Impact of abiotic stresses on the protection efficacy of defence elicitors and on metabolic regulation in tomato leaves infected by *Botrytis cinerea*

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Abstract

Finding sustainable plant protection strategies is a major challenge for agriculture. Taking advantage of the plant natural immune system by using plant defence elicitors is an interesting avenue to explore. However, transfer to field application is often difficult, mostly due to the complexity of interactions between plants and their environment, involving biotic and abiotic stresses. The protection efficacy against gray mold and the modes of action of potential elicitors were studied on tomato. Modulation of plant defense was studied using both global and targeted metabolic profiling. We identified seven potential elicitors showing good plant protection efficacy and able to trigger the oxylipin pathway, including jasmonic acid production, after inoculation with *Botrytis cinerea*. Following preliminary assays, seven elicitors including two well-studied elicitors (Bion 50WG® and BABA) showing good plant protection efficacy and low fungitoxic effect were selected to assay the effect of abiotic stresses (wounding, water stress and nitrogen deficiency) on their protection efficacy. Our results showed that the protection efficacy of all products was reduced when plants were exposed to abiotic stresses, suggesting an antagonistic interaction between the tomato responses to abiotic stresses and product treatments. We found that responses to leaf cuttings and product treatments induced metabolic changes in a time-dependent manner, and that both of which mainly activated the oxylipin and JA pathway. However, the negative effects of wounding on tomato protection efficacy of defence elicitors

suggest that interplay with other antagonistic signalling pathways is also involved in the tomato responses to this combination of stress.

Keywords

Stress combinations
Wounding
Gray mold
Induced resistance

Electronic supplementary material

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Introduction

Plant disease control in the 21st century faces multiple challenges (Walters et al. 2005). Plant pathogens are continuously evolving, leading to the selection of strains able to circumvent host resistance genes or resistant to fungicides when repeatedly used. In addition, there are increasing concerns related to the environmental effects of widespread use of chemicals. In this context, alternative approaches of plant disease control need to be implemented in a sustainable way.

Plants naturally have the ability to defend themselves thanks to their immune system (Jones and Dangl 2006). Following perception of the pathogen by the plant, a complex signalling network takes place, in the centre of which the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the key players (Sanchez et al. 2012). These signalling events ultimately lead to the establishment of plant defences, including the production of antimicrobial compounds and cell wall modifications. Among available biocontrol strategies, the induction of resistant mechanisms, notably through the application of plant defence elicitors (PDE), represents a promising alternative to chemical fungicides (Ballester et al. 2011). PDE can be defined as natural or synthetic compounds with no direct antifungal activity which, by mimicking natural signalling compounds, induce defence mechanisms in plants, in turn resulting in enhanced resistance to pests and pathogens upon attack (Fu and Dong 2013; Dufour et al. 2013). Alternatively, some compounds do not immediately trigger plant defence mechanisms but provide

resistance through a faster and/or stronger induction of defence mechanisms following pathogen and pest infection. This phenomenon, named priming, should be preferred to strategies based on direct activation of plant defences because associated costs are reduced (Pastor et al. 2013). Since the end of the 80s, plant treatments with elicitors were reported to induce defence mechanisms and allow broad spectrum resistance to pests and pathogens such as algal, microbial or plant extracts (Trouvelot et al. 2008; Klarzynski et al. 2000), plant hormones, their analogues or other chemicals (Cohen et al. 1994~~, 2001~~; Métraux et al. 1990; Brisset et al. 2000), mycorhiza or microbial antagonists (Pozo and Azcón-Aguilar 2007). Since they generally have no toxic effects on the environment, elicitation or priming of plant defence may allow, in combination with other alternative solutions, effective and sustainable disease management in the field (Walters et al. 2005).
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However, elicitors used in real field conditions often show a reduced efficacy when compared to results obtained under laboratory conditions, mainly because interactions with the environment affect the outcome of the induced resistance mechanisms. Indeed, plants are exposed to multiple biotic and abiotic stresses in combination rather than experiencing one stress at a time and they respond to such stresses through various signalling pathways that may interact or inhibit one another (Anderson et al. 2004; Asselbergh et al. 2008; Niinemets 2010). Such crosstalk may therefore interfere with PDE-induced resistance mechanisms and can partially account for the limited success of such a strategy under field conditions.

The fungus *Botrytis cinerea* is a plant necrotrophic pathogen responsible for gray mold on a broad range of crops, including grapevine, tomato, soft fruit and vegetable plants (Mansfield 1980). It is a major pathogen affecting tomato production worldwide and it causes severe yield losses in both pre- and post-harvest. Current control methods mainly rely on prophylactic practices, such as the disinfection of tools and greenhouses and the application of preventative fungicides. Elicitor treatments able to induce good protection efficacy against *B. cinerea* have been well documented, mainly under greenhouse conditions, including laminarin and chitosan in grapevine (Aziz et al. 2003, 2006), oligandrin in tomato (Lou et al. 2011), a *Fusarium* crude elicitor fraction on pepper (Veloso and Díaz 2013), oligogalacturonides from plant cell walls (Ferrari et al. 2007) and rhamnolipids (Sanchez et al. 2012) in *Arabidopsis*. However, to our knowledge, no study has yet focused

on the performance of plant defence elicitors against *B. cinerea* when plants were subjected to various abiotic stresses. Notably, during tomato growth, leaf removal regularly performed by growers or insect attacks often results in wounded plants while nitrogen deficiency and/or water stress can also be of concern for field-grown tomatoes.

The aims of this study are: i) to determine the protection efficacy and the mode of action of 11 products of various origins including four well-known PDE: Bion 50WG, β-amino-butyric acid (BABA), Methyl jasmonate (MeJA) and Chitosan; ii) to evaluate the influence of abiotic stresses (wounding, nitrogen deficiency and water stress) on the protection efficacy of these agents; and iii) to better understand the plant physiological responses to a combination of abiotic and biotic stresses including leaf wounding, treatments with the products and inoculation with *B. cinerea*, using both global and targeted metabolic profiling by U-HPLC-MS/MS.

Materials and methods

Plant material

Experiments were done on tomato *Lycopersicon esculentum* (L.), using a variety (African) susceptible to *B. cinerea*. Plants were grown in semi-controlled greenhouse in pots containing compost until they reach a four-leaf stage, which approximately corresponds to 4 weeks after sowing. Plants were exposed to 14 h of light per day, temperature was set to 22 ± 5 °C and hygrometry was not regulated. Every plant was watered with about 200 ml every 4 days.

Leaf treatments with products

The products used in this study were dissolved at different concentrations in water as indicated in Table 1 and sprayed onto leaves using a fine glass atomizer (about 3 ml per plant), 5 days prior to inoculation (see Online Resource, Fig 1). Based on literature, four agents for which the ability to induce plant defence mechanisms has been previously demonstrated were used here as positive "PDE controls": Bion 50WG®, BABA, Chitosan and methyl jasmonate (MeJA) (Amborabe et al. 2004; Azami-Sardooei et al. 2013; Dufour et al. 2013; Eyre et al. 2006; Zhu and Tian 2012). In addition to those "PDE controls", the protection efficacy against *B. cinerea* of seven potential PDE were investigated (coded A to G; Table 1). A positive chemical reference, Rovral (see Table 1), was used in all experiments and always

presented a protection efficacy of 100 % (data not shown).

Table 1

List of products tested against *Botrytis cinerea*

Product code	Active material and /or composition	Dosage
A	Coproduct of L-glutamic acid production by fermentation	0.5–1 %
B	Green tide algae *Ulva* sp. raw extract	8 %
C	P2O5 (40 % w/v); K2O (26.9 % w/v); MgO (0.21 % w/v); CaO(0.21 % w/v)	
D	*Saccharomyces cerevisiae* extract	0.15 %
E	P2O5 (40 % w/v); K2O (26.9 % w/v); MgO (0.21 % w/v); CaO(0.21 % w/v); CaO.SiO2 (0.21 % w/v)	
F	Eugenol	0.5 %
G	KH2PO3	0.2 %
MeJA	Methyl Jasmonate	800 mM
Chitosan	Chitosan	2–4 %
Bion50WG®	S-methyl benzo [1.2.3] thiadiazole-7-carbothioate (BTH)	0.08 %
BABA	b-amino-butyric acid	15 mM
Rovral®	Iprodione	0.17 %

Plant inoculation with *B. cinerea*

To prepare the spore suspension, 10 ml of PDB (Potato Dextrose Broth, Conda Laboratory, Spain) and Tween® 80 at 0.05 % (Sigma-Aldrich, USA) were deposited on 7 day-old PDA culture of the strain DSM No. 877 (Strain designation : N51, Germany). The inoculum harvested from the plate was then filtered (using a sieve of 150 µm mesh size) to separate the mycelium and spores. The suspension was then calibrated with a Malassez cell (Preciss, France), and the spore concentration was adjusted to 10^3 spores ml^{-1}. Plants were inoculated with implants of *B. cinerea* 5 days after treatments with each product (see Online Resource, Fig 1). In order to prepare the implants, 1 ml of spore suspension was deposited on a new PDA plate. Thirty minutes later, a 4 mm diameter piece was cut and deposited on the top of each of the first two

leaves (spores contact the leaf). Leaves were previously sprayed with water to promote the adhesion of the implants. Two to four leaflets per leaf of each plant were inoculated. After inoculation, plants were placed in trays in the growth cabinet calibrated to 25 ± 5 °C at night / 25 ± 5 °C during the day; HR > 85 %, and 18 h of darkness. The trays were covered with a plastic cover for 18 h to favour spore germination. Disease evaluation was carried out 5 days after inoculation by measuring the length of the necrosis caused by *B. cinerea* (see Online Resource, Fig 1).

Experimental design

The assays were organized into four technical replicates. Each replicate contained the plants treated with each of the products randomly distributed, with one to two plants per tested product. Two to three independent biological experiments were carried out depending on the product.

For each experiment, the protection efficacy (PE) of tested products was calculated compared to water-treated controls and as the mean of replicates. For each replicate i (rep i), PE was calculated as follows: PE rep i (%) = [(mean necrosis length on water-treated control in rep i – mean necrosis length on product-treated plants in rep i) / mean necrosis length on water-treated control inrep i] × 100.

Biocide effect of tested products on spore germination and mycelial growth

Five-hundred µl of a fresh spore suspension at 4×10^3 spores ml^{-1} were mixed with 500 µl of each product (diluted either at the concentration used in the disease assay or ten times this concentration), sprayed onto PDA and incubated at 20 °C for 17 h. The percentage of germinating spores was evaluated and compared to that found in water treated controls (i.e., when spores were mixed with 500 µl of sterilized water). Mycelial growth was examined as well. 80 µl of each tested product were placed on two pads of sterile filters, placed on the edge of the PDA dishes, and then an implant of spores was placed in the middle of the dishes at room temperature. The fungicidal effect was evaluated by measuring the colony diameter of *B. cinerea* 3 days after inoculation. Each assay was arranged in four technical randomized replicates (i.e., four dishes). At least two independent biological experiments were carried out per assay.

Application of abiotic stresses

The impact of three abiotic stresses (water stress, nitrogen deficiency and wounding) on the protection efficacy of nine agents (Products A-G and BABA and Bion 50WG® as PDE controls) was studied. For all abiotic stresses, plants were treated, inoculated and scored for disease development as described before. Two independent biological experiments were carried out.

Wounding the apical portion of each leaflet of stressed plants was cut off (see Online Resource, Fig 1) 2 days before treatment. To determine whether timing of wounding influences the protection efficacy of the products, a comparison with wounding applied after treatment or just prior inoculation, was also performed for products A, E and PDE controls.

Water stress All tested plants were irrigated with 200 ml of water per plant every 4 days, from sowing until 1 week before treatment with products. Irrigation of stressed plants was stopped 1 week before treatment. After treatment, stressed plants were irrigated with 10 ml of water every 2 days until scoring (see Online Resource, Fig 1). Irrigation of the non-stressed plants was maintained with 200 ml of water per plant every 2 days from sowing until disease evaluation.

Nitrogen deficiency Two nutritive solutions, at 100 and 7.5 % of nitrogen requirements, were prepared to feed plants grown in vermiculite. The complete nutritive solution (100 %) contained: Ca(NO3)2: 9.7 g, KNO3: 5.3 g, MgSO4: 4.9 g, KH2PO4: 2.5 g, Fer EDDHA: 225 mg, OligoMix: 56 mg in 10 l of water. The solution used for nitrogen deficiency (7.5 %) contained: Ca(NO3)2: 0.5 g, MgSO4: 4.9 g, KCl: 3.8 g, CaCl2: 4.1 g, Fer EDDHA: 225 mg, OligoMix: 56 mg in 10 l of water. 200 ml per plant of the complete and deficient solutions were respectively applied to unstressed and deficient plants every 4 days, from sowing until disease scoring.

U-HPLC-MS/MS metabolite profiling

For metabolic profiling studies, plants were treated with water as negative control, and Bion® and BABA as PDE controls and inoculated with *B. cinerea* as previously described. Leaf samples were collected at four time points: 5 days before inoculation (5dbi), just prior treatment (3dbi), the day of inoculation, i.e., just prior inoculation (d0) and 4 days after inoculation (4dpi), when necrosis was large enough (see Online Resource, Fig 1). The 3 mm part surrounding the necrotic region was harvested (see Online Resource, Fig 1). In addition to analyzing PDE controls, we analyzed the 4dpi metabolic..

profiling of samples treated by products A, B, D, E and G.

To explore the mechanisms of action of putative PDE in inducing resistance against *B. cinerea* and those responsible for the loss of efficacy in pre-stressed plants, we chose to conduct global and targeted metabolomic studies using U-HPLC-MS/MS on tomato plant leaves, under wounding stress, pre-treated or not with different putative PDE before infection with *B. cinerea*. Bion 50WG® and BABA were first used as PDE positive controls, to study the combined effects of wounding and PDE-treatments during a time-course. The tomato metabolic profiling were established in samples collected 2 days after cutting (i.e., 5 days before inoculation with *B. cinerea* (5dbi), and just before PDE-treatment), 3 days before inoculation (3dbi), just before inoculation (d0) and 4 days after *B. cinerea* inoculation (4dpi) (see Online Resource, Fig 1). Using all the metabolic data, a principal component analysis (PCA) was performed to analyze the relationships between the three variables (i.e., PDE-treatment, wounding stress and sampling time). Each sample was obtained from a pool of four leaflets from two different plants per sampling date, and the experiments contained three technical replicates. After being collected, samples were immediately frozen in liquid nitrogen, freeze-dried and stored at −20 ° C. Samples were ground in CK-Mix tubes using a Precellys grinder (Bertin Technologies, Montigny-le-Bretonneux). Extraction was carried out by adding 1 ml [MeOH/H_2O (8:2)] containing 1.25 µg 12-OH-Lauric acid as internal standard and by shaking for 1 h at 4 °C. Extracts were centrifuged for 10 min at 17 500 × g at 4 °C and supernatant was transferred in a glass vial stored at −80 °C until analysis. Sample fingerprinting was performed on a Dionex Ultimate 3000 RSLC system including an autosampler, a tertiary pump and coupled to a Finnigan LTQ-Orbitrap™ hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was performed on an Acclaim 120 C18 column (100 × 2.1 mm × 2.2 µm particle size, Dionex). Mobile phase consisted in water containing 0.1 % acetic acid (A) and acetonitrile containing 0.1 % acetic acid (B). The used elution gradient (A:B, v/v) was as follow: 80:20 from 0 to 5 min; 95:5 at 15 min and hold for 10 min; 80:20 at 26 min and hold for 4 min. The injected volume was 5 µl, the flow rate was 0.25 ml min^{-1} and the temperature of the column was maintained at 20 °C. The UHPLC column was connected without splitting to the electrospray interface operating in the positive ion mode. The electrospray voltage was set to 3.5 kV, the capillary voltage to 45 V, and the tube lens offset to 130 V. The sheath and auxiliary gas flows (both nitrogen) were set to five arbitrary units (a.u.), and the drying gas

temperature was set to 300 °C. Mass spectra were recorded from 50 up to 1000 *m/z* at a resolution of 30 000 (FWHM at *m/z* 400). Mass spectra were acquired in the centroid mode. For data processing, following their acquisition by Xcalibur® software (Thermo Fisher Scientific), metabolomic fingerprints were deconvoluted to allow the conversion of the three-dimensional raw data (*m/z*, retention time, ion current) to time- and mass-aligned chromatographic peaks with associated peak areas. Massmatrix File Conversion was used to convert the original Xcalibur data files (*.raw) to a more exchangeable format (*.mzXML). Data processing was then performed using the open-source XCMS software. XCMS parameters for the R language were implemented in an automated script. CentWave was used for the peak picking. The interval of *m/z* value was set to 0.1, the signal to noise ratio threshold was set to 10, the group band-width was set to 10 and the minimum fraction was set to 0.75. SIMCA 13.0 (Umetrics, Malmö, Sweden) was used for statistical analysis. Identification of the different metabolites was carried out using the Metlin database and in-house standards. Xcalibur was later used for the quantification of chosen metabolites.

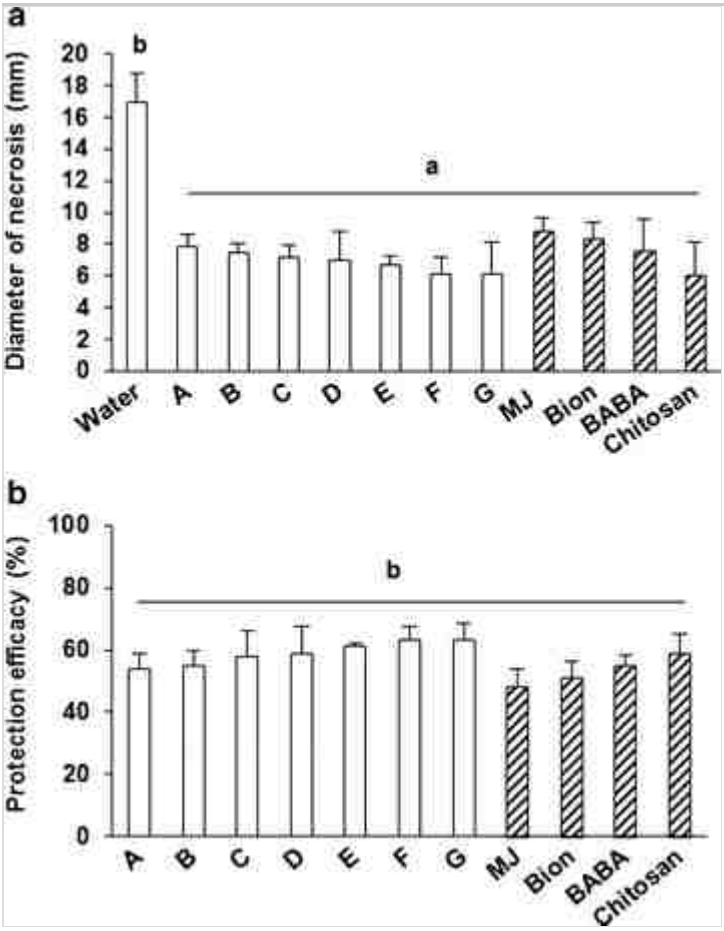
Statistical analyses

Statistical analysis was performed using Splus software v.6.1 (TIBCO Sofware Inc., USA) for ANOVA and multiple comparisons and using R v2.15.0 (http://www.r-project.org) for Principal Component Analysis (PCA). Targeted metabolite data was log10-transformed as log10(13-HpODE + 0.01), log10(13-HOTrE + 0.1), log10(JA + 0.001) and log10(SA + 0.001) to ensure normal distribution and homogeneity of variance. Differences between treatments were determined with multiple mean comparisons with the control's mean (e.g., water treatment) using the simulation-based method (determined by software as the best procedure with the smallest critical point among all valid methods). For the protection efficacy assays, a Bonferroni adjustment was used and the level of significance for multiple comparisons between treatments was set at $p < 7{,}57 \times 10^{-4}$ (0.05/66 combinations; Fig. 1).

Fig. 1

Protection efficacy of seven putative PDE or PDE references against gray mold development on tomato. Mean necrosis length measured 5 days after inoculation with *B. cinerea* (**a**) on tomato plant treated with water, seven putative PDE or PDE references. Protection efficacy of these putative PDE (*A-G*, *white bars*) was evaluated and compared to water-treated controls (**b**). Four reference alternative products (*dashed bars*): Methyl Jasmonate (MeJA), BABA, Bion

50WG and Chitosan were included. Standard deviation was calculated on two to three independent experiments. Differences between treatments were determined with multiple comparisons using the Bonferroni adjustment ($\alpha = 7{,}57 \times 10^{-4}$)



Results

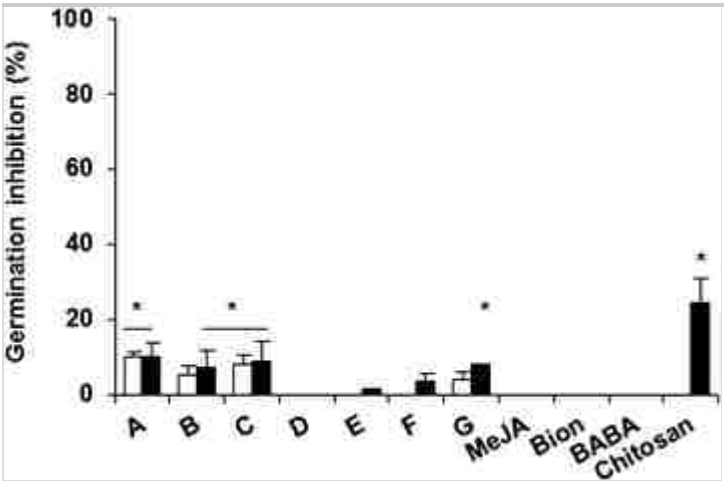
Identification of potential PDE efficient against tomato gray mold

We investigated the protection efficacy against *B. cinerea* of four well-known PDE and seven potential PDE (coded A to G, Table 1). The mean length of necrosis in water-treated controls was 16 mm ± 2.2 (Fig. 1a). Treatments with the four PDE controls induced a significant smaller necrosis length compared to water-treated plants (p-value = 10^{-6}), (Fig. 1a) with the following protection efficacy: Chitosan (59 %), BABA (55 %), Bion® (51 %) and MeJA (48 %) (Fig. 1b). Each of our potential PDE was also able to significantly reduce disease development compared to water-treated plants with the following protection efficacy: A (54 %), B (55 %), C (58 %) D (59 %), E (61 %), F (63 %) and G (63 %), (p-value = 10^{-6}). The fungicidal effect of all products was then evaluated at two doses (the dose used in the disease assay

and ten times this dose, below refer to as dose 1× and 10×) on both mycelial growth of *B. cinerea* and spore germination, in comparison to water-treated controls (with a mean mycelial growth of 40 mm ± 1.5, 3 days post-inoculation and a spore germination rate of 100 %). No fungicidal effect on mycelial growth was observed with any of the products whatever the concentration tested, when compared to water-treated controls (p-value = 0.14). Regarding the impact on spore germination (Fig. 2), Chitosan among the four PDE induced a significant effect, compared to water-treated controls with 25 % of spore germination inhibition at dose 10× (p-value = 5.10^{-6}). In addition, four products (A and C at the two doses; B and G at dose 10×) showed a significant negative effect on *B. cinerea* spore germination but at a lower level than chitosan (percentage of spore germination inhibition ranging from 7 to 10 %) (p-value = 10^{-6}) (Fig. 2). The other products did not show any significant effect on spore germination at the two doses tested (p-value = 0.1).

Fig. 2

B. cinerea spore germination inhibition caused by seven putative PDE and PDE references, tested at two doses. Seven products and four reference alternative products (Methyl Jasmonate (MeJA), BABA, Bion 50WG and Chitosan) were tested. Two product doses were assayed, 1× (*white bars*) and 10× (*black bars*). Germination inhibition of products was calculated as a ratio of germinated spores in water control. Standard deviation was calculated on two independent experiments. The *star* indicates significant differences with the water-treated controls, for which 100 % of the spores germinated (ANOVA, $\alpha = 0.05$)



Influence of abiotic stress on the protection efficacy of PDE

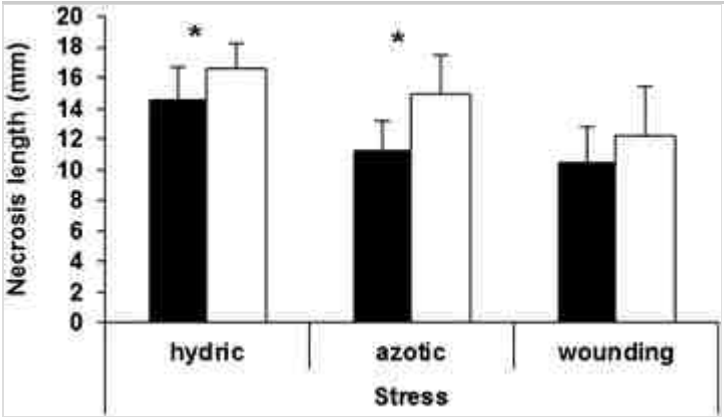
The influence of abiotic stress on the protection efficacy of PDE was

examined for three types of stress: leaf wounding, water stress and nitrogen deficiency.

First, the effects of abiotic stresses were evaluated on disease development in plants treated with water. Necrosis lengths caused by *B. cinerea* were always higher on plants previously exposed to an abiotic stress when compared to non-stressed plants, with significant differences found for water stress and nitrogen deficiency (p-value = 0.0081, < 0.0001 and 0.0881 for water stress, nitrogen deficiency and wounding, respectively) (Fig. 3). Under our experimental conditions, the ability of *B. cinerea* to colonize the host was therefore enhanced on pre-stressed plants.

Fig. 3

Mean necrosis length 5 days after inoculation with *B. cinerea* on tomato water-treated plants previously stressed or not and treated with water. The histograms (*white*: stressed; *black*: non-stressed plants) represent the mean of three independent assays for the wounding experiment and two assays for water stress and nitrogen deficiency experiments. The *bar* represents the standard deviation of the two or three independent experiments. The *star* indicates if there is significant difference between pre-stressed plants infected with *B. cinerea* and plants only infected with *B. cinerea* (ANOVA, $\alpha = 0.05$)

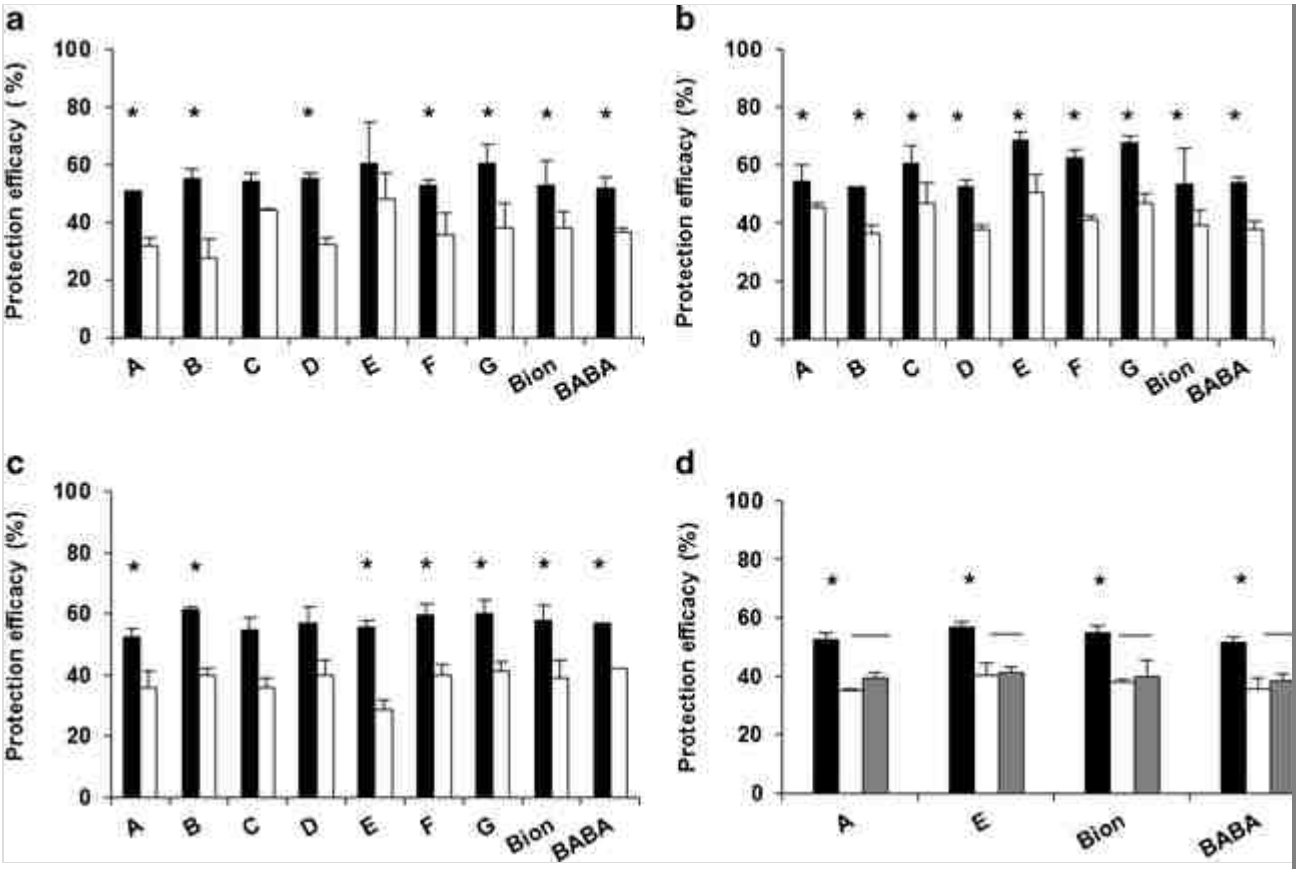


We then studied the influence of abiotic stresses on the efficacy of two PDE controls (BABA and Bion) and the products A-G (Fig. 4). Whatever the nature of the abiotic stress, the ANOVA indicated a significant effect of both the products (p-value = 10^{-6}, for water stress, nitrogen deficiency and wounding, respectively) and the application of the abiotic stress or not (p-value = 3.10^{-5}, 10^{-6} and 10^{-6}, for the three types of stress, respectively) on the necrosis length with no significant two-way interaction. In addition, whatever the nature of the abiotic stress, the protection efficacy of the

products was always lower on pre-stressed plants compared to non-stressed plants, with significant differences for most of the products (Fig. 4a–c). The reduction in protection efficacy between pre-stressed and non-stressed plants ranged from 9 to 27 %, depending on the product and the nature of the abiotic stress. Overall, our results clearly highlight that protection by PDE and potential PDE is affected in plants which have been previously exposed to wounding, nitrogen deficiency or water stress. Note however that treatments with the products were still able to significantly reduce disease development on pre-stressed plants compared to water-treated controls (p-value = 0.001).

Fig. 4

Protection efficacy of seven putative PDE and two PDE references against gray mold development. Protection efficacy of seven selected products (*A–G*), and PDE references products (Bion 50WG® and BABA) were assayed by measuring disease development 5 days after inoculation and compared to the protection efficacy observed in water-treated plants inoculated with *B. cinerea*. Plants were treated 5 days before inoculation (5 dbi) and were either stressed (*white and gray bars*) or not (*black bars*). Water stress (**a**), nitrogen deficiency (**b**) and wounding (**c** and **d**) were studied. Wounding was either applied 2 days before treatment (i.e., 7 days before inoculation) (**c**), or at two different time-points, 2 days before treatment (*white bars*) or at the moment of inoculation (*gray bars*) (**d**). Standard deviation was calculated on two independent exepriments. *Stars* indicate significant difference between non-stressed and pre-stressed plants (ANOVA, $\alpha = 0.05$). *Dashes* above the histograms indicate that no significant difference (**c**)



The timing of wounding effect was also studied: plant were stressed, either 2 days before treatment (i.e., 7 days before inoculation), or just prior to the inoculation (d0), (see Online Resource, Fig 1) with no significant differences in disease development. Both timing of wounding led to a similar loss of efficacy, whatever the product tested (Fig. 4d).

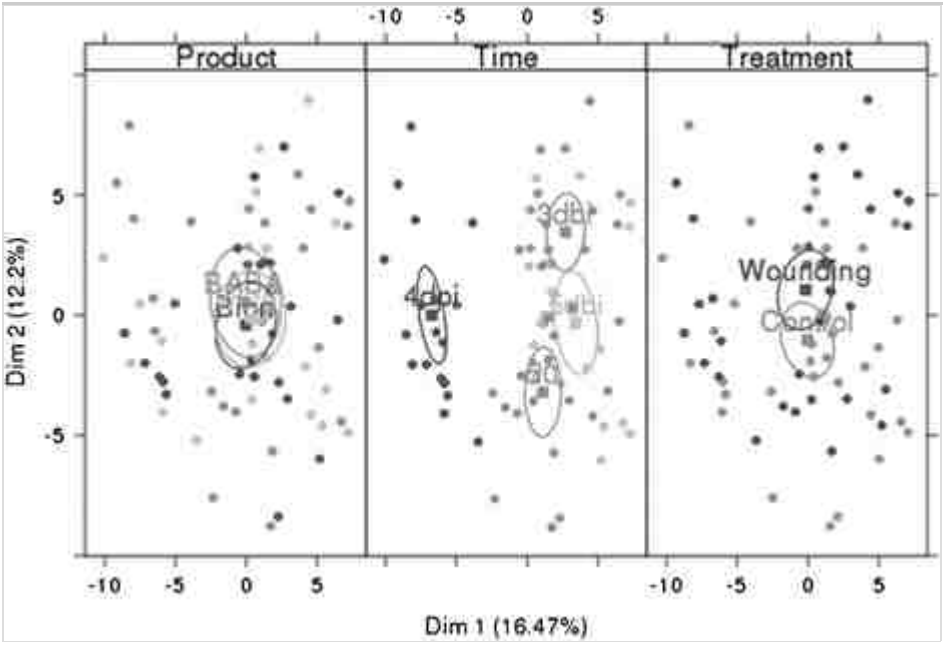
Studies of tomato metabolic regulation under wounding, PDE treatments and *B. cinerea* infection

The tomato metabolic profiling was established in samples collected as previously mentioned at four sampling times. Using all the metabolic data, a principal component analysis (PCA) was performed to analyze the relationships between the three variables, (i.e., PDE-treatment, wounding stress and sampling time). The plots showed that the first (16 %) and second components of the total variance (12 %) for all the pooled data were mostly explained by the sampling time, and in a smaller proportion by the wounding pressure, but not by the PDE-treatments (Fig. 5). Because of the strong effect of sampling time, PCA were then performed independently at each time point to analyze the relationships between PDE-treatment (Product) and wounding stress on metabolic responses (see Online Resource, Fig. 2). These statistical analyses showed that before infection (at 5dbi and 3dbi, see Online Resource, Fig. 1), the most important loadings on the first component (respectively, 22

and 17 %) were the Wounding and Control variables, whereas 5 days after PDE-treatment (d0) and moreover 4 days after inoculation by *B. cinerea* (4dpi) the two first components of the total variance were better explained by the PDE-treatments (Product variable), especially by the BABA-treatment, than by stress application (Treatment variable) (see Online Resource, Fig. 2).

Fig. 5

Principal Component Analysis (PCA) on metabolic profiling of tomato leaves harvested at different times, after wounding, PDE-treatments and *B. cinerea* inoculation. The PCA was performed on 71 samples, described by 130 metabolites and three qualitative variables (Product: water, Bion WG®, BABA; Time: 5 dbi, 3 dbi, d0, 4 dpi; Stress: Wounding, Control); (see Online Resource, Fig. 1)



To study the effect of wounding on metabolic regulation, we specifically analyzed the metabolic responses of tomato leaves, harvested 2 days after wounding (5dbi). When compared to non-stressed leaves, 10 upon the first 15 metabolites shown to feature significant increases in all wounded leaves (p-value ≤ 0.024; 1.1 to 3.6 fold) were identified as oxylipins (see Table 2; Online Resource, Table 1). Moreover the metabolic profiling of these 10 oxylipins strongly discriminated control and wounded samples in a partial least squares discriminant analysis (PLS-DA) (see Online Resource, Fig. 3).

Table 2

List of the 10 metabolites, significantly up-regulated in wounded leaves compared to control plants, harvested 5 days before inoculation and 2 days after wounding

XCMS name	Fold change	*P*-value	Formula	Metlin ID
M293T11	2.1	0.001	C18H30O3	13-HOTrE
M235T11	3.4	0.001	C15H24O2	Fragment from Octadecatetraenoic acid
M265T11	3.6	0.002	C16H26O3	Fragment from 13-HODE
M295T12	1.7	0.002	C18H32O3	13-HODE
M275T11	1.7	0.003	C18H28O2	Octadecatetraenoic acid
M593T11	2.0	0.006	C27H47O12P	PI(18:3/0:0)
M407T24	1.9	0.007	C19H35O7P	Palmitoylglycerone phosphate
M291T12	1.6	0.008	C18H28O3	12-oxo-PDA
M433T24	1.3	0.017	C21H39O7P	Lysophosphatidic acid
M431T24	1.1	0.024	C26H50NO7P	1-linolenoyl-sn-glycero-3-phosphate

In order to further study resistance mechanisms against necrotrophic pathogens, we also performed a time-course metabolite profiling of jasmonic acid (JA), salicylic acid (SA) and two targeted oxylipins, 13-hydroperoxy-octadecadienoic acid (13HpODE), 13-hydroxy-octadecatrienoic acid (13HOTrE), chosen as marker of these defence reactions. Production levels of the four compounds were compared between inoculated plants treated with PDE products (A, B, D, G, Bion, BABA) or with water (Table 3). The A, B, D and G products were selected because they showed good protection efficacy and relatively low direct effect. Significant increases of 13HpODE and/or JA compared to water control were detected at 4dpi for all treatments while no significant differences of 13HOTrE levels were found between the water treatment and the product treatments (Table 3). In our conditions, BABA mainly repressed or did not modulate the production of the selected metabolites (ratios mainly ranging between 0 and 1), with the exception of a significant increase of 13-HpODE at 4dpi (Table 3). Regarding SA, increased levels (over 2-fold changes) were found in Bion-, A-, B- and D-treated leaves

compared to water control at d0 and 4dpi, with significant differences only detected in Bion-treated leaves at d0. Significant decreased levels of SA were found in G-treated leaves at 4dpi (Table 3).

Products	Time	Metabolites			
		13-HpODE	13-HOTrE	JA	SA
BION50WG®	3dbi	5.3	2.4	ND	1.9
	d0 *	0.2	0.4	0.9	9.0
	4dpi	9.3	1.8	7.4	2.7
BABA	3dbi	3.6	2.3	ND	1.3
	d0	0.5	0.4	0	1.1
	4dpi	2.8	0.6	0	0.5
G	3dbi *	4.8	0.5	ND	1.5
	d0 *	0.9	0.4	3.1	1.0
	4dpi *	5.3	1.3	2.2	0.1
D	3dbi	19.0	1.2	ND	2.9
	d0	3.2	0.6	1.3	2.8
	4dpi	16.9	1.7	11.7	3.5
A	3dbi *	2.9	1.3	ND2	1.6
	d0 *	0,6	0.7	7.1	3.2
	4dpi *	7.4	1.3	4.9	1.9
B	3dbi	16.5	0.6	ND	1.7
	d0	1.3	0.3	3.0	2.0
	4dpi	8.3	2.1	4.3	2.3

Table 3

Relative metabolic ratio in tomato leaves pre-treated with PDE controls and putative PDE, compared to water-treated controls

Leaves were harvested 2 days after treatment, 3 days before inoculation (3 dbi) with *B. cinerea*, at the time of the inoculation (d0) and 4 days post inoculation (4 dpi). Two PDE controls (BION 50WG® and BABA) and four putative PDE (A, B, D and G) were tested. Metabolites were either not significantly induced (white), significantly induced (gray) or significantly repressed (black)

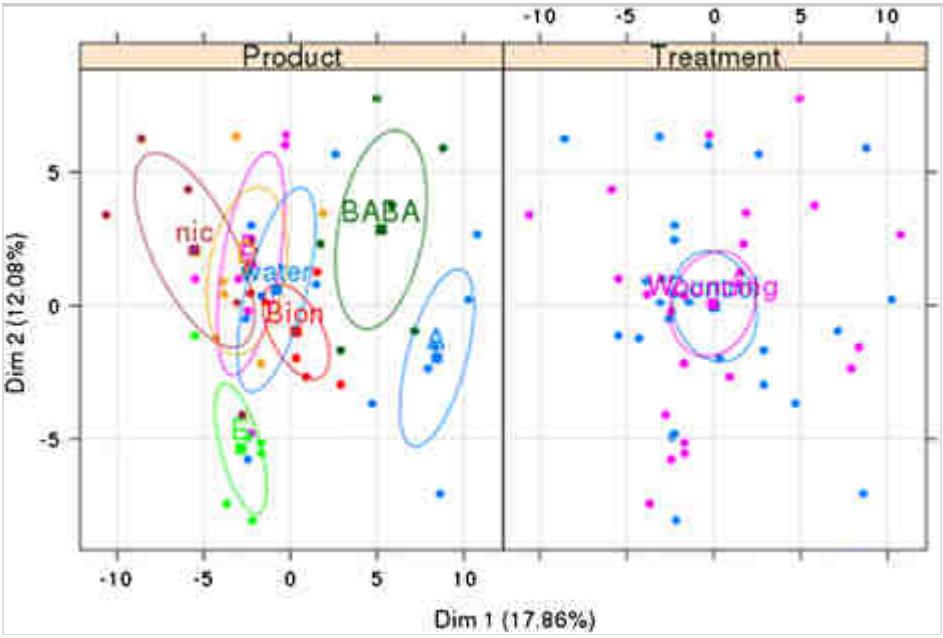
ND not detected in both product-and water-treated samples. *ND2* not detected in water-treated samples but detected in A-treated samples. Values are mean of fold changes in product-pretreated samples compared to water-treated samples ($n = 3$; *: $n = 2$). *13-HpODE* 13-hydroperoxy-octadecadienoic acid (oxylipin; fatty acid oxygenated), *13-HOTrE* 13-hydroxy-octadecatrienoic acid (fatty hydroxyl), *JA* Jasmonic Acid, *SA* Salicylic acid

Finally, we also investigated the action mode of new potential PDE (A, B, D, and E) and analyzed the metabolic profiling of tomato leaves harvested 4 days after inoculation by *B. cinerea*, following wounding and PDE treatment.

Using PCA, the global metabolic responses were compared between control and wounded plants, and non-inoculated (nic) and inoculated plants after the different PDE-treatments (including Bion and BABA) (Fig. 6). As previously shown for water, Bion and BABA treatments (see Online Resource, Fig. 2), at 4dpi, the Wounding/Control variables (Treatment) were not significantly different, while some Product variables were important loadings on the first two components of the total variance. The non-inoculated plant profiles (nic) appeared well separated from all inoculated tomato samples. Some PDE-treated plant metabolic profiling, e.g., with B, D, and Bion, were not significantly distinct from the water-treated samples. On this first axis of the total variance (18 %), A- and BABA-treated samples were the most important loadings, whereas on the second axis (12 %), it was the E-treated plants (Fig. 6).

Fig. 6

Principal Component Analysis (PCA) on metabolic profiling of tomato leaves harvested 4 days post-inoculation with *B. cinerea* and previously submitted to wounding and different PDE-treatments. The PCA was performed on 48 samples, described by 130 metabolites and two qualitative variables (Product: nic, A, B, D, E; water, Bion, BABA; Stress: Wounding, Control). *nic* non-inoculated control; (see Table 1)



Discussion

In this paper, we studied the protection efficacy of four established PDE (Bion 50WG®, BABA, MeJA, and Chitosan) against *B. cinerea* and seven new

potential PDE, which had no or few biocide effect on mycelial growth and spore germination of *B. cinerea.* Using a specific bioassay, these new products were able to reduce gray mold disease development, similarly to the PDE Bion 50WG®, BABA, Chitosan and MeJA. Under controlled conditions, a protective effect against *B. cinerea* was already demonstrated using BABA on *Arabidopsis* (Zimmerli et al. 2001; Cohen et al. 1994), Bion 50WG® on both *Arabidopsis* and tomato (Azami-Sardooei et al. 2013; Audenaert et al. 2002; Zimmerli et al. 2001), Chitosan on vine (Amborabe et al. 2004) and MeJA on tomato fruit (Zhu and Tian 2012; Eyre et al. 2006). To the exception of Chitosan which partly inhibited fungal germination (Fig. 2), their efficacy is not due to a direct fungicidal effect, but rather to the stimulation of plant defence reactions, leading to plant protection (Azami-Sardooei et al. 2013; Audenaert et al. 2002; Zimmerli et al. 2001; Cohen et al. 1994). Our study also characterized new potential PDE products for tomato protection against *B. cinerea* among which Eugenol, a phosphite, and extracts from seaweeds (*Ulva* spp.) and yeast (*S. cerevisiae*). Some of these were already characterized as plant defence elicitors in other plant/pathogen interactions, such as phosphite in *Arabidopsis* / *Hyaloperonospora arabidopsidis* (Massoud et al. 2012), and Eugenol in tomato/yellow leaf curl virus (Wang et al. 2013). Despite a good efficacy of those PDE under laboratory conditions, transfer to field conditions is sometimes found inconclusive or too variable (Small et al. 2012; Dinh et al. 2007), most probably because, in the field, many external factors can influence the efficacy of elicitor treatments. For example, protection by BTH or MeJA was shown to be ineffective in field on Geraldton waxflower (Dinh et al. 2007). To address these issues, we assayed, under controlled conditions, the impact of abiotic stresses commonly encountered during tomato growth (wounding, water stress and nitrogen deficiency) on gray mold development. First, in plants treated with water, pre-stressed plants were found to be more sensitive to *B. cinerea* than non-stressed plants. It has already been demonstrated that abiotic stresses influence the outcome of a pathogenic interaction, either negatively or positively, depending on the pathogen, timing, nature and severity of each stress (Suzuki et al. 2014; Atkinson and Urwin 2012). Some publications showed that depending on the type of pathogens, nitrogen deficiency can increase plant resistance (Stout et al. 1998; Sandermann 2004; Teng 1994; Dietrich et al. 2005). Concerning drought stress, it increases *Arabidopsis* susceptibility to *Pseudomonas syringae* (Mohr and Cahill 2003) but enhances resistance to *B. cinerea* in *Arabidopsis* (Chassot et al. 2008) and in tomato (Achuo et al. 2006), in contrast to our results. The discrepancy between the latter studies and our

results may be due to the timing and intensity of the abiotic stress applied. Indeed, in Achuo et al. (2006), the drought stress was applied by suspending irrigation of 3-week-old plants until they wilted, mildly irrigating for recovery, then allowing them to wilt again three times. Plants were then inoculated with the pathogens within 18 h after the last recovery irrigation. In our study, pre water stressed plants were beginning to wilt and no recovery was allowed before inoculation. Therefore, it is likely that, at the time of inoculation, the magnitude of the water stress was stronger in our study than in theirs. Secondly, the protection efficacy of the products and the PDE controls was always reduced when plants were subjected to an abiotic stress prior to treatments, whatever the nature of the abiotic stress. This result suggests that the tomato responses to abiotic stresses and to treatment with the products interact negatively.

AQ4

At a molecular level, the mechanisms involved in the plant responses to combination of biotic and abiotic stresses are controlled by various mode of signalling pathways including the hormone signalling (Atkinson and Urwin 2012; Suzuki et al. 2014). Abscissic acid (ABA) and JA are the main hormones involved in response to abiotic stresses while plant signalling pathways against pests and pathogens involve mostly SA, JA, ET and ABA (Curvers et al. 2010; Kettner and Dörffling 1995; Audenaert et al. 2002; Ryan and Moura 2002; Salt et al. 1986; Ton et al. 2009). Under our experimental conditions, we found that wounding induced significant metabolic changes, including the activation of the JA pathway with a 1.1 to 3.6-fold increase for 10 oxylipins in wounded leaves 2 days after stress application and before treatment with products. It has been previously shown that JA appeared very quickly after the wounding (<5 min) and not only in *Arabidopsis* wounded leaves, but also in the leaves distal to the wounded sites in plants (Glauser 2010). Over time, the metabolic changes associated with wounding could not be discriminated from non-wounded plants, from the day of inoculation (d0) until 4 days post-inoculation. Instead, differential tomato metabolic responses were identified depending on the product treatments and fungal inoculation. In other words, the metabolic changes occurring in tomato leaves could be first attributed to wounding. Seven days after the prior abiotic stress application, the effects of wounding on metabolic regulation were then outcompeted by the effects of other external factors, the product treatment and *Botrytis* inoculation. Moreover, if the wounding effect on metabolic profiling disappeared 4 days after inoculation, the interaction between product

treatment and infection with *Botrytis* did not lead to similar metabolic regulations (Fig. 6), suggesting a different mode of action, at least for BABA, the products A and E.

More specifically, the tomato responses associated with product treatments were studied using target metabolic profiling with the time-course of two oxylipins (13HpODE and 13HOTrE), JA and SA. Interestingly, it is worth underlining that the induction of defence pathways, compared to water controls, was significantly amplified after inoculation at 4 dpi. It seems therefore that Bion 50WG®, BABA, A, B, D and G act therefore more as priming agents than direct elicitors. In addition, we showed that treatment with products A, B, D and G and with Bion50WG® induced significant higher production of JA and 13-HpODE compared to water-treated controls, at 4dpi. Considering that we showed no or low direct effect of these products on the pathogen, protection efficacy conferred by these products may probably rely on their ability to induce the JA pathway, known to be involved in the resistance against necrotrophic pathogens. In addition, we also found that treatments with Bion, A, B and G led to increased SA levels at d0 and 4 dpi, with significant difference for Bion-treated leaves only detected at 0 dpi. This is not surprising given that Bion is a chemical analogue of SA and is classically used to induce SA biosynthesis in plants. The role of SA in the plant resistance against *B. cinerea* is rather complex. Using SA-deficient tomato plants, several lines of evidence indicate that SA contributes to the susceptibility of tomato plants against *B. cinerea* (El Oirdi et al. 2011) while a recent study supports a major involvement of the oxylipin pathway, in agreement with our study, along with a role of SA in both the basal response and the hexanoic-acid priming effect against *B. cinerea* (Angulo et al. 2014). Although our results cannot rule out the involvement of the SA pathway in the resistance mechanisms of tomato plants against *B. cinerea*, they underline that the activation of the JA-signalling pathway probably contribute the most to the product-induced resistant mechanisms, with the exception of BABA-induced resistant mechanisms. Indeed, under our conditions, BABA repressed or did not modulate the production of the selected metabolites except for 13-HpODE at 4 dpi, in agreement with a different action mode. Likewise, the global metabolite profiling of BABA-treated leaves was found to be significantly different compared to Bion- and water-treated plants at 4 dpi (see Online Resource, Fig. 2). BABA-induced resistant mechanisms against plant pathogens have been the focus of previous studies (Jakab et al. 2001; Zimmerli et al. 2001; Ton and Mauch-Mani 2004). In the latter study,

the authors discovered that BABA treatment in *Arabidopsis* primed callose accumulation, which is controlled by an ABA-dependent defence pathway and induced resistance to two necrotrophic pathogens, *Alternaria brassicicola* and *Plectosphaerella cucumerina*. In addition, results from Zimmerli et al. (2001) also suggested that BABA enhances resistance in *Arabidopsis* against *B. cinerea* through potentiation of SA-dependent defence responses but not via the JA/ET signalling pathway.

AQ5

Because plant resistance to *B. cinerea* notably occurred through the activation of the JA pathway, we can wonder why, under our experimental conditions, the effects of wounding, which also induced the JA-signalling pathway, interacted negatively with the effects of the products, resulting in a lower resistance to *B. cinerea*. It is classically considered that SA can antagonize JA and vice-versa (Mur et al. 2006; Truman et al. 2007). Based on our results which indicate that SA does not seem to contribute to the product-induced resistant mechanisms against *B. cinerea*, it cannot be hypothesized that the SA-JA antagonism may account for the decreased protection efficacy of the products on pre-stressed plants. Instead, other antagonist signalling pathways may be involved that were not studied here. For example, it has been shown that plant treatments with ABA increase plant susceptibility to various pathogens such as *B. cinerea* in tomato (Audenaert et al. 2002) and *P. syringae* in *Arabidopsis* (Mohr and Cahill 2003). Also, the ABA-deficient tomato mutant, sitiens, was found to be more resistant to *B. cinerea* (Audenaert et al. 2002; Curvers et al. 2010). Based on these results, it can be hypothesized that the application of the abiotic stress may have also led to the activation of the ABA signaling pathway, in turn resulting in an antagonistic interaction with JA/ET signalling pathway triggered by the products. Alternatively, hormone signalling interplay might not be the only explanation to a reduced efficacy of PDE under stressed conditions. Plant defences may be impaired after prolonged exposure to abiotic stress leading to enhanced susceptibility to subsequent biotic stresses (Suzuki et al. 2014), as suggested by the significant increase of the necrosis symptoms in stressed tomato leaves compared to unstressed controls (Fig. 3). Similarly, another study pointed that nitrogen deficiency combined with Bion 50WG® treatments led to a metabolic competition between the processes involved in the defence and plant growth, resulting in a lower fitness (Heil et al. 2000). Here, the exposure of tomato plants to wounding, nitrogen deficiency or water stress has probably resulted in the increase of the metabolic costs associated with

plant development, resulting in the weakening of plant defences and decreased efficacy of PDE.

In conclusion, we identified several promising nontoxic products and potential priming agents able to reduce gray mold development on tomato, notably through the activation of the JA pathway, but also through other signalling and regulation pathways (i.e., those involved in BABA-induced resistance). In addition, our work provides clear evidence that prior exposure of tomato plants to abiotic stress (leaf wounding, water stress or nitrogen deficiency) alters the protection efficacy of PDE treatments, suggesting an antagonistic interaction between the tomato responses to abiotic stresses and product treatments. Under our experimental conditions, we also showed that leaf cuttings and product treatments induced metabolic changes in a time-dependent manner, and that both of which activated the oxylipin and JA pathway. Interplay with other signalling events may also be involved that were not identified here, such as processes involved in plant growth or in other hormone signalling pathways. In turn, these interplays may have resulted in competitive interactions and ultimately led to an increased susceptibility of the plant to *B. cinerea* under stressed conditions. The outcomes of biotic and abiotic stress combination most probably vary according to the severity of the abiotic stress conditions, the plant species or genotype (Suzuki et al. 2014). The synergic or antagonist molecular mechanisms involved in the plant responses are also highly complex and mostly unknown (Suzuki et al. 2014). Future challenges will involve deciphering such mechanisms notably through the application of advanced omics technologies.

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Electronic supplementary material

Below is the link to the electronic supplementary material.

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