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Benzothiadiazole and BABA improve resistance to *Uromyces pisi* (Pers.) Wint. in *Pisum sativum* L. with an enhancement of enzymatic activities and total phenolic content

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Abstract Benzothiadiazole (BTH) and DL-β-aminobutyric acid (BABA) induced systemic resistance was investigated in susceptible and resistant pea genotypes against *Uromyces pisi*. Resistance was characterized by reduced infection frequency mainly due to decreases in appressorium formation, stomatal penetration, growth of infection hyphae and haustorium formation. Changes in β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase and peroxidase activities and in total phenolics content, demonstrate that *U. pisi* resistance is induced by BTH and BABA treatments at early and late stages of the fungal infection process, but that the chemicals operate via different mechanisms. In fact, our study showed that BTH treatment primed the activity of pathogenesis related-proteins such as β-1,3-glucanase, chitinase and peroxidase in both susceptible and resistant genotypes. On the other hand, BABA treatment did not increase the enzymatic activities in the studied genotypes, but significantly increased their total phenolic contents.

Keywords Chitinase · Glucanase · Pea rust · Peroxidase · Phenolics · PR-protein

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Abbreviations

BABA	DL-3-amino-n-butanoic (DL-β-aminobutyric) acid
BTH	Benzo [1,2,3]thiadiazole-7-carbothionic acid-*S*-methyl ester (Bion®)
PR	Pathogenesis-related (proteins)
TLC	Thin layer chromatography

Introduction

Plants can be sensitized for a more rapid or more intense mobilization of defence responses leading to enhanced resistance to biotic or abiotic stresses (Beckers and Conrath 2007). Systemic acquired resistance (SAR) is a phenomenon which can be induced by limited pathogen infection, avirulent pathogens, certain non-pathogenic bacteria, and certain chemicals, and which primes the plants to be more resistant to a subsequent pathogen infection (van Loon 2001; Lin et al. 2008). SAR has been described to be effective against a broad spectrum of pathogens including viruses, fungi, bacteria and nematodes (Beckers and Conrath 2007). During SAR reactions, different defence mechanisms are known to be induced in plants. These mechanisms include changes in structural defences, e.g., strengthening of the cell wall through deposition of lignin and callose on the plant cell wall where the pathogen attempts to penetrate (Prats et al. 2002), enhancement of enzymatic activity related to

defence processes such as chitinases and glucanases, and immediate production of reactive oxygen species (Radman et al. 2003).

Pea rust has become an important pathogen of pea (*Pisum sativum* L.), with yield losses up to 30% particularly in warm and humid weather (Barilli et al. 2009a). The disease has been reported to be caused either by *Uromyces viciae-fabae* (Pers.) J. Schröt (syn. *U. fabae* (Pers.) de Bary) or *U. pisi* (Pers.) Wint. Despite the fact that most previous reports refer to *U. viciae-fabae*, commonly referred as faba bean rust, as the major causal agent of pea rust (Kushwaha et al. 2006) the increasing importance of *U. pisi* is becoming evident (Emeran et al. 2005; Barilli et al. 2009b). The high potential of pea under Europe, India, Australia and Mediterranean conditions, emphasizes the need for resistance to solve rust problems (Emeran et al. 2005). Breeding for resistance is the most economical and environmentally friendly method of rust control, but low resistance is available in pea so far (Barilli et al. 2009a). Partial resistance, not associated with host cell death, has been found. This is macroscopically characterized by an increased latent period and a decreased infection frequency and microscopically by a reduction of haustorium formation and significant levels of early aborted colonies (Barilli et al. 2009c).

In order to validate alternative pea rust control methods, a preliminary study on induction of resistance in this plant-pathogen interaction was initiated using both biotic (*U. pisi* and *U. appendiculatus*) and abiotic (salicylic acid (SA), benzo[1,2,3]thiadiazole-7-carbothionic acid (BTH) and DL-β-aminobutyric acid (BABA)) inducers (Barilli et al. 2009d). Significant reductions of infection levels locally and systemically with BTH and BABA foliar treatments were observed, whereas neither biotic inducers nor SA significantly inhibited rust development.

Over the past few years, there have been several reports on BTH and BABA-induced inhibition of rusts (Prats et al. 2002; Iriti and Faoro 2003; Amzalek and Cohen 2007; Barilli et al. 2009d), but knowledge of their underlying mechanisms of action were not clarified, especially for BABA induction. In addition, the effect of both BTH and BABA on the same host/pathogen interaction has not been studied.

BTH is a chemical inducer against a wide range of pathogens even though its effect may vary with the concentrations used and the pathosystems considered (van Loon 2001). Expression of BTH-induced resistance has been associated with transcriptional activation of genes encoding pathogenesis-related (PR)-proteins promoted by endogenous accumulation of salicylic acid (SA) (Van Loon 1997; Jiang et al. 2008). In the *Puccinia helianthi*-sunflower and *P. triticina*-wheat interactions, BTH-induced protection has also been associated with excretion of phytoalexins to the leaf surface, which inhibited urediospore germination and appressorium formation (Görlach et al. 1996; Prats et al. 2002).

The cellular and molecular mechanisms through which BABA exerts its action are not so well studied as those of BTH. Also, its capacity to confer protection against basidiomycetes in general, and rusts in particular, is contradictory (Amzalek and Cohen 2007). Unlike BTH, BABA does not appear to have any inhibitory effect on *Puccinia helianthi* in sunflower on the events prior to stomatal penetration (Amzalek and Cohen 2007), suggesting that the protection induced by these two chemicals operates via different pathways.

This work aims to clarify the underlying mechanisms during BTH and BABA-induced defence response in the pea-*U. pisi* interaction. Two different genotypes of pea including the susceptible genotype Messire and genotype PI347321 with partial resistance against *U. pisi* (Barilli et al., 2009c) were used to carry out histological studies. Biochemical studies testing activities of specific enzymes (PAL, peroxidase, scopoletin peroxidase, β-1,3-glucanase and chitinase) as well as total phenolic content were also carried out, since their presence was previously related to rust resistance (Anguelova-Merhar et al. 2002; Prats et al. 2002).

Materials and methods

Plant growth and treatments

Experiments were conducted with two pea genotypes: commercial genotype Messire and genotype PI347321, which were previously found to be susceptible and partially resistant, respectively, to *U. pisi* infection (Barilli et al. 2009c). Plants were raised in pots (6×6×10 cm) filled with a potting mixture (sand/perlite, 1:1 *v*/*v*) in a growth chamber at 20±2°C and 65% RH under cycles of 14 h light/10 h darkness,

with a light intensity of 150 $\mu Em^{-2}s^{-1}$ supplied by high-output white fluorescent tubes (Lumilux Cool White FQ 80 W//840 HO; OSRAM, Germany).

When plants had the two first pairs of leaves completely expanded and the third pair was expanding, they were treated with the inducers benzo[1,2,3]thiadiazole-7-carbothionic acid-*S*-methyl ester (BTH, also named acibenzolar-*S*-methyl (ASM)) or the non-protein amino acid DL-3-amino-n-butanoic acid (DL-β-aminobutyric acid [BABA]). BTH was purchased in the form of Bion 50 WG (50% active ingredient) from Syngenta AG, (Basel, Switzerland), and BABA was purchased from Sigma-Aldrich, (Missouri, USA) (product code: A1754). Concentrations used were 10 mM for BTH and 50 mM for BABA, concentrations previously shown to be effective in this pathosystem (Barilli et al. 2009d). Solutions were prepared in sterile water to which Tween 20 (0.03%, *v*/*v*) was added. Then, 3 droplets (15 μl/drop) of each solution were applied on each leaflet at first node from the base. Control plants were treated with sterile water plus Tween 20.

Inoculation

U. pisi monosporic isolate UpCo-01 from the fungal collection belonging to the Institute for Sustainable Agriculture-CSIC (Córdoba, Spain) was used for the experiments. Inoculum was multiplied on plants of the susceptible genotype "Messire" and was collected and stored at −80°C until use. Seedlings of both genotypes Messire and PI347321 were inoculated 5 days after treatments by dusting the plants with rust urediospores (2 mg spores plant $^{-1}$) mixed with pure talc (1: 10) using a spore settling tower. Plants were incubated for 24 h at 20±2°C in complete darkness and 100% RH, then returned to growth chamber conditions, to allow disease development. Infection frequency (IF), recorded as the number of pustules·cm^{-2}, was determined 10 days after inoculation on the third pair of leaves. Recordings were made 10 days after inoculation (dai) on 1 cm^2 marked area using a pocket lens (magnification×7).

Microscopic assessment

Two days after inoculation (dai), two leaves per plant and genotype were used to assess the different pathogen developmental stages during the infection process before stomatal penetration. Leaves were placed on paper moistened with acetic acid/ethanol (1:3, v:v) until chlorophyll was removed. After that, leaves were placed on paper moistened with distilled water for 2 h and finally, placed on paper saturated with lacto-glycerol (5:2, *v*/*v*) until used. In order to monitor the developmental stages after stomatal penetration, two additional leaves were harvested at 2 dai and stained with Trypan blue (Sillero and Rubiales 2002). Thus, leaves were fixed in acetic acid/ethanol (1:3, *v*:*v*) for 30 min, stained by boiling in 0.05% Trypan blue in lactophenol/ethanol (1:2, *v*:*v*) for 10 min, and cleared in a nearly saturated aqueous solution of choral hydrate (5:2, *w*/*v*) to remove Trypan blue from the chloroplast membranes. The different stages of the infection process were assessed using a phase contrast Leica DM LS microscope at×400 magnification (Leica Microsystems, Wetzlar, Germany). Necrosis was identified by uptake of Trypan blue by the plant cells.

Two additional leaves per plant and genotype were harvested at 6 dai in order to measure the colony size (CS) (Sillero and Rubiales 2002). Size was measured on 25 randomly chosen established colonies per leaf using a Leica DM LS microscope at X100 magnification. The length (L) and width (W) were established with an eyepiece micrometer. CS was calculated using the formula CS=1/4 π LW. The presence of host cell necrosis associated with the colonies was also assessed.

In vitro *urediospores germination assay*

For assessing the direct effect of inducers on germination ability, *U. pisi* urediospores were placed on glass slides where BTH and BABA solutions (10 mM and 50 mM, respectively) had been sprayed. The slides were placed in a 100% RH chamber at 20± 2°C in dark. Percentage of germinated spores was measured microscopically after 24 h.

Enzyme assays

At 2 dai, two leaflets per third node per plant were harvested, weighed and protein extracted for enzymatic activity determination. Protein content was determined using the Bradford reagent (Sigma) with BSA as the protein standard as recommended by the manufacturer.

For β-1,3-glucanase and chitinase assays, samples (0.1 –0.2 g FW) were ground with a plastic pestle in a

2 ml microcentrifuge tube with 1% w/w PVPP (Polyvinilpolipirrolidone) and 1.0 mL 50 mM potassium acetate buffer, pH 5.0, containing 1 mM EDTA and 5 mM reduced glutathione that was added immediately prior to homogenization. Extracts were centrifuged at 9000 g for 5 min and supernatants transferred to 1.5 ml microcentrifuge tubes.

β-1,3-glucanase was assessed according to Dann and Deverall (2000). The assay is based on the release of a soluble and measurable dye when the substrate, azurine-crosslinked pachyman (AZLC-Pachyman, Megazyme, Wicklow, Ireland), is hydrolyzed by endo-β-1,3-glucanase. For substrate preparation, 15 mg of product was suspended in 1.0 ml double-deionised water and stirred to obtain a homogeneous suspension. Potassium acetate buffer (0.4 ml, 10 mM, pH 5.0) and 0.1 ml of a diluted crude leaf extract were added to a 1.5 ml microcentrifuge tube and allowed to equilibrate at 30°C for 3 min. The reaction was initiated by the addition of 0.1 ml of the substrate suspension and stopped after 10 min by addition of 0.7 ml 20% *w*/*v* Tris buffer. The tubes were vortexed, maintained at room temperature for 5 min, vortexed again and centrifuged at 9000 g for 2 min. Aliquots of supernatant (0.35 m) were transferred to wells of a micro-titre plate and colour development was measured with a spectrophotometer (Synergy HT, Bioteck, Winooski, VT, USA) at 610 nm. Results were calculated as a change in optical density at 610 nm.

The assay to determine the chitinase activity used carboxy-methyl chitin linked with the dye Remazol Brillant Violet 5R (Wirth and Wolf 1990). Potassium acetate buffer (0.2 ml, 0.1 M, pH 5.0) and 0.1 ml of a suitably diluted crude leaf extract were added to a 1.5 microcentrifuge tube and allowed to equilibrate to 37°C. The reaction was initiated by adding 0.1 ml aqueous CM-Chitin-RBV (2 $mgml^{-1}$ solution, Loewe Biochemica GmbH, Sauerlach, Germany). After 30 min, the reaction was terminated by adding 0.1 ml 2 N HCl, which precipitated the un-degraded substrate. Tubes were placed on ice for 10 min then centrifuged for 5 min at 9000 g. Aliquots of supernatant (0.35 ml) were transferred to wells of a micro-titre plate and colour development was measured with a spectrophotometer (Synergy HT, Bioteck, Winooski, VT, USA) at 550 nm. Results were calculated as a change in optical density at 550 nm.

Phenylalanine ammonia-lyase (PAL) activity was measured according to Prats et al. (2003). 0.3 g frozen pea leaves were homogenised in liquid nitrogen and the powder extracted with 50 mM Tris-HCl buffer (pH 8.5) containing 1 mM PMSF, 14 mM 2-Mercaptoethanol and 5% insoluble PVPP. The extract was centrifuged at 10000 g for 15 min at 4°C and the supernatant desalted with NAP 5 columns (GE Healthcare, Buckinghamshire, UK), following the instructions from the manufacturer. Fifty μl from the enzymatic extract were transferred to micro-titre plate wells and mixed with 150 μl 12.1 mM of L-Phenylalanine (D-Phenylalanine for the control). Samples were incubated during 90 min at 37°C. During this time, the absorbance was measured every 10 min with a spectrophotometer at 290 nm. Enzyme activity was calculated as the increase of absorbance units per minute per gram fresh leaf weight.

Activity of total soluble peroxidase and scopoletin peroxidase were determined by measuring the appearance of pink/brown colour resulting from scopoletin oxidation in the presence of hydrogen peroxide at 470 nm (Prats et al. 2006). Briefly, leaves were homogenated in Tris-HCl buffer (0.1 M, pH 7.4) 1:1 (*v*/*w*) with 30% of PVPP. The samples were centrifuged at 10000 g and 4°C during 15 min. Aliquots of 25 μl of plant extract were transferred to wells of a micro-titre plate and mixed with 5 μl of 100 mM H_2O_2 and 220 μl of 0.1 M potassium-phosphate buffer pH 6.5. Finally, 10 mM guaiacol or scopoletin was added to 5 μl of substrate. Changes in absorbance were measured with a spectrophotometer during 180 s at 470 and 595 nm for total peroxidase and scopoletin peroxidase, respectively.

Phenolic content

For phenolic determination, tissue samples were extracted as described by Prats et al. (2002). Frozen tissues (leaves collected at the third node) were homogenized with pre-chilled (-20°C) 1:10 (w/v) acetone in a mortar. After filtering off the solvent, the residues were further sequentially extracted with a similar volume of acetone:methanol (1:1, *v*/*v*). The combined solvent extract was concentrated on a rotavapor, re-dissolved in 1 ml of methanol and cleared by centrifugation. Total phenolic compounds were determined by using the Folin-Ciocalteu reagent, as reported by Prats et al. (2002). Thin layer chromatography (TLC) analysis of the methanolic extract was performed according to Gutiérrez-Mellado et al. (1996), by using Silicagel 60 F_{254} plates (Merck)

and ethyl acetate as the mobile phase. Plates were visualized under UV light (254 nm) lamp in a Vilber Lourmat transiluminator (VL-TCP-20 LC). The coumarins scopoletin and ayapin, and the phytoalexins medicarpin and pisatin were identified as fluorescent spots at λ=254 nm by comparison of the retention factor (Rf) values with pure samples (reference standard).

Statistical analyses

Each experiment had four replications, each consisting of four plants per treatment and the experiments were repeated three times. For ease of understanding, raw data are presented in the tables (means of a single representative experiment). However, for statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value=180/π × arcsine [√(%/100)]) to normalize data and stabilize variances throughout the data range, and subjected to analysis of variance using GenStat 7th Edition, after which residual plots were inspected to confirm data conformed to normality. Values for colony area were transformed to square roots to obtain a linear rather than a quadratic value for colony size.

Results

Macroscopic observations

Treatment with BTH and BABA gave a significant reduction of infection frequency (IF) ($p<0.001$) in both the resistant and the susceptible genotype, with no differences between compounds (Table 1), albeit the protection level reached was higher in the resistant (8 –10 fold reduction in IF) than in the susceptible genotype (3 fold reduction). No symptoms of phytotoxicity were observed on treated pea plants for either product at the concentrations tested.

Fungal development

Urediospore germination in a given genotype was unaffected by treatments with any of the inducers studied (Table 2). However, *U. pisi* urediospore germination was significantly lower on the resistant genotype PI347321 compared with genotype Messire ($p<0.001$)

Table 1 Infection frequency (IF), measured as number of of pustules cm^{-2} 10 days after inoculations (dai) on pea genotypes inoculated with *U. pisi*

Genotype	Treatment	IF[a]
Messire	Control	169.0 (0)
	BTH	58.8 (65.2)***
	BABA	54.8 (67.6)***
PI347321	Control	84.2 (0)
	BTH	14.8 (82.4)***
	BABA	8.8 (89.5)***

***, **, * indicates significance of the treatments respect to the control within each genotype at $p<0.001$, $p<0.01$ and 0.05, respectively

Numbers in brackets represents the reduction of IF in percent compared to the control

When germination succeeded, the germ tube grew towards a stoma, over which it formed an appressorium. The percentage of germ tubes that formed appressoria was significantly lower in the resistant genotype PI347321 than in genotype Messire ($p<0.001$). Interestingly, treatment with BTH and BABA significantly decreased this percentage in both genotypes (Table 2). After appressorium formation, a penetration hypha penetrated through the stoma and formed a substomatal vesicle from which a haustorial mother cell (HMC) elongated and attempted to penetrate into the mesophyll cells. In non-treated control plants of both genotypes, most appressoria penetrated the stomata. However, after BTH and BABA treatment, the percentage of appressoria that formed a substomatal vesicle significantly decreased. The same trend was observed when the percentage of substomatal vesicles that developed HMC was assessed, except for BABA treated PI347321 plants (Table 2).

Following contact of a HMC with the mesophyll, a peg penetrated the cell and developed a haustorium. Both inducers decreased the percentages of germlings producing haustoria and reduced the number of haustoria per colony in the susceptible genotype (Table 3). In the resistant genotype PI347321, BTH treatment, but not BABA treatment, gave a lower number of haustoria per colony (Table 3). Non-treated control plants of PI347321 had a lower number of germlings associated with haustoria and a lower number of haustoria per colony than the susceptible control Messire ($p<0.001$ for both parameters).

Table 2 Histological components of pre-penetration inhibition of *Uromyces pisi* in pea genotypes, under controlled conditions at 2 dai

Genotype	Treatment	Percent spores germinated	Percent germ tubes forming an appressorium over the stoma	Percent appressoria over a stoma forming SSV[a]	Percent SSV forming HMC[b]
Messire	Control	84.5	77.5	97.2	95.1
	BTH	84.5 ns	70.8*	78.3***	51.5***
	BABA	85.5 ns	67.3***	90.0**	85.5*
PI347321	Control	70.5	66.0	95.0	74.9
	BTH	66.7 ns	51.7**	84.0**	40.3***
	BABA	67.2 ns	53.2**	84.2**	79.0 ns

***, **, * indicates significance of the treatments respect to the control within each genotype at $p<0.001$, $p<0.01$ and 0.05, respectively; ns: not significant

[a] SSV: substomatal vesicle

[b] HMC: haustorial mother cell

Furthermore, in PI347321, there was an inhibition in the number of hyphal tips per colony and a reduced colony size compared to genotype Messire ($p<0.05$). Both parameters were reduced after BTH and BABA treatment in both genotypes (Table 3).

In vitro urediospores germination

The percentage of urediospores germinating on glass slides sprayed with water, BTH or BABA solutions ranged between 96 and 98.5%. The differences observed between treatments were not significant (data not shown).

Enzyme activities

The β-1,3-glucanase activity significantly increased in Messire control plants following *U. pisi* inoculation ($p<0.01$), whereas there was no significant difference in genotype PI347321 (Fig. 1). BTH-treated plants of either genotype, showed a significant increase in β-1,3-glucanase activity both in healthy and *U. pisi*-inoculated plant compared to their respective controls ($p=0.002$ and $p<0.001$, respectively). Furthermore, there was a significant increase in β-1,3-glucanase activity after fungal inoculation ($p=0.034$ and $p=0.038$) in Messire and PI347321 BTH treated plants, respectively). BABA-treated plants irrespective of genotype or inoculation did not show increased β-1,3-glucanase activity compared to the non-treated controls. However, β-1,3-glucanase activity was significantly increased by fungal infection in both genotypes ($p=0.04$ and $p=0.031$ for Messire and PI347321, respectively) (Fig. 1).

Chitinase activity in non-treated control plants increased significantly after inoculation in Messire plants ($p<0.01$), whereas there was no significant

Table 3 Histological components of post-penetration inhibition of *Uromyces pisi* in pea genotypes, under controlled conditions at 2 dai (6 dai for colony size)

Genotype	Treatment	Percent germlings associated with haustoria	Haustoria/ colony	Hyphal tips/ colony	Colony size (mm^2) 6 dai
Messire	Control	90.2	2.4	4.3	0.20
	BTH	18.7***	1.2***	2.4***	0.07***
	BABA	63.7***	1.5***	3.9*	0.08***
PI347321	Control	64.0	1.5	3.7	0.14
	BTH	19.5***	1.3**	2.9**	0.07***
	BABA	59.4***	1.6 ns	3.0*	0.09***

***, **, * indicates significance of the treatments respect to the control within each genotype at $p<0.001$, $p<0.01$ and 0.05, respectively; ns: not significant

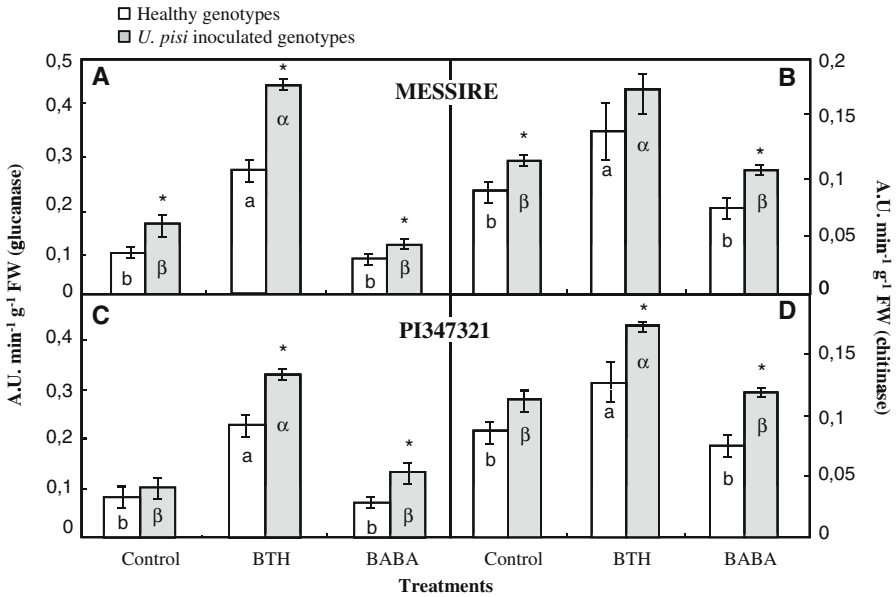

Fig. 1 **Total** β-1,3-glucanase (**a** and **c**) and chitinase (**b** and **d**) activity. Values are expressed as Absorbance Units $min^{-1} g^{-1}$ fresh weight and are means of four replications. Different roman letters within an open bar indicate significant differences ($p<0.05$) within the healthy plants. Different Greek letters within a solid bar indicate significant differences ($p<0.05$) within the inoculated plants. Asterisks indicate significant differences ($p<0.05$) between healthy and inoculated plants for each treatment. Error bars indicate standard errors

increase in genotype PI347321 (Fig. 1). In both genotype Messire and PI347321, treatments with BTH gave rise to a significant increase in chitinase activity both in healthy and *U. pisi*-inoculated plant compared to their respective non-treated controls ($p=0.014$ and $p<0.001$ for Messire and $p=0.004$ and $p=0.002$ for PI347321, respectively). However, fungal inoculation only increased chitinase activity in genotype PI347321. As for β-1,3-glucanase, following BABA treatment, plants did not show significantly increased chitinase activity compared to their respective non-treated controls in either genotype. However, chitinase activity was induced by the presence of the fungus in both genotypes ($p=0.038$ and $p=0.029$ for Messire and PI347321, respectively) (Fig. 1).

There were no significant changes in PAL activity in uninoculated and inoculated pea leaves in either genotype or between treatments (data not shown).

In genotype Messire, total peroxidase activity was not significantly increased after either treatment or *U. pisi* inoculation (Fig. 2a). However, both non-inoculated and inoculated PI347321 plants treated with BTH had increased peroxidase activity compared with their respective non-treated controls ($p<0.05$ and $p<0.01$, respectively). In addition, in genotype PI347321 treated with BTH, there was a significant increase of peroxidase activity in inoculated compared with healthy plants ($p=0.031$; Fig. 2c). In general total peroxidase activity did not increase in healthy or infected plants following BABA treatment (Fig. 2a and c). Scopoletin peroxidase increased following BTH treatment in both non-inoculated and *U. pisi*-inoculated Messire and PI347321 plants compared to their respective non-treated controls ($p<0.01$ and $p=0.003$ for Messire, $p<0.001$ and $p<0.001$ for PI347321, respectively) (Fig. 2b and d). In addition, fungal challenge increased the scopoletin peroxidase activity of both Messire and PI347321 BTH-treated plants ($p=0.026$ and $p=0.032$ for Messire and PI347321, respectively). In parallel with total peroxidase activity, scopoletin peroxidase activity did not increase in healthy or infected plants following BABA treatment.

Total phenolics content

Non-treated control plants of PI347321 showed a higher constitutive level of total phenolics than the susceptible control Messire ($p<0.01$) (Fig. 3). In healthy Messire plants, an increase in the content of phenolic compounds was found following BTH

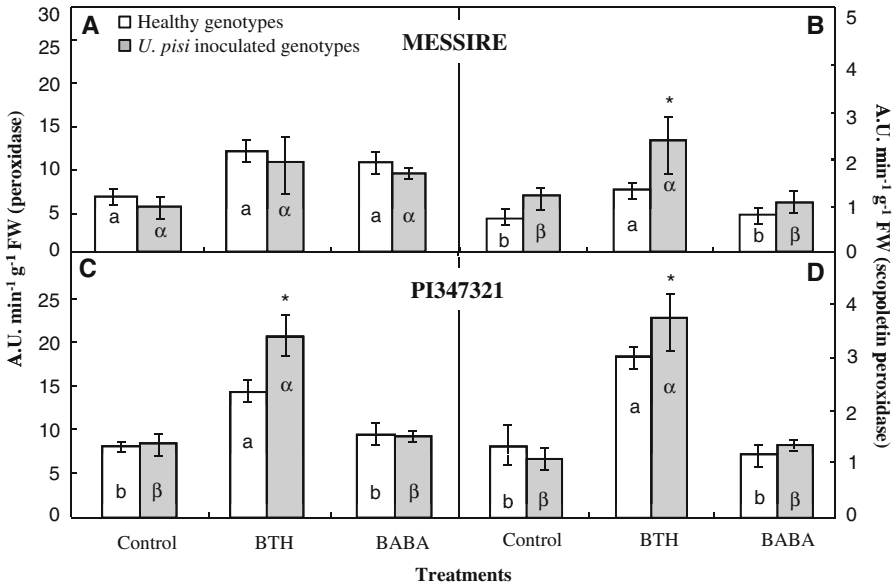

Fig. 2 Total peroxidase (**a** and **c**) and scopoletin peroxidase (**b** and **d**) activity. Values are expressed as Absorbance Units $min^{-1}g^{-1}$ fresh weight and are means of four replications. Different roman letters within an open bar indicate significant differences ($p<0.05$) within the healthy plants. Different Greek letters within a solid bar indicate significant differences ($p<0.05$) within the inoculated plants. Asterisks indicate significant differences ($p<0.05$) between healthy and inoculated plants for each treatment. Error bars indicate standard errors

treatment ($p<0.01$) whereas in PI347321 an increase was observed following BABA treatment. Furthermore, in PI347321, both BTH and BABA treatment had increased total phenolic content after fungal inoculation ($p<0.01$ and $p<0.05$, respectively).

Discussion

The protective effect of BTH and/or BABA have been previously reported in a number of plant-pathogen interactions (Dann and Deverall 2000; Prats et al. 2002; Amzalek and Cohen 2007; Roldán-Serrano et al. 2007). However, not much is known about the effect of these inducers in pea against *U. pisi* (Barilli et al. 2009d). We showed for the first time that both BTH and BABA induced resistance against *U. pisi* in a susceptible and partially resistant pea genotype by enhanced specific enzymatic activity.

The disease reduction observed after treatment with the inducers was not complete. However, treatment with 10 mM BTH and 50 mM BABA effectively reduced the infection frequency, with this reduction being higher in the partially resistant than in the susceptible genotype. The reduction in IF cannot be attributed to the toxic effect of the chemicals on the fungus, as neither of them showed a fungistatic activity against *U. pisi* uredospores (data not shown), Furthermore, the protective effect observed was related to triggering of defence responses, as reported for other plant-pathogen interactions (Prats et al. 2002; Iriti and Faoro 2003; Amzalek and Cohen 2007).

The detailed microscope study showed that resistance induced by the chemicals acted both prior to stomatal and mesophyll cell penetration. Following BTH treatment, a lack of success in appressorium formation and penetration through stomata together with an increase in the number of substomatal vesicles which did not form haustorial mother cells was observed. The effect of BABA was restricted to a reduction of appressorium formation and a reduced ability to form a subsequent substomatal vesicle (SSV), particularly in the resistant genotype. These results are in agreement with Prats et al. (2002) who found that BTH interfered with pre-penetration events in the sunflower-rust interaction. Previously, there has apparently not been any report of BABA impairing the early stages of fungal development. Inhibition of the early stages of fungal development has been associated with the excretion of specific antifungal plant metabolites, including phenolics, to the leaf surface that interfere with fungal development (Prats et al. 2006). Phenolics have been shown to play an

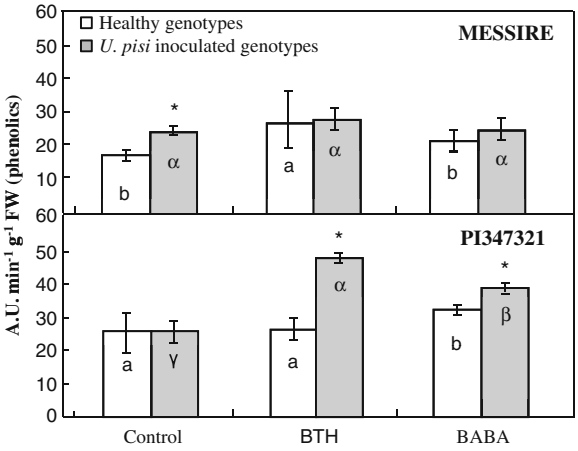

Fig. 3 Total phenolic contents. Values are expressed as Absorbance Units $min^{-1} g^{-1}$ fresh weight and are means of four replications. Different roman letters within an open bar indicate significant differences ($p<0.05$) within the healthy plants. Different Greek letters within a solid bar indicate significant differences ($p<0.05$) within the inoculated plants. Asterisks indicate significant differences ($p<0.05$) between healthy and inoculated plants for each treatment. Error bars indicate standard errors

important role in disease resistance both at the early and late stage of the infection process (Prats et al 2003, 2007). They may exert a direct toxic effect on the pathogen and contribute the cell wall strengthening through cross-linking and lignin formation (Hückelhoven 2007). Phenylalanine ammonia-lyase (PAL) catalyses the first reaction of the phenylpropanoid pathway from which the different families of phenolics (e.g. coumarins, flavonoids and lignins) derive. The induction of PAL activity preceding an increase in phenolics content has been observed in several BTH- and BABA-mediated induced resistances, as in cucumber-powdery mildew and grapevine-downy mildew interactions (Lin et al. 2008; Slaughter et al. 2008). However, our study showed no induction of PAL activity in either genotype following treatment (data not shown). This might be explained by the tissue assessed and the sampling time. We sampled inoculated and healthy leaves 2 dai from the third node. PAL activity has been described to be increased locally, in the same leaves treated by inducers, but not systemically (Dann and Deverall 2000); and in addition, 2 dai could be too late to see an induction of phenolic excretion to the leaf surface prior to stomatal penetration. Work is in progress to isolate different metabolites excreted to the leaf surface that could exert an inhibitory effect on the early fungal development stages.

Haustorium development following fungal penetration into plant mesophyll cells is one of the most critical steps in pathogenesis (Iriti and Faoro 2003). In the present work, both inducers considerably increased the penetration resistance of the mesophyll cells. Penetration resistance is a key component of basal defence against pathogens and is associated with cell wall strengthening and specific development of papillae (cell wall appositions) under the site of attempted penetration (Hückelhoven 2007). Formation of cell wall appositions is achieved following signal transduction pathways, rapid reorganization of actin microfilaments, actin dependent transport of secretory products to the infection site and local activation of callose synthesis (Prats et al. 2006; Hardham et al. 2007).

In the present work, the higher mesophyll penetration resistance promoted in both genotypes by BTH suggests that the observed resistance might be due in part to the physical cell wall strengthening or/and to generation of antimicrobial compounds limiting fungal development. This hypothesis is supported by the fact that BTH treatment enhanced peroxidase, particularly in PI347321 plants. In addition, the specific scopoletin peroxidase activity was found to be significantly higher in both genotypes following BTH treatment. Peroxidases are enzymes involved in the cell wall strengthening through protein and other polymer oxidative cross-linking and have been associated to resistance in several plant-pathogen interactions (Roldán-Serrano et al. 2007). The roles in defence of scopoletin peroxidase include oxidation of these coumarins for participation in cross-linking with polysaccharides and of extensin monomers, lignifications and suberization (Chittoor et al. 1997). Systemic induced peroxidase activity has been previously reported in pea-*U. viciae-fabae* pathosystems, where the enzyme was induced only at the local level after BTH treatment (Dann and Deverall 2000). Interestingly, BABA treatment did not elicit total peroxidase nor scopoletin peroxidase activities in either of the genotypes, suggesting that the protection exerted by this inducer is not directly related with cell wall strengthening by these enzymes.

In addition to the physical barriers mentioned above, induction of PR-proteins has been reported to influence fungal post-penetration development

through a direct toxic effect (Mauch et al. 1988; Cohen et al. 1999). Our results show that both chemicals effectively decreased both the number of haustoria and hyphal tips per colony with a consequent reduction in the colony size of *U. pisi* pustules. This effect might be due, at least in part, to the enhancement of β-1,3-glucanase, chitinase and peroxidase activity observed following BTH treatment. The enhanced activities of β-1,3-glucanase and chitinase could contribute to such resistance via their hydrolytic action on β-1,3-glucans and chitin present in cell walls of many pathogenic fungi as demonstrated *in vitro*. In addition, both enzymes release oligosaccharides that are active elicitors and responsible for the amplification of the defence responses (Anguelova-Merhar et al. 2002; Amzalek and Cohen 2007). In contrast, we did not observed induction of β-1,3-glucanase and chitinase following BABA treatment. Different and varying results have been related to BABA-induced PR-proteins depending on the pathosystem considered since they have been enhanced in some (Cohen et al. 1994), but not all species (Amzalek and Cohen 2007). In tomato, PR-proteins were strongly induced by foliar spray or root application of BABA (Cohen et al. 1994), whereas in tobacco and pea, they were induced by foliar spraying, but not by stem injection or root application (Cohen et al. 1994; Barilli et al. 2009b). We found an increase of plant resistance following BABA treatment which impairs fungal development at both early and late stages. However, we cannot relate this resistance to the assessed PR-proteins as demonstrated for BTH treatments.

Interestingly, our data showed an increase of total phenolic content, particularly in inoculated PI347321 plants following BABA treatment. This increase was also observed in BTH treated plants. In addition, preliminary results showed differences in the amount and nature of particular phenolic compounds, excreted to the leaf surface following treatment with both inducers (unpublished). This suggests a role for phenolic compounds in the induced resistance exerted by both BTH and BABA. It has been well documented in different pathosystems that phenolic compounds can play an important role in disease resistance, limiting fungal germ tube development or appressorium formation and contributing to cell wall strengthening (lignins), thus preventing plant tissue colonisation (Nicholson and Hammerschmidt 1992; Prats et al. 2002). The induction of the phenolic biosynthesis pathway by both inducers might therefore actively contribute to the resistance to *U. pisi*.

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