygenase (LOX) activities in wheat leaves treated with HSA

		CAT (nkat mg prot^{-1})	LOX (nkat mg prot^{-1})
30% Ethanol control	ni	0.89	1.15
	i	0.79	0.7
HSA	ni	0.68*	1.72*
	i	0.67	2.22*

First leaves were sprayed and assessed for their CAT and LOX activities every day over a 4 days period, in both inoculated (i) and non inoculated (ni) conditions. Data presented were obtained 4 days after treatments. For the (i) samples, inoculation was performed 48 h after treatment. Values with an asterisk are significantly different from control. Data represent means of three independent series of replicates.

on day 2 allowed this treatment to result in a 13% decrease in inoculated (i) samples (see Table 2). On day 4, Iodus 40 was still able to induce a significant effect on lipid peroxidation, with a 11% decrease in (ni) conditions and a 15% decrease in (i) conditions. The trehalose treatment resulted in no significant effect over the 4 days period in both conditions (data not shown). However, the HSA/SA experiment allowed us to observe an increase of the lipid peroxidation level. This increase was measured on days 4, 5 and 7 in both non inoculated and inoculated conditions. HSA resulted on day 4 in 32% and 29% increases in non-inoculated and inoculated conditions, respectively. On day 5, 78% and a 88% increases were observed in both conditions, whereas on day 7, 154% and 93% increases were recorded (Table 3).

2.3. Iodus 40 and trehalose decreased haustorium formation and Iodus 40 altered conidia germination in vitro

Conidia and penetrated cells were first examined on infiltrated plants. The induced accumulation of compounds such as phenolics at the penetration sites of AGTs was observed using fluorescence microscopy. When plants treated with Iodus 40 and trehalose were compared to the control ones, the only significant effect was an increase of the percentage of penetration events associated to a strong

Table 2
Effect of treatments on lipid peroxidation level measured as MDA content in Iodus 40-treated plants

		MDA (nmol g fw^{-1})		
		Day 1	Day 3	Day 4
Water control	ni	107.23	93.27	67.34
	i		95.34	77.10
Iodus 40	ni	78.52*	74.47*	60.15*
	i		82.89*	65.31*

First leaves were sprayed and examined every day (except on day 6) over a 7 days period, in inoculated (i) and non-inoculated (ni) conditions. For the (i) samples, inoculation was performed 48 h after treatment. Data represent means of three independent replicates. Values with an asterisk are significantly different from the corresponding control. Data represent means of three independent replicates.

Table 3
Effect of treatments on lipid peroxidation level measured as MDA content in HSA-treated plants

		MDA (nmol g fw^{-1})			
		Day 3	Day 4	Day 5	Day 7
30% Ethanol control	ni	107	82.8	55.8	38.8
	i	82.4	64.6	40.7	37.1
HSA	ni	113	109*	99.7*	98.6*
	i	80.8*	83.4*	76.8*	71.4*

First leaves were sprayed and examined every day (except on day 6) over a 7 days period, in inoculated (i) and non-inoculated (ni) conditions. For the (i) samples, inoculation was performed 48 h after treatment. Data represent means of three independent replicates. Values with an asterisk are significantly different from the corresponding control. Data represent means of three independent replicates.

fluorescence and to an absence of fungal haustorium (from 46% to 58% and 55%, respectively, Fig. 3a). The same effect was induced by SA (from 27% to 72%, Fig. 3b). We also noticed a slight decrease of percentage of penetration events associated to both a weak fluorescence and the absence of haustorium for Iodus 40 (from 4% to 0.5%, Fig. 3a). HSA had no significant effect (data not shown).

While studying conidia germinating on Petri dishes, five classes of conidia could be distinguished on the water control dishes: non-germinated conidia (66%), conidia forming

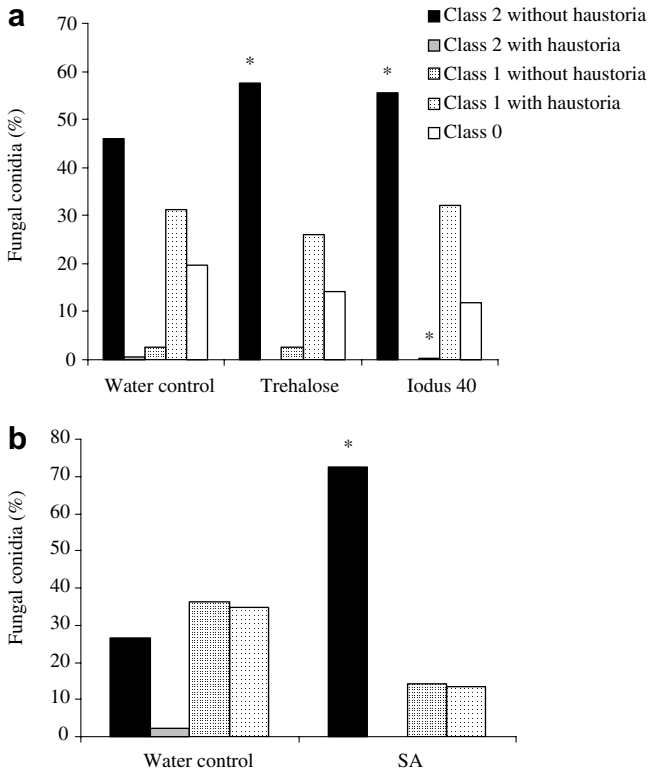

Fig. 3. Effect of treatments on papillae autofluorescence, fungal haustorium formation in infiltrated epidermal cells. (a) Effect of Iodus 40 and trehalose. (b) Effect of SA. Each intensity class was compared to the corresponding control. Bars with an asterisk are significantly different from the control. Data represent means of three independent series of experiments.

one short germ tube (12%), conidia forming a long germ tube (8%), conidia with several germ tubes (4%) and conidia showing only very short abortive germ tubes (10%). On Petri dishes supplemented with each of the inducers, the same types of germinating conidia were observed. However, on Iodus 40 treated dishes, conidia forming one short GT increased from 12% to 20% whereas conidia forming a long GT dropped from 8% to 3% (Fig. 4). No significant effect was found for the HSA, SA and trehalose treatments (data not shown).

3. Discussion

3.1. Iodus 40, SA and trehalose induce partial protections whereas HSA is highly effective

Previous knowledge about efficacies of the resistance inducers studied here was partial and disparate. Nothing was known concerning Iodus 40 in wheat, although it was released as a resistance inducer against *B. graminis* f. sp. *tritici*. Trehalose has been already reported as a partial resistance inducer against wheat powdery mildew (Reignault et al., 2001; Muchembled et al., 2006). In a previous work, using a different wheat cultivar and distinct experimental procedure, HSA was reported to lead to a 70% protection only against *B. graminis* with a single slight spraying of a 1 g l^{-1} solution. In these conditions, a SA solution failed to induce any resistance (Muchembled et al., 2006). No data about the effect of single or double up-to-run-off sprayings of SA or double sprayings of HSA were reported before this work was done. Our work allowed us to compare the efficacy level of the four different inducers using a single experimental procedure in controlled conditions. Clearly, two classes of inducers were underlined in this work. Firstly HSA, when sprayed in up-to-run-off assays, showed an almost total efficacy with only one spraying. The residual infection level completely disappeared after two sprayings. Secondly, the three other inducers tested here, Iodus 40, SA and trehalose, showed partial and similar efficacies after one spraying and the repetition of the treatment did not alter the partial nature of these resistances. Since, Iodus 40 is now used by farmers in field conditions, our work suggests a possible use of SA and trehalose in control strategies of wheat powdery mildew. The very high efficacy exhibited by HSA appears as even more promising. However, this inducer is not a natural product and one must keep in mind any potential impact on the environment.

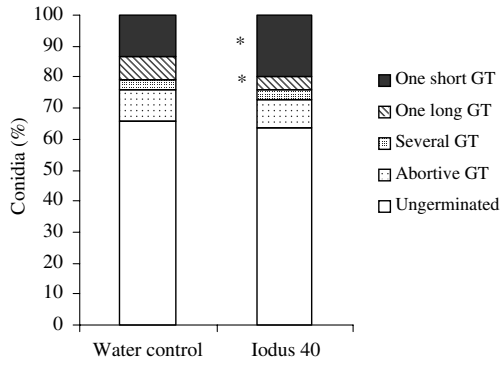

Fig. 4. Effect of Iodus 40 on *in vitro* conidia germination of *Blumeria graminis* f. sp. *tritici*. Data are means from three experiments, each consisting of a set of three replicates. AGTs longer than 50 μm were considered as long, whereas shorter ones were considered as short. Percentages were obtained from sets of 100 conidia and germinating types with an asterisk ("one short GT" and "one long GT") were significantly different from the control. Data represent means of three independent series of experiments.

3.2. HSA exhibited more numerous biological activities than the closely related SA

We showed that HSA exhibited the largest extent of biological activities on wheat: it increased H_2O_2 accumulation at the whole cell level, decreased the CAT activity, increased the LOX activity as well as the lipid peroxidation level. We suggest that the decrease of CAT activity led to an increase of H_2O_2 concentration and therefore contribute to the increases of LOX activity and lipid peroxidation. However, HSA did not interfere with fungal germination and haustorium formation (data not shown). We still have to explain the increase of H_2O_2 accumulation observed at the whole cell level and not at the fungal penetration point, as seen with Iodus 40 and trehalose. One could speculate that such an accumulation may be an initial step towards death of the entirely stained cell, as it is observed during incompatible interactions between cereals and powdery mildews. As shown by Hückelhoven et al. (1999), hypersensitive cell death in resistant barley attacked by the powdery mildew fungus was associated with H_2O_2. On the other hand, SA showed far less numerous biological activities than HSA: indeed, SA had only an effect on H_2O_2 accumulation and on autofluorescence. Such a result, as well as a protection efficacy lower than that of HSA, showed the effectiveness of the modification of SA by its esterification with heptanoic acid. It could be suggested that the short fatty acid chain formed by heptanoyl acid helps the penetration efficiency of this product through the plant cuticule and therefore significantly increases the extent of the responses observed in the plant.

3.3. Iodus 40 and trehalose induced common effects

Iodus 40 was previously shown to induce the release of H_2O_2, to increase phenylalanine ammonia lyase (PAL) and LOX in tobacco cells (Klarzynski et al., 2000). It was also reported to induce an oxidative burst, chitinase and glucanase activities and phytoalexin production in grapevine (Aziz et al., 2003). However surprisingly, although this product is released as targeting wheat diseases, no cytological data nor data about biological activity of Iodus 40 on wheat defence physiology were available. This work is the first report of Iodus 40 physiological targets associated with an induced resistance in wheat.

Iodus 40 and trehalose increased H_2O_2 accumulation only at the penetration sites. In addition, they both induced an increase of the frequency of strong fluorescence at the fungal penetration point and a decrease of haustorium formation. With comparable protection efficiencies, Iodus 40 and trehalose therefore showed, at least in the case of the wheat/*B. graminis* f. sp. *tritici* pathosystem, several similarities in their mode of action, except for lipid peroxidation. Moreover, Iodus 40 shares two biological activities with Milsana, another commercially released resistance inducer: Iodus 40 had the same effect on lipid peroxidation, i.e. an increase of the LOX activity but also a partial decrease of lipid peroxidation level (Randoux et al., 2006). This effect was also measured in this work in infectious conditions. In addition, we previously showed that Milsana inhibited conidia germination *in vitro*. No such drastic effect occured with Iodus 40 *in vitro*, but we observed a small significant decrease in the proportion of conidia forming long germ tubes, in parallel with a small increase in the proportion on conidia forming short germ tubes. However, in addition to laminarin, this commercial preparation contains other components such as formulating agents that might contribute to this effect.

Altogether, our data show for HSA a mode of action based on ROS metabolism (accumulation of H_2O_2 at the whole cell level, decrease of CAT and increase of LOX activities) and increase of lipid peroxidation. On the other hand, Iodus 40 and trehalose act through accumulation of H_2O_2 at the penetration site and phenolics accumulation in papillae. They also decrease of lipid peroxidation and impair haustorium formation. However, the good efficacy of these products did not correlate with several potential biological activities that were tested in this work. For example, trehalose, although leading to a 60% protection after two sprayings only altered H_2O_2 and phenolics accumulations as well as haustorium formation. In addition, a biological activity does not always occur as an induction. Several parameters such as CAT activity and lipid peroxidation were down-regulated by HAS and Iodus 40, respectively. Finally, resistance inducers do not necessarily act through the modulation of plant defence only. When considering Iodus 40 and our previous results with Milsana, we know that a resistance inducer may also lead to an alteration of fungal germination.

4. Experimental

4.1. Plant culture, pathogen maintenance, and chemicals

Wheat (*Triticum aestivum* L.) cultivar Orvantis was kindly provided by Benoît C.C. (Orgerus, France). This cultivar is fully susceptible to the MPEBgt1 powdery mildew isolate of *Blumeria graminis* f. sp. *tritici* used in this study. The fungus was both inoculated and maintained on Orvantis plants in a room separate from healthy plants under constant light, at 20 °C. Heavily sporulating leaves were shaken above 2-week-old plants and inoculated plants were left in the room until new fully sporulating leaves were obtained, usually within 8–10 days. Wheat caryopses were soaked overnight in water in order to facilitate their germination. They were then grown on compost in a Phytotron (day temperature 18 °C, night temperature 12 °C, RH 70%) under a 12 h photoperiod (light intensity 250 μmol m^{-2} s^{-1}). Ten-days-old wheat plantlets were treated as indicated below, when the first two primary leaves were expanded. Iodus 40 was kindly provided by Goëmar (Saint-Malo, France), HSA (99.9 purity) was obtained from Dr. D. Couturier (Molecular Engineering Laboratory, USTL, Villeneuve d'Ascq, France), and trehalose was a gift from Amcan (Le Chesnay, France). Unless otherwise stated, the different chemicals used in this study were purchased from Sigma (Saint-Quentin Fallavier, France). "Fluorinert" FC 43 was obtained from 3 M (Cergy-Pontoise, France).

4.2. Treatment applications, plant inoculation and infection level assessment

For infection tests and enzymatic activities (CAT, LOX and OXO) , plantlets were treated with solutions of Iodus 40 (laminarin, 1 g l^{-1}), SA (1 g l^{-1}) or trehalose (15 g l^{-1}) (Reignault et al., 2001; Muchembled et al., 2006) or distilled water as the corresponding control. HSA could not be dissolved in distilled water and a minimum of 30% ethanol had to be added in order to get a solution (1 g l^{-1}). In the treatment experiments, its corresponding control was therefore a 30% (v:v) ethanol solution (Muchembled et al., 2006). Treatments consisted in up-to-run-off sprayings. Two days after treatment, inoculation-challenged plantlets were sprayed with *B. graminis* f. sp. *tritici* conidia suspended in "Fluorinert" FC 43. The inoculum concentration was adjusted to 250,000 spores ml^{-1} and conidia were sprayed on leaves in order to apply approximately 20 conidia cm^{-2} leaf area. For two treatments, two sprayings were performed, at 48 h intervals before inoculation. Eight days after inoculation, 10 treated and inoculated plantlets were randomly harvested in order to count the fungal colonies of powdery mildew, when the colonies were still separated and before coalescence has occurred. The protection against powdery mildew was determined by assessing the percentage of sporulating colonies on treated primary leaves compared with controls as soon as symptoms were visible. A variability in fungal fitness and in rapidity of the infection process was observed from a set of experiments to another and the mean number of sporulating colonies on the control leaves ranged between 70 and 140 according to the experiment.

For the DAB staining and the autofluorescence experiments, approximately 50 μl of treatment solutions and the corresponding controls were infiltrated into a 2 cm long central part of the leaves (abaxial face, fully expanded first leaf of 10 days-old plants) using a hypodermic syringe without needle. This procedure allowed to cover an area of 1 cm^2 in the central part of the leaves which could be

easily visualized since it had a darker green colour. This area was delineated with a marker pen. Inoculum concentration was adjusted to 500,000 spores ml^{-1} and conidia were sprayed on leaves in order to apply approximately 40 conidia cm^{-2} leaf area.

4.3. Light microscope detection of H_2O_2

Iodus 40, HSA, SA, trehalose, 30% ethanol and distilled water were infiltrated as described above and leaves were inoculated with *B. graminis* conidia 48 h later. Plants were kept in the phytotron until further microscopic examination. The detection of H_2O_2 was carried out using the DAB (3,3′-diaminobenzidine) staining method according to Thordal-Christensen et al. (1997). Seventeen hours after inoculation, the first leaves were excised and the cut end was immersed in a solution containing 1 mg ml^{-1} DAB dissolved in water (pH 3.8 with HCl). The leaves were kept in this solution, allowing DAB uptake for 8 h. DAB-treated leaves were then transferred in acetic acid–ethanol (1:3) to fix the DAB staining and bleach the leaves. These were stored in lactoglycerol (1:1:1, lactic acid:glycerol:water, v/v). A 1.5 cm-length segment was cut around the infiltrated leaf area and used for microscopy to visualize the H_2O_2 accumulation at the site of fungal penetration. A staining intensity scale was applied to identify the degree of hydrogen peroxide accumulation (Fig. 5a): (i) class 0, the TGA penetrated the epidermal cell but there was no visible DAB staining, (ii) class 1, weak DAB staining intensity, (iv) class 2, average intensity, and (iii) class 3, strong intensity. In addition, as shown in Fig. 5b, we also visualized whole cell DAB staining.

4.4. Lipid peroxidation assay

The level of lipid peroxidation was measured in sprayed primary leaves, either inoculated or non-inoculated by *B. graminis* conidia. It was measured in terms of content of malondialdehyde (MDA), a product of lipid peroxidation. The thiobarbituric acid method (Dhinsa et al., 1981) was used to assess spectrophotometrically the MDA formation. Foliar segments were homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000*g* for 5 min. Four millilitres of 20% TCA containing 0.5% thiobarbituric acid were added to 1 ml of supernatant. The extract was incubated at 95 °C for 30 min, ice-cooled immediately, and centrifuged at 10,000*g* at 4 °C for 10 min. The absorbance of the supernatant was measured at 532 nm and the value for the non-specific absorption at 600 nm was substracted. The concentration of MDA was calculated using its extinction coefficient value of 155 mM^{-1} cm^{-1}.

4.5. Enzyme activities

LOX assay was performed according to Todd et al. (1990), Avdiushko et al. (1993) and Xing and Chin (2000) with some slight modifications. The formation of conjugated dienes from linoleic acid, a substrate of lipoxygenase, was assayed spectrophotometrically at 235 nm to

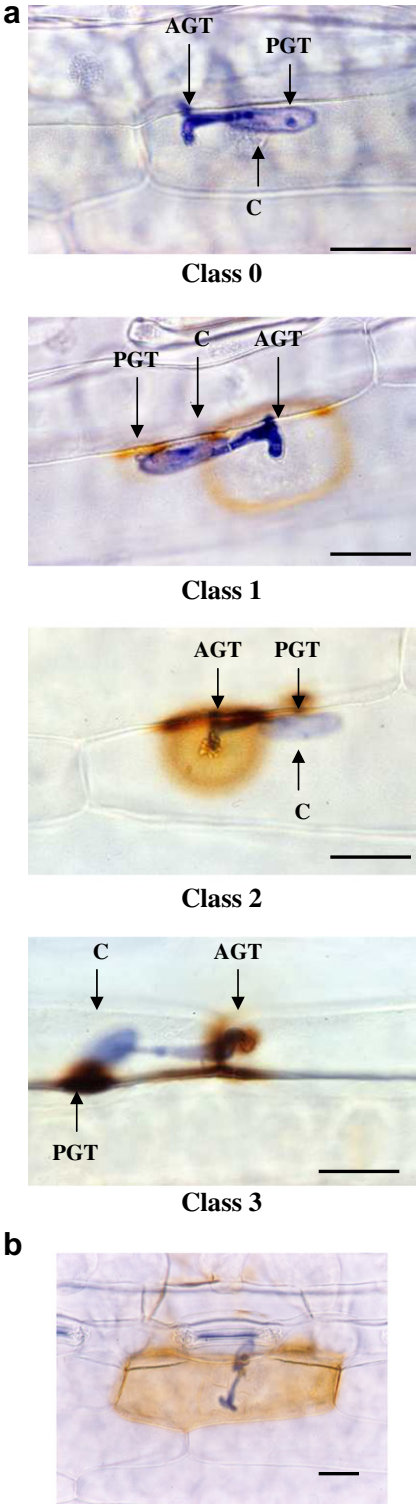

Fig. 5. (a) H_2O_2 accumulation at the *B. graminis* penetration sites on wheat epidermal cells visualized after DAB staining. C, conidium; PGT, primary germ tube; AGT, appressorial germ tube; class 0, no visible DAB staining; class 1, weak intensity; class 2, average intensity; class 3, strong intensity (scale bar = 25 μm). (b) Whole cell H_2O_2 accumulation in cells attacked by *B. graminis* visualized after DAB staining (scale bar = 25 μm).

determine LOX activity. Leaf tissues were ground in ice-cold 0.1 M potassium phosphate buffer pH 7.6 containing 9 mM octylthioglucopyranoside, 2% polyvinylpolypyrrolidone (PVPP) and 1 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was centrifuged at 10,000*g*, at 4 °C for 15 min and was tested for its LOX activity. Substrate was prepared by dissolving 100 µl of Tween 20 in 20 ml of MOPS (pH 7.0), adding 20 µl of linoleic acid, mixing thoroughly, and incubating the substrate mixture under N_2 for 10 min at 20 °C. The reaction mixture contained 286 µl of substrate, 28 µl of enzyme extract in a final volume of 1 ml of 50 mM MOPS (pH 7.0). Changes in absorbance at 234 nm were followed over a 5 min period. Proteins were measured by Bradford (1976) method using bovine serum albumin as standard (Sigma). One unit of enzymatic activity was defined as the amount of enzyme that catalyses the conversion of one mole of substrate per second ($\varepsilon = 23{,}000\ M^{-1}\ cm^{-1}$). The other activities, e.g. OXO and CAT, were measured according to Sugiura et al. (1979), and Garcia-Limones et al. (2002), respectively.

4.6. *B. graminis* conidia germination and penetration

The effects of treatments and corresponding controls on conidia germination and penetration have been investigated both *in vivo*, during the early steps of the interaction, and *in vitro*. In the first case, plantlets were infiltrated and inoculated as described above. Leaf segments (1 cm) were cut and bleached in ethanol:acetic acid (3:1, v/v) and stored in lactoglycerol. Samples were stained with trypan blue, mounted on glass slides and observed by fluorescence and transmitted light microscopy. For fluorescence microscopy, observations were made using a Nikon (Champigny-sur-Marne, France) fluorescence microscope (blue exciter filter, maximum transmittance 400 nm, dichroic mirror and barrier filter with a 500–800 nm transmittance range). Fluorescence intensity of papilla associated to AGTs was recorded as either strong or weak. Papilla fluorescence, primary germ tubes (PGT), appressorial germ tubes (AGT) and haustoria were easily observed using this procedure. An intensity scale was used to quantify the observed fluorescence (Randoux et al., 2006): (i) class 0, no fluorescence; (ii) class 1, weak fluorescence; (iii) class 2, strong fluorescence. The *in vitro* germination behaviour of conidia was compared on Petri dishes supplemented with 12 g l^{-1} agar and covered either with 500 µl of the treatment solution or with 500 µl of the corresponding control. After spreading and drying, a fresh 8-days-old inoculum was shaken above Petri dishes that were then placed in the phytotron. The length and the number of germ tubes were measured 24 h later under the microscope.

4.7. Statistical analysis

Performed experiments (infection tests, DAB staining, *in planta* conidia penetration and *in vitro* germination) involved distinct and independent series of data and several factors: day of the kinetics, type of activity, type of treatment, inoculated or non-inoculated condition, three independent repetitions (OXO, CAT and LOX activities, lipid peroxidation). We therefore performed multi-factorial ANOVA to compare the data from the treated samples with those from the corresponding control. Analysis of variance were carried out with the statistical program Xlstat 5.1 v2 (Addinsoft, Paris, France). The test of Dunnet was used to compare the means obtained in the treated plantlets with those obtained with the corresponding treated controls and to check statistical significance ($p \leqslant 0.05$). ANOVA was performed on transformed data in order to fulfil its pre-requisite. For the enzymatic activities, data were submitted to a logarithmic transformation, whereas for the infection level, DAB staining and autofluorescence intensities, they were submitted to the arcsin square root transformation.

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