Biological Control 134 (2019) 53–62

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Extract of *Xanthomonas axonopodis* induces resistance in *Solanum tuberosum* against *Pectobacterium atrosepticum*

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ARTICLE INFO

Keywords:
Antioxidant enzymes
Biotic elicitor
Defense-related enzymes
Soft rot

ABSTRACT

Potato (*Solanum tuberosum*) is affected by many pathogens, such as the necrotrophic bacterium *Pectobacterium atrosepticum* (Pba), resulting in substantial economic losses. The induction of natural disease resistance in crops using biological and chemical elicitors has received increasing attention in recent years, due to the low environmental toxicity of this method of disease management. We evaluated the ability of the autoclaved suspension of *Xanthomonas axonopodis* (XTH) to modulate the defense metabolism and retard the disease caused by *P. atrosepticum* in detached leaves of *S. tuberosum*. Our results demonstrated that XTH slowed the progression and ameliorated the disease symptoms caused by *P. atrosepticum*. This effect may be related to the early activation of antioxidant enzymes such as catalase (CAT) at 0.5-h post treatment (hpt), as well as to the activation of the defense-related enzymes, phenylalanine ammonia lyase (PAL) and chitinase at 24 hpt and 12 hpt, respectively. Leaves treated with XTH + Pba also showed increase in polyphenol oxidase activity at 96 hpt. Leaves pretreated with the biotic elicitor did not show increase on free SA level, differing from the those inoculated with Pba. Our results indicated that XTH induces the plant defense metabolism and delays the progression of disease in potato caused by *P. atrosepticum*.

1. Introduction

Pectobacterium atrosepticum, formerly named *Erwinia carotovora* subsp. *atroseptica,* is a causal agent of bacterial soft rot and black leg, affecting both yield and quality of potato tubers. *P. atrosepticum*, a necrotrophic pectinolytic Gram-negative bacterium, produces extracellular enzymes such as pectate-lyases, pectinases, cellulases and proteases, resulting in tissue maceration and rot symptoms which affect postharvest storage and causing significant economic losses (Saubeau et al., 2014).

The induction of natural disease resistance in crops using biological and/or chemical elicitors is an auxiliary tool for plant disease management. Elicitors are compounds specifically recognized by plants, that induce defense responses against pathogens or herbivores. These compounds may lead to systemic expression of a wide variety of genes coding antimicrobial molecules in distal tissues, protecting the entire plant from secondary infection. This phenomenon, known as systemic acquired resistance (SAR), has been reported to be effective against diverse pathogens (Gozzo and Faoro 2013; Oliveira et al. 2017).

Over the years, the use of agrochemicals (fungicides and pesticides) to control crops pathogens has been found to constitute an environmental hazard and causes bioaccumulation of toxic substances in the food chain (Enebe and Babalola, 2019). Therefore new strategies are required to permit sustainable crop production with minimal impact on natural resources, as well as, safety for users and consumers (Luna-Diez, 2016). In this context, the use of biotic inducers has been shown to be a good eco-friendly alternative for disease control (Borges et al., 2019).

Different classes of biotic inducers are recognized by the cell surface–localized pattern-recognition receptors (PRRs) through pathogen-associated molecular patterns (PAMPs). Common features of PAMPs are their highly conserved structures, such as bacterial lipopolysaccharide (LPS), lipoproteins and flagellin. They are functionally important for microorganisms and are present in a broad range of microbial species (Casabuono et al., 2011). Elexa® (chitosan) and Messenger® (harpin from *Erwinia amylovora*) are commercial biotic inducers based on these natural defense responses which have been used to protect a range of crops against important pathogens. In our previous studies, an autoclaved extract from the bacterium *Xanthomonas axonopodis* pv. *citri* (inducer XTH) was efficient in promoting the defense metabolism of *S. tuberosum* plants sprayed with this elicitor and challenged with *Pectobacterium* spp. (US8932844B2). Potato plants sprayed with XTH

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https://doi.org/10.1016/j.biocontrol.2019.03.012
Received 21 December 2018; Received in revised form 10 March 2019; Accepted 13 March 2019
Available online 14 March 2019
1049-9644/

formulation showed over 60% reduction of disease symptoms caused by *P. atrosepticum*, and neither hypersensitivity responses (HR) nor plant toxicity were observed (CN102256495A, US8932844B2). On the other hand, the interaction of the cell suspension of *X. axonopodis* pv. *citri* with various nonhost plants, such as cotton, bean, tobacco, tomato, pepper and potato, resulted in pathogenic gene expression (*hrp*) and plant HR symptoms (Dunger et al., 2005; Poiatti et al., 2009).

The spread of disease in a plant is limited by plant's ability to induce a complex array of defense responses after the detection of a microorganism, via the recognition of elicitor molecules released during the plant–pathogen interaction. Following elicitor perception, the activation of signal transduction pathways leads to the production of reactive oxygen species (ROS). The most biologically important ROS are the free radicals H_2O_2 (hydrogen peroxide), O_2^- (superoxide) and OH^- (hydroxyl radicals). Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APx), are responsible for the maintenance of the ROS balance, and therefore, increased activities of antioxidant enzyme is an important component in the defense strategy (Oliveira et al., 2016; Noctor et al., 2017). In addition, hormone signals, such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) are activated in the cell after ROS imbalance. These signals lead to increased activity of defense-related enzymes, such as phenylalanine ammonia lyase (PAL), polyphenol oxidases (PPO) and peroxidases (POX), as well as the accumulation of pathogenesis-related (PR) proteins (β-1,3-glucanase and chitinase) (Mahesh et al., 2017). All these defense reactions, often associated with localized cell death, are important responses for limiting the pathogen growth. However, if these defense reactions do not occur within a suitable time to impair the pathogen, the infection will spread successfully (Tsuda and Katagiri, 2010).

The present study evaluated the ability of the biotic inducer XTH to modulate the defense metabolism and to retard the disease caused by *P. atrosepticum* in detached leaves of *S. tuberosum*. The cellular metabolism involved in this response was analysed through the changes in the activity of antioxidant (SOD, CAT and APx) and defense-related enzymes (PAL, PPO, POX, β-1,3-glucanase and chitinase), as well as in the levels of salicylic acid (SA) and total phenolic compounds.

2. Materials and methods

2.1. Plant material

Seed tubers of *Solanum tuberosum* cv. Agata were provided by potato growers (São Francisco de Paula, Rio Grande do Sul, Brazil, 29°26′49″S 50°34′44″W). The cv. Agata is widely cultivated in Brazil and it is highly susceptible to *Pectobacterium* spp. (Poiatti et al., 2009). Tubers were cultivated in pots (8 L) with commercial organic substrate (clay, hummus and ground calcareous rock) and sand (70:30 (v/v), and maintained in a greenhouse for three or four weeks (stolon and tuber formation phases).

2.2. Preparation of the biotic elicitor XTH

Xanthomonas axonopodis pv. *citri* extract (XTH) was used as elicitor of plant defense metabolism. *X. axonopodis* was grown in LB broth at 25 °C for 48 h under 100 rpm. The bacterial culture was centrifuged at 4000*g* for 10 min, the pellet was re-suspended in sterile distilled water and rinsed three times. Bacterial concentration was adjusted to 10^{12} cfu.mL^{-1} (OD_{600nm} = 1.0) and inactivated by autoclaving for 1 h at 121 °C. The concentration and inactivation method were determined in previous experiments (data not shown). The inactivated bacterial suspension (therein, XTH) was used as the biotic elicitor. Experiments in which leaves were challenged with the pathogen, XTH was sprayed on potato plants (pretreatment), using a low-pressure atomizer.

2.3. Culture of the phytopahogen

Pectobacterium atrosepticum (Pba) (kindly provided by Dr. Valmir Duarte, Laboratory of Phytopathology, UFRGS, Brazil) was grown in LB broth at 25 °C for 16 h under 100 rpm. The bacterial culture was centrifuged at 4000*g* for 10 min, the pellet was re-suspended in sterile distilled water and rinsed three times. In the final step, bacterial concentration was adjusted to 10^9 cfu.mL^{-1} (OD_{600nm} = 0.5) in sterile distilled water. The suspensions of Pba were immediately used for inoculation on detached potato leaves.

2.4. Experimental design

2.4.1. Effect of XTH as biotic inducer on potato leaves

Expanded leaves from potato plants (three to four weeks old) were detached and placed in sterile Petri dishes (15 × 2.5 cm) with two layers of moistened filter paper (15 mL of sterile distilled water). The petioles were covered with wet cotton to reduce the leaf dehydration within the plate. Two leaves were placed in each plate. All plates were maintained under photoperiod (16 h-light) and controlled temperature (25 °C). Elicitor treatment consisted in spraying 1 mL/leaf of XTH. Leaves from the control were sprayed with sterile water.

Fresh leaves were analyzed for activity of enzymes related to oxidative stress and defense metabolism, levels of free and conjugated salicylic acid (SA), and levels of phenolic compounds at different time points (Sections 2.6–2.8).

2.4.2. Effect of XTH pretreatment on potato leaves challenged with Pba

Potted potato plants (3–4 weeks old) were pretreated by spraying 5 mL.plant^{-1} of the elicitor solution (XTH), and maintained in a greenhouse. Plants sprayed with sterile distilled water were used as control-pretreatment (Cp). Four days after spraying (Poiatti et al., 2009), expanded leaves were detached (eight leaves per treatment), transferred to Petri dishes as described above (Section 2.4.1), and challenged with the pathogen by infiltration of 100 μL Pba suspension on the abaxial surface of the lateral leaflet using a needleless syringe. The petioles were covered with wet cotton in order to reduce leaf dehydration.

Treatments consisted in detached leaves that were removed from plants sprayed either with elicitor (XTH) or sterile water (Cp), and inoculated with Pba (namely, XTH + Pba; Cp + Pba, respectively). The absolute control treatment consisted in plants sprayed (Cp) and inoculated with sterile water (Ct) (Cp + Ct). All plates were maintained under a photoperiod of 16 h and at 25 °C. Detached leaves in moist chambers have been used for studying plant-pathogen interactions in many species (IAEA, 2010). This method is a routine assay for screening of potato for resistance to *Phytophthora infestans* disease, as well as physiological and molecular analyses of potato defense responses (Vleeshouwers et al., 1999, 2000)).

Fresh leaf samples (fresh mass, FM) were analysed for activity of enzymes related to oxidative stress and defense metabolism, levels of free and conjugated salicylic acid (SA), and levels of phenolic compounds at different time points (Sections 2.6–2.8).

2.5. Disease assessment of leaves pretreated with XTH

Disease progression was evaluated using detached leaves from plants submitted to the following treatments: XTH + Pba, Cp + Pba and control (Cp + Ct). Leaves were maintained in Petri dishes, as previously described. As disease symptom, chlorosis was evaluated during five days after the pathogen infection (dpi). The disease incidence was determined by estimating the leaf area (%) showing the symptom. For this purpose, the leaf was divided into three equal parts. Disease incidence in a completely chlorosed leaf was considered to be 100%. The area under the disease progress curve (AUDPC) was estimated (Jeger and Viljanen-Rollinson, 2001).

2.6. Enzyme assays

2.6.1. Antioxidant enzymes

Enzymes related to oxidative stress (SOD, CAT, and APx) were analysed in leaf samples collected at 0.5, 1, 3 and 6 h post treatment of either the biotic inducer XTH or the pathogen (hpt).

Superoxide dismutase (SOD; EC 1.15.1.1) – Leaf samples (0.5 g) were homogenized in 2.5 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 1% PVP and 0.1 mM EDTA. The homogenate was centrifuged at 3200*g* for 20 min at 4 °C and the supernatants were used for enzyme assays. SOD activity was determined according to Beyer and Fridovich (1987) in a reaction containing 50 mM potassium phosphate buffer (pH 7.8), 57 μM nitro blue tetrazolium (NBT), 9.9 mM methionine, 1% Triton X-100, 0.9 μM riboflavin and 200 μL of extract. Absorbance was recorded at 560 nm after white illumination for 15 min. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT photoreduction.

Catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APx; EC 1.11.1.11) – Leaf samples (0.6 g) were homogenized in 1.8 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1% PVP and 0.1 mM EDTA. The homogenate was centrifuged at 3200*g* for 20 min at 4 °C and the supernatants were used for enzyme assays. The activity of CAT was estimated by measuring the rate of decomposition of H_2O_2 at 240 nm (Havir and McHale, 1987). The reaction medium contained 50 mM potassium phosphate buffer (pH 7.0), 12 mM H_2O_2 and 50 μL extract. Total APx activity was estimated by monitoring the decrease in absorbance at 290 nm (Nakano and Asada, 1981). The reaction contained 50 mM potassium phosphate buffer (pH 7.0), 2 mM H_2O_2, 20 μL ascorbate (2 mM) and 50 μL extract. One unit of APx was defined as the amount of enzyme required to oxidize 1 μmol of ascorbate. min^{-1}.

2.6.2. Defense-related enzymes

The enzymes PAL, PPO, POX, β-1,3-glucanase and chitinase were analysed in leaf samples collected at 12, 24, 72 and 96 hpi.

Phenylalanine ammonia lyase (PAL; EC 4.3.1.24) – Leaf samples (0.3 g fresh mass) were grounded in 3 mL of 50 mM Tris-HCL (pH 8.8) containing 1 mM EDTA, 1% polyvinylpyrrolidone (PVP), and 0.2% Triton X-100. Extracts were centrifuged at 3200*g* for 20 min at 4 °C, and the supernatants were used for enzyme assays. PAL activity was evaluated following Navarre et al. (2013), with modifications. The reaction containing 200 μL of extract, 0.2 M phenylalanine and 50 mM sodium borate buffer (pH 8.8) was incubated for 1 h at 37 °C. The enzyme-activity unit was defined as the amount of enzyme needed to produce 1 nmol of cinnamic acid. mL^{-1} of reaction. $min^{-1}.mg^{-1}$ of protein, at 290 nm.

Polyphenol oxidase (PPO; EC 1.14.18.1) and peroxidase (POX; EC 1.11.1.7) – Leaf samples (0.5 g) were homogenized in 2.5 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 1% PVP. Extracts were filtered and centrifuged at 3200*g* for 20 min at 4 °C, and the supernatants were used for enzyme assays. Polyphenol oxidase activity was determined spectrophotometrically at 400 nm using chlorogenic acid as substrate (Poiatti et al., 2009). The samples were incubated for 30 min at 30 °C. The enzyme-activity unit was defined as the change in absorbance.$min^{-1}.mg^{-1}$ protein. Peroxidase activity was determined spectrophotometrically by the oxidation of guaiacol at 420 nm. The reaction mixture contained 50 mM sodium phosphate buffer (pH 6.0), 0.1 M guaiacol as substrate and 10 mM hydrogen peroxide. The enzyme-activity unit was expressed in μkatal.mg^{-1} protein. One katal (kat) corresponds to the amount of enzyme activity that transforms 1 mol.s^{-1} of substrate (Poiatti et al., 2009).

β-1,3-Glucanase (EC *3.2.1.39) and chitinase (EC 3.2.1.14)* – Leaf samples (0.5 g) were homogenized in 2.5 mL of 50 mM sodium acetate buffer (pH 5.0) containing 1% PVP. The extracts were filtered and centrifuged at 3200g for 20 min at 4 °C, and the supernatants were used for enzyme assays. The β-1,3-glucanase activity was determined according to Fink et al. (1988), with modifications. The reaction mixture consisted of 50 μL extract, 170 μL laminarin (2 $mg.mL^{-1}$) and 50 mM sodium acetate buffer (pH 5.0). The reaction was incubated at 37 °C for 1 h. Then, copper reagent was added and the solution was heated in a water bath (100 °C) for 10 min. The mixture was allowed to return to room temperature, and an arsen-molybdate solution was added. Samples were diluted in 1.5 mL of distilled water and read at 500 nm. For the determination of chitinase activity, the reaction solution consisted of 1.8 mg of chitin azure (Sigma), 140 μL of 50 mM sodium acetate (pH 5.0) and 70 μL of extract. The reaction solution was incubated at 37 °C for 24 h. Then, 100 μL of 2 M HCl and 300 μL of 50 mM sodium acetate (pH 5.0) were added to stop the reaction. Samples were centrifuged at 14,000*g* for 10 min and the absorbance of the supernatant was measured at 550 nm.

Specific activities of enzymes were calculated based on total protein concentration, which was estimated using Bradford reagent with bovine serum albumin (BSA) as standard (Bradford, 1976). A minimum of four biological repetitions with three replicates each was used for all analyses.

2.7. Phenolic compounds

Leaf samples (0.25 g) collected at 12, 24, 48 and 72 hpi were homogenized in 5 mL of 80% (v/v) aqueous methanol. Extracts were filtered and centrifuged at 4000*g* for 15 min at 15 °C. Phenolic compounds were quantified by the colorimetric technique, using Folin-Ciocalteu reagents and Na_2C0_3 (20% w/v). Absorbancy (765 nm) was determined after 30 min incubation at 25 °C in the dark (Poiatti et al., 2009). Gallic acid was used as standard. A minimum of four biological repetitions with three replicates each was used for analysis.

2.8. Salicylic acid analysis

SA extraction was adapted from Marek et al. (2010) and Verberne et al. (2002). Leaves samples (0.5 g) were macerated in liquid nitrogen and extracted with 1 mL of 90% methanol. Sample was sonicated for 5 min and after centrifugation at 14,000*g* for 10 min, the supernatant was transferred to a microcentrifuge tube. Extraction was repeated twice. Supernatants were combined and concentrated under air flow. Residue was resuspended with 0.1 M sodium acetate buffer (pH 5.5) in order to convert SA into sodium salicylate, which is less volatile. Trichloroacetic acid (5% v/v) was added and the mixture was partitioned twice with ethyl acetate:cyclohexane (1:1 v/v). The organic phase (with free SA) was concentrated. The aqueous phase, with the glucose conjugated SA (salicylic acid 2-O-β-D-glucoside), was subjected to acid hydrolysis by adding 8 M HCl and heating at 80 °C for 2 h. The hydrolyzed fraction was partitioned and concentrated as previously described. Recovery rate of SA, after extraction steps, was determined by analysis of a sample with a known concentration of SA (internal control). The recovery rate was calculated and used for correcting the extracted SA concentration. SA quantification was performed by HPLC performed using a Luna C18 (150 × 4.60 mm i.d.; 3 μm) column from Phenomenex (Torrance, CA, USA) with a Phenomenex SecurityGuard pre-column. and spectrofluorometric detector operated at an emission wavelength of 407 nm and an excitation wavelength of 305 nm. Sample was eluted with 0.2 M sodium acetate buffer (pH 5.5) and methanol (90:10 v/v) at a flow-rate of 0.8 mL min^{-1}. A minimum of four biological repetitions was used; each repetition was analyzed in one replicate for the chromatographic analysis.

2.9. Statistical analysis

Means were determined from four to six replicates of a pool of eight leaves per treatment. The homogeneity of variances was determined by Levene's test and when necessary data were transformed to adjust to the normal distribution. The occurrence of extreme values was determined by BoxPlot test. Means were compared using analysis of variance

Table 1
Disease progression (Area Under the Disease Progress Curve; AUDPC) in detached leaves of *S. tuberosum* pretreated with extract of *X. axonopodis* and inoculated with *P. atrosepticum* (XTH + Pba); pretreated with sterile water and inoculated with *P. atrosepticum* (Cp + Pba); pretreated with sterile water and inoculated with sterile water (Cp + Ct). Evaluation was carried out for five days.

Treatments	AUDPC
Control (Cp + Ct)	111.00 ± 80.65b
Cp + Pba	496.72 ± 314.22 a
XTH + Pba	233.10 ± 151.50b

Mean ± standard error with different letters indicate significant differences between the treatments (Duncan's test, $p \leq 0.05$).

(ANOVA) complemented by Duncan's test, with $p \leq 0.05$. The correlation between activities of antioxidative enzymes was determined using Pearson correlation coefficient, considering the all the time points of analysis. Data were analyzed using the SPSS software v. 18.

3. Results

3.1. Promotion of detached leaf resistance by XTH

Treatment with the biotic elicitor XTH resulted in alterations of the disease progression. Detached leaves from the XTH + Pba treatment showed a significant reduction in disease progression compared to the treatment Cp + Pba and it was similar to the control (Cp + Ct) leaves (Table 1; Fig. 1a). In addition, the challenge with Pba of plants that had no previous treatment with biotic elicitor (Cp + Pba) resulted in 55.5% of disease symptoms evaluated by the area of leaf chlorosis at the third

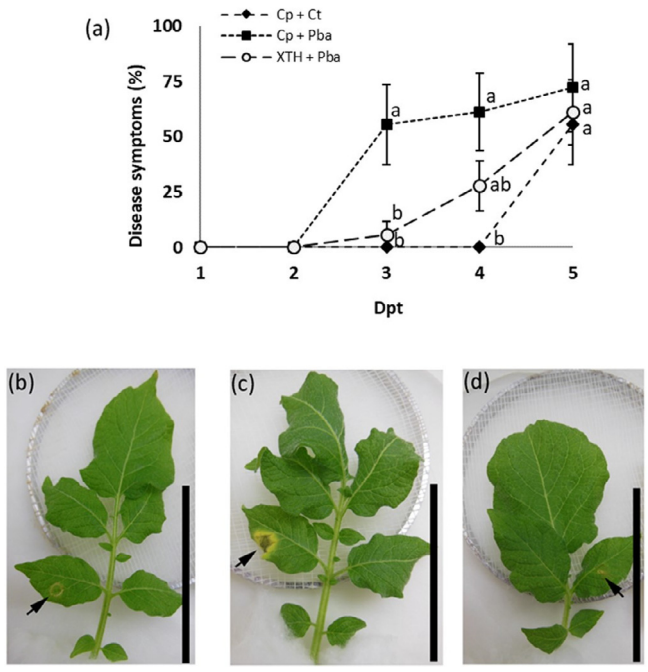

Fig. 1. (a) Disease symptoms in detached leaves of *S. tuberosum* pretreated with extract of *X. axonopodis* and inoculated with *P. atrosepticum* (XTH + Pba); pretreated with sterile water and inoculated with *P. atrosepticum* (Cp + Pba); pretreated with sterile water and inoculated with sterile water (Cp + Ct). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$). Representative images of leaves at 3 dpi: (b) Cp + Ct, (c) Cp + Pba, infected leaf with necrotic spot in the leaflet, (d) XTH + Pba. Arrows indicate the site of bacterial inoculation. Bar = 5 cm.

day after challenging (Fig. 1a and c). Leaves of XTH + Pba did not differ statistically and visually from the control (Fig. 1a, b and d). Our previous results showed that XTH do not impair Pba growth, regardless the density of inactivated bacteria suspension used (Supplementary material, S1). Nevertheless, at 5 dpi disease was observed in the treatments with Pba, regardless the pretreatment with XTH (Fig. 1a). Leaf senescence (chlorosis symptoms) of detached leaves was observed at the fifth day post-detachment in the control treatment (Fig. 1a). It is noteworthy that spraying plants with XTH did not induce HR (Fig. 1d).

3.2. Antioxidant enzymes

The activity of enzymes related to oxidative stress responded to the treatment with the biotic elicitor XTH although the time of response varied according to each enzyme.

When the effect of XTH on potato leaves was tested, activity of SOD was lower or similar to the control in most of the time points, although an increment was observed at 1 hpt (Fig. 2a). CAT activity in the leaves sprayed with XTH was higher than in control leaves within the first 3 hpt, while APx activity increased only at 1 hpt (Fig. 2b and c). A negative correlation ($r = -0.503$, $p < 0.05$), representing increase in CAT activity simultaneous with reduction of SOD activity, was observed in leaves treated with XTH. On the other hand, correlation between the activities of APx and CAT was determined as positive over the time of analysis ($r = 0.742$, $p < 0.01$).

Leaves pretreated with XTH and challenged with Pba (XTH + Pba) showed lower SOD activity at 0.5-h post treatment (hpt) then leaves from the Cp + Pba and Cp + Ct treatments. Moreover, SOD reached the significantly highest level of activity in leaves from Cp + Pba at 3 hpt (Fig. 3a). Detached leaves pretreated with the elicitor XTH and challenged with the pathogen Pba (XTH + Pba) presented increased SOD activity at 1 and 6 hpt when compared to non-pretreated leaves (Cp + Pba). CAT activity in detached leaves from the treatment XTH + Pba showed a markedly increase at 0.5 hpt when compared to the other treatments (Fig. 3b). However, no difference was detected later in the evaluation, from 1 to 6 hpt (Fig. 3b). Similar to the other analyzed enzymes, APx activity was modulated by pretreating detached leaves with XTH and a peak of enzymatic activity was seen at 6 hpt (Fig. 3c). Leaves XTH-untreated but infected with Pba (Cp + Pba) showed significant increase in APx activity at 3 hpt, similar to the response observed with the SOD enzyme.

Correlation between SOD and CAT activities in leaves from XTH + Pba treatment followed the same tend observed when XTH was used alone ($r = -0.491$, $p < 0.05$). Otherwise in the Cp + Pba ($r = -0.620$, $p < 0.01$) the negative correlation represented increase on SOD activity simultaneous to the decrease of CAT activity. A strong positive correlation was observed between CAT and APx in XTH + Pba ($r = 0.709$; $p < 0.01$). No correlation was detected in the control treatment (Cp + Ct) for the enzymes analysed.

3.3. Defense-related enzymes and phenolic compounds

Modulation of plant defense metabolism involving changes in the activity of enzymes, such as PAL, PPO and POX, as well as in the production of phenolic compounds were observed in detached leaves of *S. tuberosum*. Treatment with XTH alone did not alter the concentration of phenolic compounds when compared to the control leaves (Cp) (Fig. 4a). One the other hand, when leaves were treated with either XTH + Pba or Cp + Pba, phenolic compounds were accumulated at 96 hpt (Fig. 5a).

In contrast to the phenolic compounds, activity of defense-related enzymes varied significantly in detached leaves along the time course analyzed. An early response related to PAL activity was detected in leaves treated with XTH and XTH + Pba, beginning at 24 hpt compared to the control treatments (Cp or Cp + Ct) (Figs. 4b and 5b). A markedly increase on PAL activity was also recorded on XTH + Pba at 96 hpt,

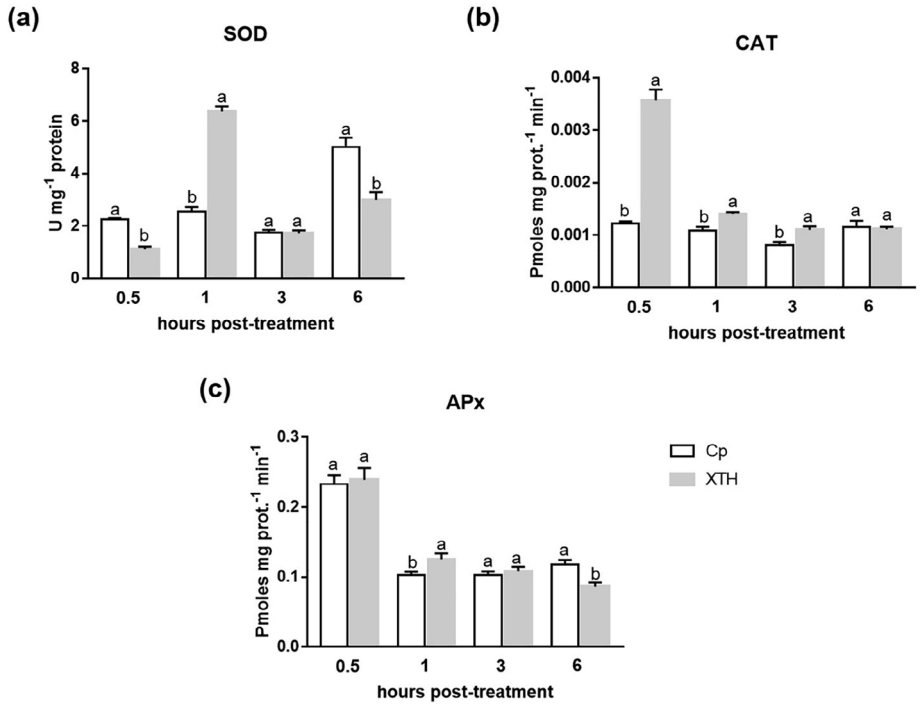

Fig. 2. Activity of antioxidant enzymes (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) ascorbate peroxidase (APx) in potato detached leaves. Plants were treated with extract of *Xanthomonas axonopodis* (XTH) or sterile water (Cp). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

significantly different from control and Cp + Pba (Fig. 5b). On the other hand, a punctual variation in PPO activity was observed among treatments at 24 hpt and 96 hpt, when its activity increased in leaves elicited with XTH and XTH + Pba treatments, respectively (Figs. 4c and 5c). At the 96 hpt the lowest PPO activity was recorded on Cp + Pba leaves (Fig. 5c). However, in the POX analyses, only the treatment Cp + Pba positively affected the enzyme activity at 72 and 96 hpt, while no difference was noted in detached leaves from XTH or XTH + Pba compared to the control treatments (Figs. 4d and 5d).

XTH alone differently affect the PR proteins β-1,3-glucanase and chitinase. A decrease on β-1,3-glucanase activity was observed from 24 hpt to 96 hpt (Fig. 4e). On the contrary, chitinase activity increased at 12 hpt (Fig. 4f). On the other hand, in detached leaves treated with Cp + Pba, β-1,3-glucanase showed evident effect of the pathogen infection, increasing activity at 24 hpt (Fig. 5e). At the following time points the activity of β-1,3-glucanase decreased in both treatments (XTH + Pba and Cp + Pba) when compared to control (Fig. 5e). Moreover, the pretreatment with XTH strongly stimulated the activity of chitinase at 12 hpt (Fig. 5f). In general, the activity of β-1,3-glucanase and chitinase decreased in all treatments at 96 hpt compared to the control treatments (Figs. 4 e, f and 5 e, f).

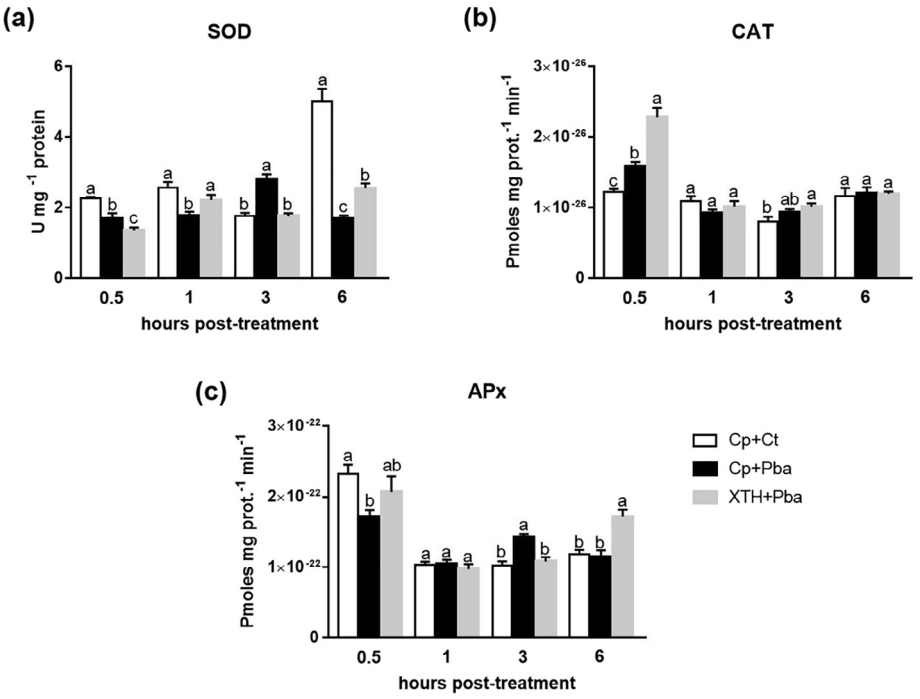

Fig. 3. Activity of antioxidant enzymes (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) ascorbate peroxidase (APx) in potato detached leaves. Plants were pretreated with extract of *Xanthomonas axonopodis* (XTH) or sterile water (Cp). After four days, leaves were detached and treated with *P. atrosepticum* (Pba) or sterile water (Ct). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

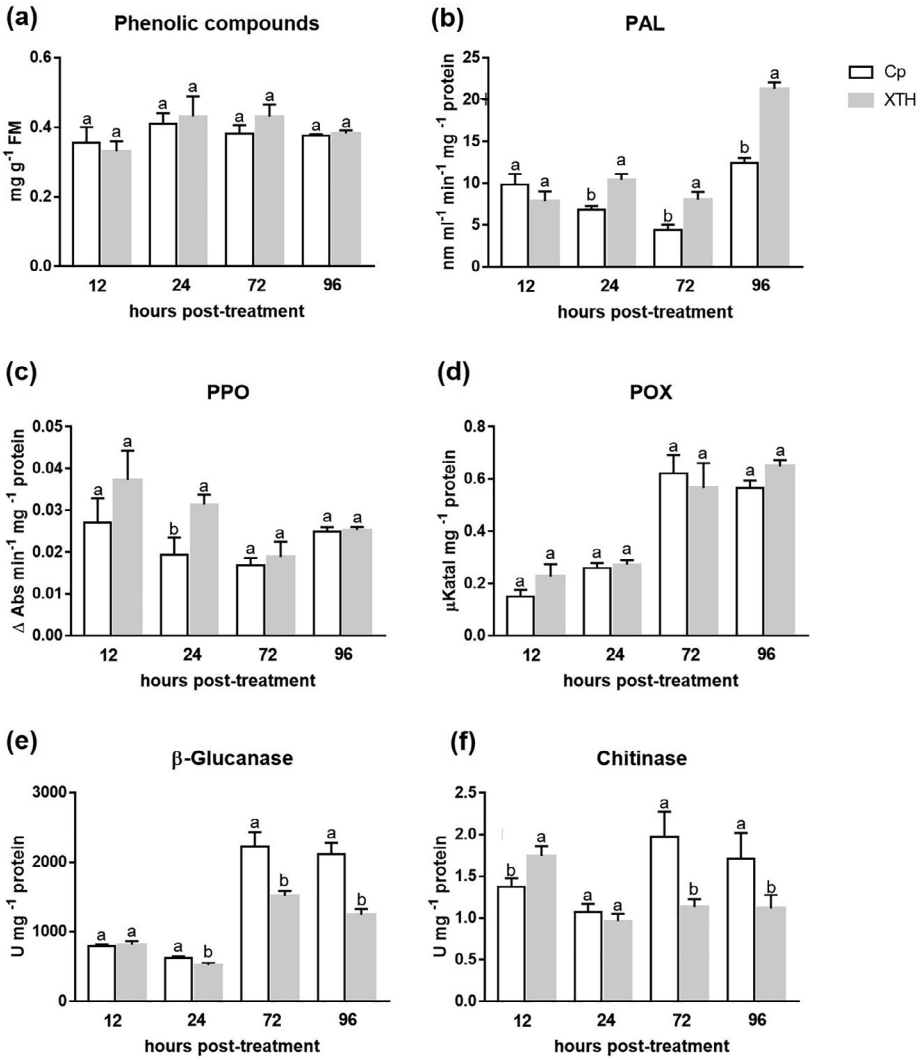

Fig. 4. Levels of (a) phenolic compounds and activity of (b) phenylalanine ammonia lyase (PAL), (c) polyphenol oxidase (PPO), (d) peroxidase (POX), (e) β-1,3-glucanase and (f) chitinase in potato detached leaves. Plants were treated with extract of *Xanthomonas axonopodis* (XTH) or sterile water (Cp). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

3.4. Salicylic acid

The levels of free and conjugated salicylic acid (SA) were determined at 12, 24, 72 and 96 hpt. XTH alone did not increase free SA (Fig. 6a). The free SA was only accumulated in detached leaves from Cp + Pba treatment at 12 and 72 hpt, compared to the other treatments (Fig. 7a). At 96 hpt leaves from both Cp + Pba and XTH + Pba contained high concentration of free SA compared to Cp + Ct (Fig. 7a). On the other hand, increased levels of conjugated SA were detected in the detached leaves treated with XTH alone (Fig. 6b), Cp + Pba and XTH + Pba only at 96 hpt (Fig. 7b), compared to the control treatments. No correlation between free-SA and conjugated-SA was observed for any of those treatments analyzed in the present study (data not shown).

4. Discussion

The use of avirulent or attenuated strains of either pathogenic or saprophytic microorganisms to induce SAR in vegetative host tissue has been relatively well documented (Terry and Daryl, 2004). According to our previous study, an inactivated extract of *X. axonopodis* pv. *citri* (XTH) played a role as an inducer, modulating the defense metabolism in leaves of *S. tuberosum* (CN102256495A, US8932844B2). Moreover, the present study demonstrated that XTH was able to induce the activity of antioxidant enzymes (SOD, CAT and APx) and some enzymes related to defense response as PAL, PPO and chitinase.

Pretreatment of susceptible plants with avirulent pathogens (biotic inducers) can enhance resistance to subsequent attack, not only at the site of treatment, but also in tissues distant from the initial infection sites. Typically, this inducible resistance system, known as systemic acquired resistance (SAR), is effective against diverse pathogens including viruses, bacteria, and fungi (Oliveira et al., 2016). In this present study, we showed that leaves pretreated with the biotic inducer XTH four days before the inoculation with *P. atrosepticum* significantly delayed the disease progression and decreased the symptoms caused by the phytopathogen. However, the mechanism involved in the defense metabolism during the infection by *P. atrosepticum* in leaves pretreated with XTH is complex and remains unclear. In this regard, it has been reported that lipopolysaccharides (LPS), indispensable components of the cell surface of Gram-negative bacteria, trigger certain plant defense-related responses, such as SAR and PR-proteins accumulation (Neyen and Lemaitre, 2016). In addition, studies with LPS from *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* demonstrated that these molecules are involved in activation of the immune response (Casabuono et al., 2011).

Plants respond to pathogen attack by inducing a range of responses

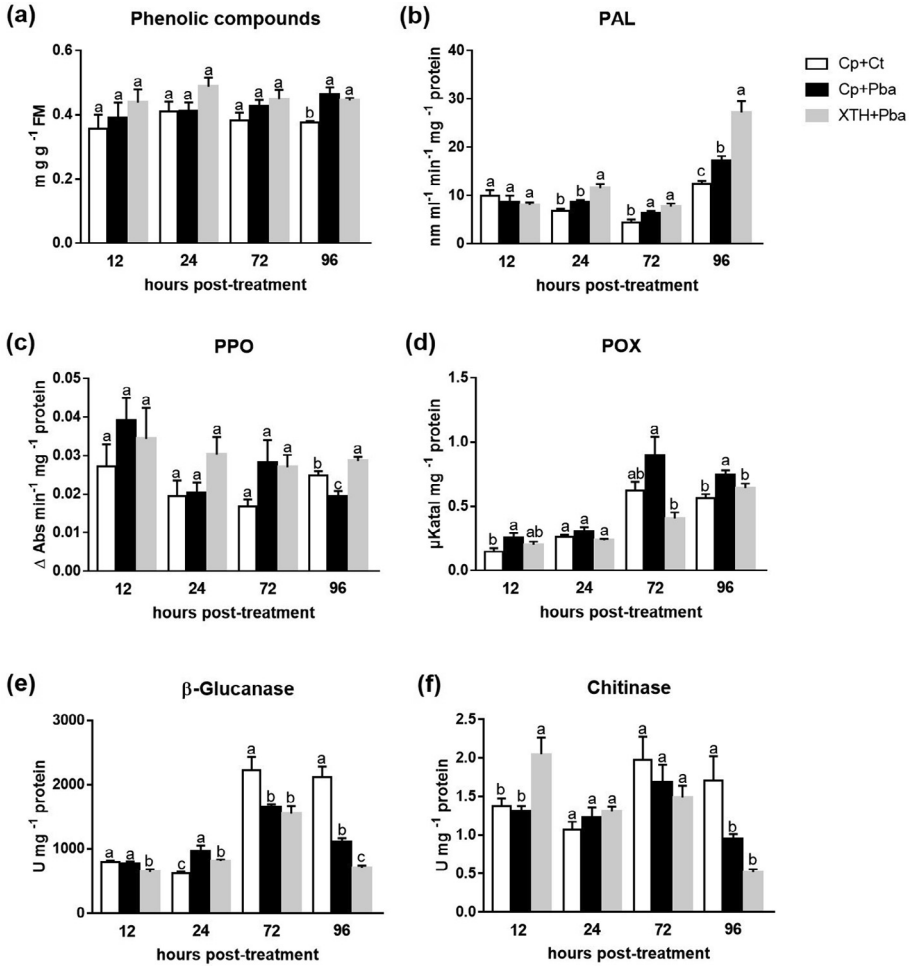

Fig. 5. Levels of (a) phenolic compounds and activity of (b) phenylalanine ammonia lyase (PAL), (c) polyphenol oxidase (PPO), (d) peroxidase (POX), (e) β-1,3-glucanase and (f) chitinase in potato detached leaves. Plants were pretreated with extract of *Xanthomonas axonopodis* (XTH) or sterile water (Cp). After four days, leaves were detached and treated with *P. atrosepticum* (Cp + Pba) or sterile water (Ct). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

that include the production of reactive oxygen species (ROS). Generation of ROS is thought to be an early event that can fundamentally influence the balance of the interaction between the plant and the pathogen (Oliveira et al., 2016). In order to evaluate this first line of plant defense, we analyzed the activity of antioxidant enzymes SOD, CAT and APx in potato detached leaves. It is noteworthy that some significant decrease on SOD activity was obtained at the earliest time point analyzed (0.5 hpt) when leaves were elicited with XTH and infected with Pba. An opposite response was observed regarding the activity of CAT, in which leaves from either XTH alone or XTH + Pba showed an increased activity at the same time point. The treatments XTH and XTH + Pba stimulated APx activity at 1 and 6 hpt, respectively. Considering leaves from the Cp + Pba treatment, CAT and APx activities in were mostly similar to the control leaves (Cp + Ct). The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of superoxide radicals and H_2O_2 (Mittler, 2002; Cavalcanti et al., 2006). SOD catalyzes the production of O_2 and H_2O_2 from superoxide (O_2^-), which results in less dangerous reactants and plays an important role in plant pathogenesis since H_2O_2 accumulation induces programmed cell death (PCD) and HR in plants (Mittler, 2002), whereas CAT and APx act to protect the cell from the detrimental effects of H_2O_2 accumulation by scavenging this molecule (Dat et al., 2000). The negative correlation observed between SOD and CAT activities in the XTH alone and XTH + Pba treatments indicated an increase in the enzymatic system of hydrogen peroxide degradation in the detached leaves. Moreover, a positive correlation was detected between CAT and APx in the same treatments, evidencing induction of both antioxidant enzymes by the elicitor XTH, result that may be related to the early and efficient response to the *Pectobacterium* attack. In *Arabidopsis thaliana,* CAT inhibited cell death and conferred evident disease resistance against the necrotrophic bacterium *Pectobacterium carotovorum* (Ahn, 2007). Indeed, the increase in CAT and APx activities was described in lines of *Brassica napus* that are resistant to the necrotrophic pathogen *Sclerotinia sclerotiorum* (Wen, 2012). On the other hand, leaves from the Cp + Pba treatment showed increased SOD activity at 3 hpt with corresponding increase of APx, but not of CAT activity, at this time. ROS accumulation leads to activation and acceleration of cell death (Ahn, 2007), result that was observed in our study when leaves were infected with Pba, without pretreatment with XTH. HR and ROS such as H_2O_2 have been reported to benefit infection by necrotrophic pathogens, which may be able to produce ROS themselves or stimulate the host to do so. Bacterial necrotrophs such as members of the genus *Pectobacterium* could induce cell death and ROS accumulation in order to infect tissues (Davidsson et al., 2013). Therefore, we hypothesize that the lack of increment in SOD activity coincident with the early increase of CAT in potato leaves from the XTH + Pba treatment results from a low oxidative burst in plants pretreated with the elicitor and challenged with the pathogen.

Plant resistance is usually correlated with activation of defense-related enzymes such as PAL, PPO, POX, chitinase and β-1,3-glucanase.

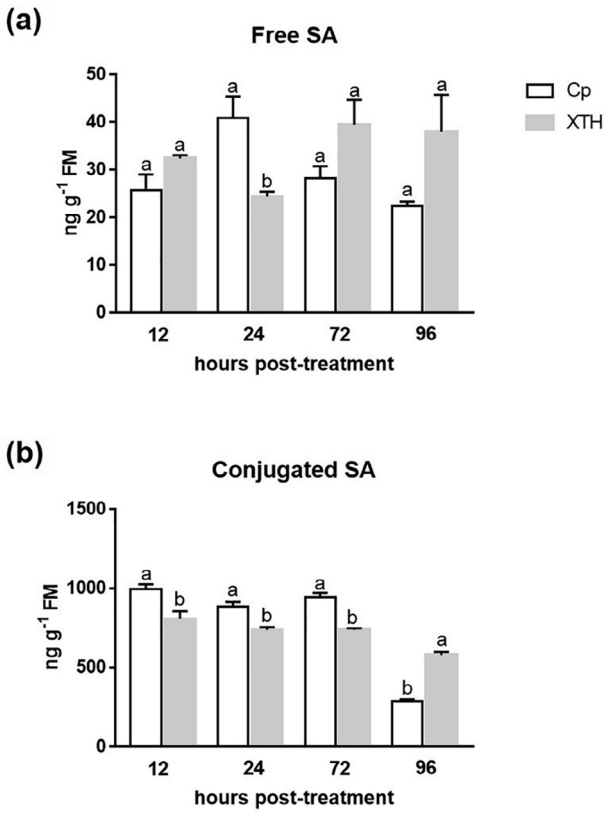

Fig. 6. Levels of (a) free and (b) conjugated salicylic acid (SA) in potato detached leaves. Plants were treated with extract of *Xanthomonas axonopodis* (XTH) or sterile water (Cp). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

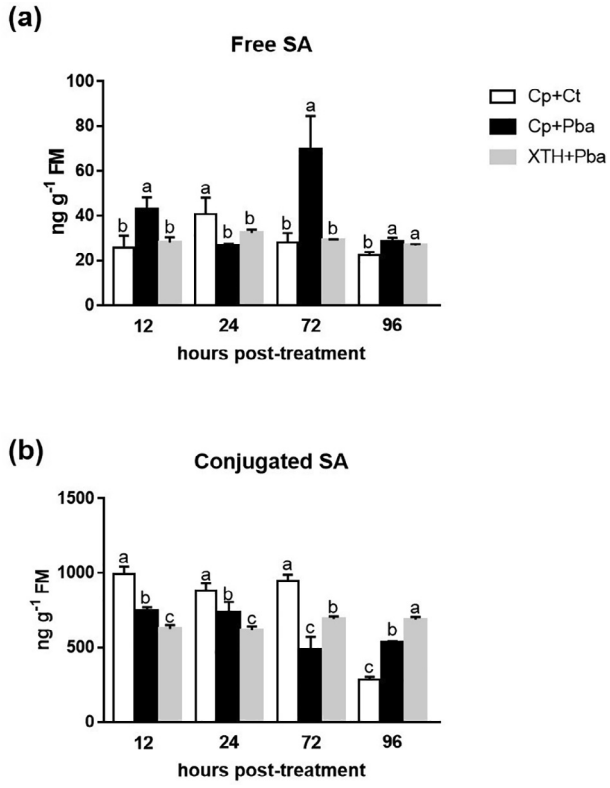

Fig. 7. Levels of (a) free and (b) conjugated salicylic acid (SA) in detached leaves. Plants were pretreated with extract of *Xanthomonas axonopodis* (XTH) or sterile water (Cp). After four days, leaves were detached and treated with *P. atrosepticum* (Pba) or sterile water (Ct). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

These enzymes represent the next phase of defense after the signaling and ROS-scavenging enzymes. Our results demonstrated that PAL activity increased early in the time course of defense promoted by elicitation with XTH. The resistance of plants to attempted microbial attack is usually associated with more-rapid and more-intense induction of defense responses (Newman et al., 2007). In Solanaceae, plant defenses are generally induced earlier and to a greater extent in incompatible than in compatible interactions (Desender et al., 2007). It has been demonstrated that an increase in PAL activity was associated with a decrease in the severity of disease caused by P. atrosepticum (Kröner et al., 2011; Saubeau et al., 2014). PAL is the key enzyme of the phenylpropanoid pathway catalyzing the transformation of phenylalanine to cinnamic acid, which is the core molecule for the synthesis of phenolics. Phenolics have been shown to be involved in defense reactions, either as physical and chemical barriers or by acting as signal molecules (Kröner et al., 2012). The present results showed an increase in the levels of total phenolic compounds in the Cp + Pba and XTH + Pba treatments after 96 hpt following the increase of PAL activity, although this result is possibly more related to the presence of Pba than XTH elicitation. Phenolic compounds are often considered to play an important role in resistance to many plant pathogens, including potato resistance against *Pectobacterium*. Andreu et al. (2001) also suggested a role for phenolics in resistance of the potato against P. infestans, since the amount of phenolics induced after infection was lower in a susceptible potato cultivar, compared to a resistant cultivar. Indeed, total phenolic content was negatively correlated with the severity of tuber rot caused by P. atrosepticum (Kröner et al., 2012).

Phenolic compounds are potential substrates of POX and PPO enzymes. The present results showed an increase in PPO activity in XTH and XTH + Pba at 24 hpt and 96 hpt, respectively, but not in the Cp + Pba treatment. PPOs catalyze the oxidation of phenols to quinones, molecules that are more toxic to pathogens (Li and Steffens, 2002) and it is important in the initial stage of plant defense, since membrane damage causes release of phenols. It has been posted that PPO plays a role in resistance to soft rot caused by *P. carotovorum* in potato tubers (Wegener and Jansen, 2007). POX is also related to defense against pathogen attack through physiological processes such as lignification, suberization and cross-linking of cell-wall proteins. POX catalyzes the polymerization of phenolics to produce a variety of bioactive products, contributing to chemical as well as physical defenses (Jang et al., 2004). Differently of PPO, the results from detached leaves of potato showed increase on POX activity only when leaves were infected with Pba and not elicited with XTH, which suggests that this enzyme is involved in defense mechanism, as reported by Jang et al. (2004) on sweet potato inoculated with Pectobacterium chrysanthemi. Similar to our results, Wegener and Jansen (2007) reported that POX activity was not correlated with resistance to *P. carotovorum* in potato. Indeed, Poiatti et al. (2009) observed an increased POX activity during *Erwinia carotovora* infection in potato plants, and no differences in POX activity in plants inoculated with *X. axonopodis* pv. *citri*.

Many biochemical changes occur during systemic acquired resistance. PR-proteins including acidic β-1,3-glucanase and chitinase are secreted in the intercellular spaces, where they act against fungal and or bacterial pathogens at an early stage of the infection process (Hajhamed et al., 2007). The present results showed an early increase in chitinase activity in potato leaves treated with XTH alone and pretreated with XTH and challenged with *P. atrosepticum*, whereas Cp + Pba treatment induced only β-1,3-glucanase. The β-1,3-glucanases and chitinases act directly by degrading microbial cell-wall components. Some plant chitinases also have lysozyme activity and can therefore hydrolyze bacterial cell walls (Baysal et al., 2003). Interestingly, many studies have suggested that β-1,3-glucanases are probably not directly involved

in resistance against bacteria, while chitinases participate in early basal resistance induced by PAMPs against virulent bacteria (Baysal et al., 2003).

Levels of resistance in whole plants are also influenced by systemic signals mediated by plant hormones. Classically, salicylic acid (SA) signaling triggers resistance against biotrophic and hemibiotrophic pathogens, whereas a combination of jasmonic acid (JA) and ethylene (ET) signaling activates resistance against necrotrophic pathogens. These two pathways are mostly antagonistic: elevated biotroph resistance is often correlated with increased necrotroph susceptibility, and elevated necrotroph resistance is often correlated with enhanced susceptibility to biotrophs (Robert-Seilaniantz et al., 2011). The present results showed an increase in free SA (active form) in leaves from the Cp + Pba treatment. Free SA can promote and potentiate programmed cell death, benefiting *P. carotovorum* infection (Davidsson et al., 2013). Interestingly, leaves treated and pretreated with the elicitor XTH and inoculated with *P. atrosepticum* did not show any increase in free SA, suggesting that XTH migh impair the initial SA burst promoted by Pba. On the contrary, it has been demonstrated that potato plants have a high constitutive level of SA that does not lead to constitutive resistance in healthy plants, indicating that potato plants may have a poor SA signal-perception and/or transduction mechanism (Yu et al., 1997). Norman-Setterblad et al. (2000) suggested that lower levels of SA might have a crucial role in modulating other types of defense in *Arabidopsis*.

In conclusion, the elicitor XTH was able to delay the symptoms and progression of disease caused by *P. atrosepticum*. This resistance promoted by XTH could be explained by a previous cellular-response modulation, where *P. atrosepticum* was unable to induce an oxidative burst and plant cell death. On the other hand, this initial XTH-modulation leads to activation of defense-related enzymes such as PAL, PPO and chitinase and impaired the free SA increase after the leaves were infected with *P. atrosepticum*. Although the majority of mechanisms related to plant resistance mediated by elicitors remains unknown, these molecules have been considered a new tool for aiding plant disease control, leading to the development of new commercial products.

CRediT authorship contribution statement

Giulia Ramos Faillace: Investigation, Data curation, Formal analysis, Writing - original draft. **Eliane Romanato Santarém:** Methodology, Writing - review & editing, Supervision, Visualization. **Leandro Vieira Astarita:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgments

Funding was provided by National Council for Scientific and Technological Development (CNPq/Brazil; 403843/2013-8) and FAPERGS through the scholarship of first author. Authors thank to the Institute of Toxicology and Pharmacology (INTOX, PUCRS), and to Dr. Valmir Duarte (Laboratory of Phytopathology, Universidade Federal do Rio Grande do Sul, Brazil) for providing cultures of phytobacteria.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2019.03.012.

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