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ORIGINAL ARTICLE

Changes in polyphenol oxidase and guaiacol peroxidase enzymes and the expression of pathogenesis-related genes in benzothiadiazole, mycorrhiza-induced or genetic resistance of sunflower plants affected by *Sclerotinia sclerotiorum*

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Abstract

White rot, a major disease in sunflower cultivation caused by *Sclerotinia sclerotiorum* is difficult to control, with no completely resistant cultivars currently available. Different studies have shown that the application of benzothiadiazole (BTH) and arbuscular mycorrhizal fungi (AMF) can effectively control this disease. It has also been reported that both induced and genetically inherited resistance, elevated polyphenol oxidase (PPO) and peroxidase enzyme (POX) activities, and resistance-related genes expression. This study aimed to elucidate the biochemical and genetic responses of sunflower plants under white rot infection and different treatments by measuring the ROS-scavenging enzymes PPO and POX activities in healthy or infected sunflower plants, as well as identifying some defense genes. Sunflower cultivars with different levels of resistance to *Sclerotinia sclerotiorum* were used. Pregerminated seeds were soaked in an aqueous solution of BTH before being sown into trays and placed in a greenhouse. Before sowing, AMF was also applied to the substrate. Twenty-one-day-old plants were then inoculated with *Sclerotinia sclerotiorum*. Our results demonstrated that BTH and AMF induced some degree of systemic resistance in sunflower against white rot, especially combined. Even though the amount of pathogen detected in plants only varied between cultivars, not changing when inducers were applied, disease severity was reduced, demonstrating that treatments were effective against white rot. In addition, the activation of defense-related genes correlated well with the degree of resistance observed in plants. Therefore, our results provide insights for a better understanding of the mechanisms underlying induced-resistance to sunflower white rot infections.

Keywords *Sclerotinia sclerotiorum* · Antioxidant response · Induced resistance · Systemic acquired resistance · Arbuscular mycorrhizal fungi · Benzothiadiazole

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Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops grown in the world in temperate, subtropical and tropical climates under a wide range of environments (Debaeke et al. 2021). The contribution of sunflower oilseed to total oilseed production worldwide has remained stable in the vegetable oil market; fifth place after soybean, rapeseed, cotton, and peanut (Vorobyov and Vorobyova 2021).

Sclerotinia sclerotiorum (Lib.) de Bary, a necrotrophic fungus, is one of the most devastating plant pathogens worldwide, causing disease in more than 500 plant species (Qi et al. 2016, Bán et al. 2017). The survival propagule of *Sclerotinia sclerotiorum* is the sclerotium, which has two germination mechanisms (carpogenic and myceliogenic), allowing the fungus to behave as an air-borne and a soil-borne pathogen, respectively. White rot, a major disease of sunflower, is caused by *Sclerotinia sclerotiorum*. Depending on the type of infection, the pathogen can cause white rot on different parts of the sunflower plants (root rot, basal stalk rot, wilt, stem rot, head rot and midstalk rot). This fungus is able to survive in the soil for many years as hard melanised sclerotia, making disease control management challenging. This is further hampered by the lack of cultivar resistance; as there are currently only partially resistant sunflower cultivars available, despite intensive breeding programs focused on developing resistance in several countries (Davar et al. 2013; Qi et al. 2016; Liu et al. 2017). This major disease can cause severe yield loss in sunflower (infected plants can yield less than 50% of healthy plants) (Filippi et al. 2020). Therefore, it is important to understand potential mechanisms of plant disease resistance against this necrotrophic pathogen, to determine effective strategies that can help to control the disease. A rapidly changing climate and increasingly restrictive regulations on the use of pesticides is driving an urgent need to develop new and more sustainable strategies in integrated pest management (IPM) for crop protection.

Depending on the pathogen lifecycle, induced resistance can be classified as systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Pieterse et al. 2014; Fass et al. 2020; Sido and Hassan 2023). When in contact with pathogens or during treatment with synthetic or natural substances, SAR involves a unique defence signalling cascade pathway that occurs systemically, whereas induced systemic resistance is considered to be and was first described as a reaction triggered by plant growth-promoting rhizobacteria (PGPR), but can also be triggered by antibiotics, surfactants, or chemical inducers (Gozzo and Faoro 2013; Sido and Hassan 2023). A number of studies have shown that SAR and ISR can be promoted through the use of different inducers in plants. Mycorrhiza-induced resistance (MIR) provides systemic protection against a wide range of pathogens and shares characteristics with both SAR (after pathogen infection) and ISR (following root colonization by non-pathogenic rhizobacteria). In this way, induced resistance can be developed after pathogen infection, through symbiosis with beneficial microorganisms, or through the application of chemical agents. In plant-pathogen interactions, plants exposed to such stimuli can increase their level of basal resistance against the future attack of pathogens when compared to non-stimulated plants (Perazzolli et al. 2022). In necrotrophic plant-pathogen interactions, systemic acquired resistance (SAR) against a second infection is usually promoted, which means not only the first infected site (plant tissue), but also the rest of the healthy plant will acquire some protection (Barna et al. 2012; Na et al. 2018).

An early and regular reaction of plants to abiotic or biotic stresses is the rapid accumulation of reactive oxygen species (ROS). To eliminate the harmful effect of ROS, enzymatic and non-enzymatic antioxidants are induced in plants (Baker and Orlandi 1995; Przemyslaw 1997; Apel and Hirt 2004). Susceptibility to necrotrophic pathogens can be caused by ROS-mediated death of plant tissues. In addition, besides being important regulators of plant growth and development, the oxidative burst, associated with the production of reactive oxygen species (ROS), is also involved in limiting pathogen ingress, induction of cell death and the signalling transduction of several defence processes (Barna et al. 2012rösi et al. 2016). The elevation of antioxidant enzymes by plants can thus increase tolerance to cell death (Rajput et al. 2021). However, pathogens can also modify the defence signalling network in plants for their own benefit, changing phytohormone homeostasis and consequently activating a cascade of antioxidant enzymes and compounds (Barna et al. 2012).

Currently, control measures are unsuccessful against sunflower white rot. By treating plants with a variety of biotic (beneficial organisms or microorganisms) and abiotic (synthetic or natural stimulants) control agents, induced resistance can be improved, locally at the site of infection or systemically, to a subsequent pathogenic attack. This strategy is not recommended in isolation, but in combination with other control methods, and can result in a long-lasting and effective strategy against a broad-spectrum of pathogens (Bán et al. 2017). Even though *Sclerotinia sclerotiorum* is a necrotrophic pathogen, it is important to note that in a biotrophic plant-pathogen interaction of sunflower plants with *Plasmopara halstedii* (Farlow) Berlese & de Toni, the pathogen causing downy mildew, it was demonstrated that the application of benzothiadiazole (BTH) significantly reduced disease symptoms and enhanced some defence-related enzyme activities in susceptible sunflower plants (Körösi et al. 2011). It is known that BTH can trigger

resistance through an increase in the activity of antioxidant enzymes (Körösi et al. 2016), and BTH-induced resistance has been associated with the transcriptional activation of genes encoding pathogenesis-related (PR) proteins promoted by endogenous accumulation of salicylic acid (Barilli et al. 2010). This makes BTH a promising chemical inducer of resistance to activate natural defence systems against white rot, resulting in systemic acquired resistance (SAR). The effects of BTH may vary in relation to different concentrations, as well as to different pathosystems. However, it has been shown to activate resistance in many crops against a broad spectrum of diseases (Bán et al. 2017).

Arbuscular mycorrhizal fungi (AMF) are a class of beneficial microorganisms widely distributed in soil ecosystems that can be used for enhancing resistance (induced systemic resistance) against plant diseases. AMF can form symbionts with 80% of higher land plants, improving plant growth and development and are thus considered to be one of the most efficient biocontrol methods to antagonize soil-borne pathogens (Ezzat 2015n et al. 2017; Weng et al. 2022). AMF are known to improve the rhizosphere environment by influencing the physical and chemical properties of the soil, increasing the growth of other beneficial microorganisms and competing with pathogenic microorganisms, plus can induce the host plant to form defence systems, including improving phytohormone concentrations, inducing signal substrate production, regulating resistance gene expression, and increasing protein production (Weng et al. 2022).

Despite the positive effects of both BTH and AMF in triggering resistance against a wide range of pathogens, there is limited information about the combined use of BTH and AMF in controlling plant diseases. This study aims to elucidate the effects of the combined application of BTH and AMF on white rot disease development in three sunflower cultivars with different resistance levels to *Sclerotinia sclerotiorum*, with the aim to increase the potential use of these two resistance inducers in combination with other control methods to improve the control of white rot in sunflower cultivation.

In a previous study by Bán et al. 2017; it was shown that the application of BTH (Bion 50 WG, Syngenta Hungary,) and AMF (Symbivit®) could effectively control sunflower white rot. Although both, BTH and M can induce resistance in plants, the mechanisms which result in SAR are still poorly understood. Therefore, we further aim to characterize the defence responses taking place in sunflower plants inoculated with *Sclerotinia sclerotiorum* and treated with these two resistance inducers by measuring antioxidant enzyme activity and the expression of defence related genes, as well as disease severity. The study focuses on the roles played by PPO and POX, as well as some defence genes, in sunflower susceptible and partially resistant plants after inoculation with *Sclerotinia sclerotiorum* and treated with BTH, AMF or both (BTH + AMF) combined.

Materials and methods

Plant material and pathogen inoculum

Three sunflower cultivars with different levels of susceptibility/resistance to *Sclerotinia sclerotiorum* were used. Iregi szürke csíkos (ISZ) is a Hungarian, open-pollinated sunflower cultivar susceptible to white rot on the stem following infection by *Sclerotinia sclerotiorum*. The sunflower hybrid PR64H41 (PR64) has tolerance against white rot in the plant head, while P63LE13 (P63) is tolerant to white rot on the stalk. The *Sclerotinia sclerotiorum* strain SZ24 from a collection at the Department of Integrated Plant Protection, Hungarian University of Agriculture and Life Sciences (MATE), previously known as the Institute of Plant Protection, Szent István University, was used in this experiment. This strain was preserved on potato dextrose agar at 4 °C, and before commencing the experiment, a piece of carrot (*Daucus carota* subsp. *sativus*), one of the pathogen's hosts, was inoculated with the *Sclerotinia sclerotiorum* strain to check its virulence (Jensen et al. 2008). The pathogen inoculum was then increased on tomato agar (Aliyu, M.S. et al. 2017). After culturing for four days on tomato agar, mycelium disks (d = 8 mm) containing the latest propagulum parts were cut from the edge of the culture media for plant inoculation.

Experimental set up and plant inoculation

The experiment was carried out in a greenhouse at the Institute of Plant Protection, Department of Integrated Plant Protection, Hungarian University of Agriculture and Life Sciences (MATE), Gödöllő, Hungary. Benzothiadiazole (BTH), a long-researched chemical inducer of resistance in plants and arbuscular mycorrhiza fungi (AMF) were used to induce plant resistance to the pathogen.

Sunflower seeds were first soaked in 1.5% NaOCl for 2 min for surface disinfection, rinsed with tap water, and germinated at 21 °C on wet filter paper. Pregerminated seedlings with 3–5 mm radicle were then soaked in an aqueous solution of BTH (Bion 50 WG, Syngenta Hungary, 320 ppm) for 2 h. Control seeds were soaked in distilled water for 2 h. Treated sunflower seedlings were sown in trays (58 × 30 × 60 cm) containing horticultural perlite. Before sowing, a commercial mycorrhizal (AMF) product Symbivit®, consisting of six fungal species (*Glomus claroideum*, *Glomus etunicetum*, *Glomus geosporum*, *Glomus intraradices*, *Glomus microaggregatum*, *Glomus mosseae*), was

applied as a biological inducer to the substrate (15 g/ 25 seeds). A nutrient solution based on the fertilizer Vitaflora (Vitaflora Ltd.) was applied to plants with 0.3% of the irrigation water once per week. Plants (treated or not with BTH, AMF or BTH + AMF) were maintained for four weeks in the greenhouse and at twenty-one days after germination, plants were inoculated with the pathogen by placing mycelial discs (d = 8 mm) of the SZ24 strain at the base of the stem which was then covered with soil. The experiment was carried out in a completely randomized blocked design with three replicates (each consisting of 25 plants) resulting in a total of 75 plants/ genotype/ treatment as following:

- CONTROL (non-treated and non-inoculated plants),
- BTH (BTH-treated and non-inoculated plants),
- M (mycorrhiza-mediated and non-inoculated plants),
- BTH + M (BTH-treated and mycorrhiza-mediated, non-inoculated plants),
- INO (inoculated and non-treated plants),
- BTH + INO (BTH-treated and inoculated plants),
- M + INO (mycorrhiza-mediated and inoculated plants), and
- BTH + M + INO (BTH-treated and mycorrhiza-mediated, inoculated plants).

Arbuscular mycorrhizal fungi (AMF) colonization

In order to measure the degree of colonization by AMF, at 21 days after sowing, five plants from the AMF treatment (non-inoculated with *Sclerotinia sclerotiorum*) were used to detect AMF colonization. Samples of roots were cleaned in a 10% potassium hydroxide (KOH) solution for 15 min at 90 °C, acidified with 5% acetic acid for 2 min, and stained with 5% aniline blue in acetic acid for 1 min. For each root system, 30 fragments of 1 cm-length were randomly selected. AMF root colonization was assessed using a light microscope (×400) (Mcgonigle et al. 1990; Grünfeld et al. 2022). The following parameters of mycorrhization were measured: (i) F (%), frequency of mycorrhization (the ratio of infected and non-infected root fragments), (ii) M (%), intensity of mycorrhization (the amount of root cortex colonized by mycorrhiza) and (iii) A (%), abundance of arbuscules (the presence of arbuscules in the root system) (Bán et al. 2017). Parameters of mycorrhization were then calculated with the MYCOCALC software package (Mycocalc 2001).

Disease assessment

Disease development (as disease severity) was assessed three times at 2, 4 and 7 days after inoculation (dpi) by using the following 0–3 scale: 0 (no symptoms), 1 (brown rot on the stem base, plant is still standing), 2 (plant is wilted, laying on the ground), and 3 (plant is dead) (Bán et al. 2017). Disease severity was rated and calculated according to the following formula:

$$DS = \frac{\sum (f \cdot v)}{N \cdot X} 100$$

where DS is disease severity; f is infection class frequencies; v is the number of plants of each class; N is a total of observed plants; X is the highest value of the evaluation scale.

Antioxidant enzymes (PPO and POX) assays

Enzyme activity was determined spectrophotometrically using a SmartSpec Plus Spectrophotometer (BioRad) at 25 °C. Samples (3 from each replicate or 9 per treatment in total) were collected at 2, 4 and 7 days post inoculation with the pathogen for enzyme assays. Polyphenol oxidase (PPO, EC 1.10. 3.1) and guaiacol peroxidase (POX, EC 1.11.1.7) enzyme activities were determined using a cell-free solution (plant extract) that was prepared from the hypocotyl of the plants (3 hypocotyls/ replicate/ treatment). For the detection of oxide-reductive enzyme activities, plant tissue (0.5 g of hypocotyl) was ground and homogenized in a cold mortar pestle (4 °C) with 20% (w/v) polyvinylpyrrolidone (PVPP) and 3 ml of TRIS-HCl buffer (50 mM, pH 7.8), containing 1 mM EDTA-Na2 and 7.5% (w/v) PVPP (Sigma-Aldrich Co.), plus sterile sand (0.5 g) to increase friction. Samples were then centrifuged at 10,000 *g* for 20 min at 4 °C (Körösi et al. 2016). The supernatant was kept on melting ice and used for the enzyme assays described below.

Polyphenol oxidase

(PPO, EC 1.10. 3.1): PPO activity was determined by measuring the rate of quinone formation, as indicated by an increase in absorbance at 400 nm, according to the methodology proposed by Fehrmann and Dimond (1967). The reaction mixture contained 2.2 ml potassium-phosphate buffer (0.1 M, pH 6.0), 1 mM EDTA-Na_2, 20 mM catechol and 200 µl plant extract. Spectrophotometer measurements lasted 100 s and PPO activity was calculated using the extinction coefficient of the formed product (950 $M^{-1}cm^{-1}$), being expressed as µM catechol × g^{-1} fresh weight × min^{-1}.

Guaiacol peroxidase

(POX, EC 1.11.1.7): POX activity was determined according to Rathmell and Sequeira (1974). The reaction mixture

contained 2.2 mL potassium-phosphate buffer (0.1 M, pH 6), 100 µl of 50 mM guaiacol, 100 µl of 12 mM H_2O_2, and 100 µl plant extract. Spectrophotometer measurements lasted 100 s at 470 nm. POX activity was calculated using the extinction coefficient of the formed product (26.6 $mM^{-1}cm^{-1}$) and it was expressed as µM $H_2O_2 \times g^{-1}$ fresh weight $\times min^{-1}$.

Semi-quantitative RT-PCR (pathogen-related genes and pathogen detection in plants)

Pathogen-related genes

RNA extraction was performed using the RNeasy Plant Mini Kit for molecular genetic analysis following the manufacturer's (Qiagen) instructions. Whole seedlings were frozen in liquid nitrogen and ground with a mortar and pestle. A solution of the extracted RNA at a 1 µg/µl concentration was used for cDNA synthesis. The BioRad iScript cDNA Synthesis Kit was used for cDNA transcription following the instructions described therein. A semi-quantitative PCR method was used to detect the transcripts of interest. The cDNA replication was stopped during the exponential phase of the process and the chain reaction was performed with a separate cycle number for each specific gene. For the PCR reactions, primers (primer pairs) specific for sunflower genes were added: Ha-EF-1α, sunflower constitutive elongation factor 1α; Ha-GST, sunflower glutathione S-transferase; Ha-PDF, sunflower defensin; and Ha-PR5, sunflower PR5 gene (Radwan et al. 2005; Azpilicueta et al. 2007). The initial step of the PCR reaction was 94 °C for 3 min, followed by a repeated DNA amplification according to the corresponding cycle: 94 °C for 15 s, Tm for 15 s, 72 °C for 20 s, and a final elongation step at 72 °C for 5 min. The amplified products were separated in 1% agarose gels and visualized with ethidium bromide. Images were captured in a molecular imager gel doc system (BioRad). After staining, signals from ethidium bromide-stained gels were quantified using a molecular mass ruler and normalized over the signals from constitutive probes of the sunflower elongation factor 1 α (Ha-EF-1α) with the Quantity One software (Radwan et al. 2005). The resulting quantified data were referred to as relative transcript accumulation (e.g., Ha-PDF data/Ha-EF1 α data = Ha-PDF relative transcript accumulation). The amplification program included an initial step at 94 °C for 3 min and 25–32 cycles (Ha-EF1α, 25; Ha-GST, 26; Ha-PDF, 30; Ha-PR5, 29) each of 15 s at 94 °C, and 15 s at Tm °C (Ha-EF1 α, 58; Ha-GST and Ha-PDF, 61; Ha-PR5, 64), and 20 s at 72 °C.

Pathogen detection

To amplify the target sequence from genomic DNA of SS, a standard PCR assay was also performed. The primer pairs SSFWD (5'GCTGCTCTTCGGGGCCTTGTATGC–3') and SSREV (5'TGACATGGACTCAATACCAAGCTG–3') were used in this study to detect the presence of *Sclerotinia sclerotiorum* (SZ24 strain) in plants (Freeman et al. 2002; Almquist and Wallenhammar 2015). For the SS-specific PCR assays, a reaction mixture (25 µl) was performed containing both primers (5 pmol), 0.5 units of Platinum Taq (Life Technologies Ltd., Paisley, UK), Tris–HCl buffer (20 mM, pH 8.4), KCl (50 mM), $MgCl_2$ (1 mM), deoxyribonucleotide triphosphates (0.2 mM), 1% formamide and DNA (50 ng–2 µg from mycelium). A touchdown (TD) PCR was used with an annealing temperature range of 72–65 °C, decreasing by 1 °C after every 2 cycles with 16 cycles at the minimum annealing temperature of 65 °C. The thermal cycling conditions were set as: 95 °C for 10 min; then a total of 30 cycles of 94 °C for 30 s, annealing (as described above) for 1 min, 72 °C for 1 min; followed by a final extension of 72 °C for 10 min. The amplified products were separated in 1% agarose gel and visualized with ethidium bromide and images were captured in a molecular imager gel doc system (BioRad). After staining, the products on the gel were quantified using a molecular mass ruler with Quantity One software (Freeman et al. 2002).

Statistical analysis

After standardization (subtracted mean and divided by standard deviation), results were expressed as the mean and standard error of the mean (SEM) of three independent replicates for each parameter evaluated. The data were subjected to analyses of variance, ANOVA ($\alpha = 0.05$), and when significant, means were compared using Tukey's post hoc test at 5% probability ($P < 0.05$). Statistical analyses were performed using the software package AgroEstat (Barbosa and W. Maldonado Junior. 2015).

Results

Effect of various treatments and genotype on the amount of pathogen in sunflower plants (pathogen biomass by gene expression)

Significant differences in disease severity symptoms were not observed between the different cultivars. However, this study aimed to confirm such results using molecular genetic techniques. According to PCR analysis carried out with the pathogen specific primer, the amount of the pathogen

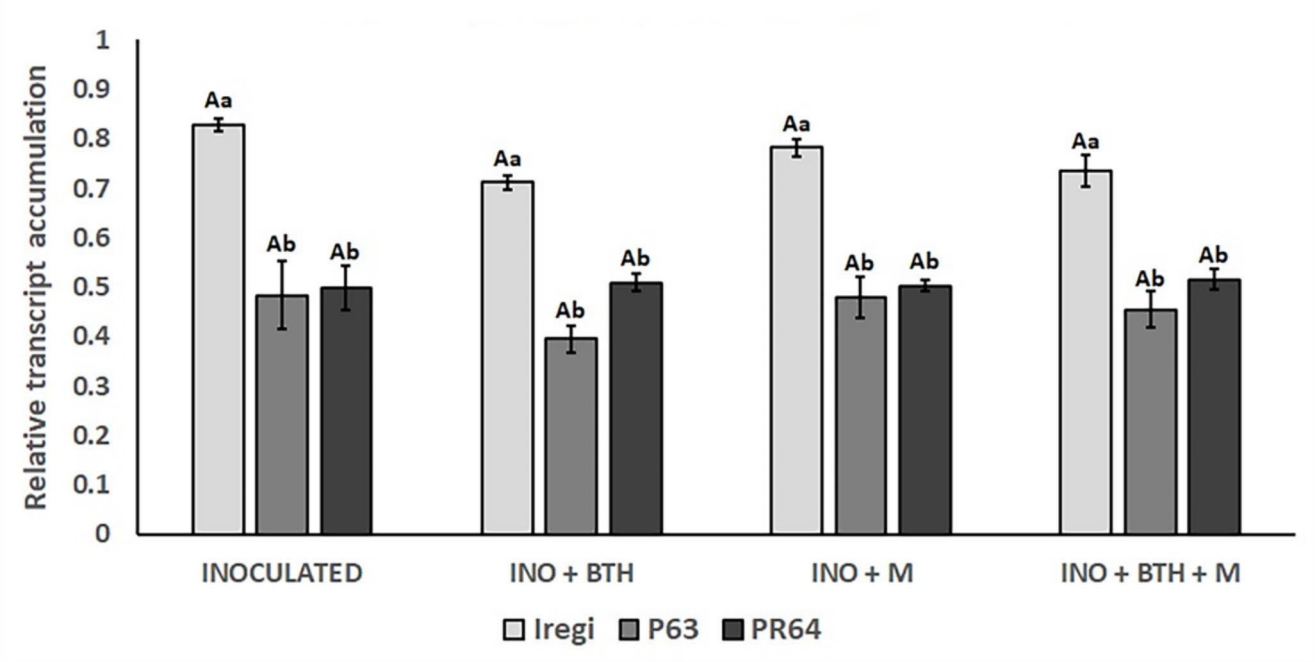

Fig. 1 Pathogen biomass determined by gene expression 5 dpi in sunflower cultivars with different levels of susceptibility/resistance to *Sclerotinia sclerotiorum* and ± treated with benzothiadiazole (BTH), arbuscular mycorrhizal fungi (AMF) or both combined: Iregi (susceptible), P63 and PR64 (partially resistant). Treatments were as follows: INOCULATED (inoculated and non-treated plants), INO + BTH (BTH-treated and inoculated plants), INO + M (mycorrhiza-mediated and inoculated plants), and INO + BTH + M (BTH-treated, mycorrhiza-mediated and inoculated plants). Different uppercase letters above columns indicate differences between cultivars, while lowercase letters indicate treatments with significant differences at $p < 0.05$ following Tukey's post-hoc test

proved to be significantly less in the partially resistant P63 and PR64 cultivars than in plants of the susceptible Iregi Szürke Csíkos cultivar. This was observed across all treatments (Fig. 1). Plants of the Iregi cultivar had a much higher pathogen biomass by gene expression (0.83) than the resistant cultivars P63 (0.48) and PR64 (0.50) in the INOCULATED treatment. However, neither of the treatments seemed to have had any effect in inhibiting or decreasing fungal transcript accumulation (pathogen development) within plant tissue in either susceptible or resistant cultivars. Although INO + BTH treatment showed some level of decrease in transcript accumulation for Iregi and P63 (0.71 and 0.39, respectively), when compared to only inoculated plants (0.83 and 0.48, respectively), this decrease was not statistically significant. The same was observed for the INO + AMF treatment for both cultivars, with Iregi having a pathogen biomass by gene expression equal to 0.78 and P63 presenting a value equal to 0.47 (Fig. 1). The amount of pathogen also did not differ amongst treatments for the PR64 cultivar, with much less fluctuation in value according to treatment (0.50, 0.51, 0.50 and 0.51) for the INOCULATED, INO + BTH, INO + M and INO + BTH + M treatments, respectively. Therefore, our results indicate significant differences with lower pathogen accumulation in the more resistant cultivars, with the different treatments having a low effect on this evaluated parameter (Fig. 1).

Polyphenoloxydase and guaiacol peroxydase enzymes activities in healthy, treated and/or infected sunflower cultivars

In the first measurement (2 dpi), PPO activity increased in heathy and treated plants being influenced by arbuscular mycorrhizal fungi colonization (C + M treatment) in plants of the PR64 genotype (7.14 µM catechol x g^{-1} fresh weight x min^{-1}). However, the highest increase in this enzyme at 2 dpi was promoted by BTH + M in combination in the P63 sunflower cultivar (12.48 µM catechol x g^{-1} fresh weight x min^{-1}), being higher than in inoculated non-treated plants of all cultivars (Fig. 2A). Inoculation alone did not result in an evident increase in PPO activity. However, BTH and BTH + M combined promoted the highest increase in PPO in inoculated plants, with the later treatment presenting the highest results for all cultivars and treatments at 2 dpi (Fig. 2A). Under plant pathogen interaction, BTH alone only increased PPO activity in the P63 genotype (15.86 µM catechol x g^{-1} fresh weight x min^{-1}) compared to plants of the INOCULATED treatment at 2 dpi (Fig. 2A). For the INO + BTH + M treatment at 2 dpi, the PR64 hybrid and the

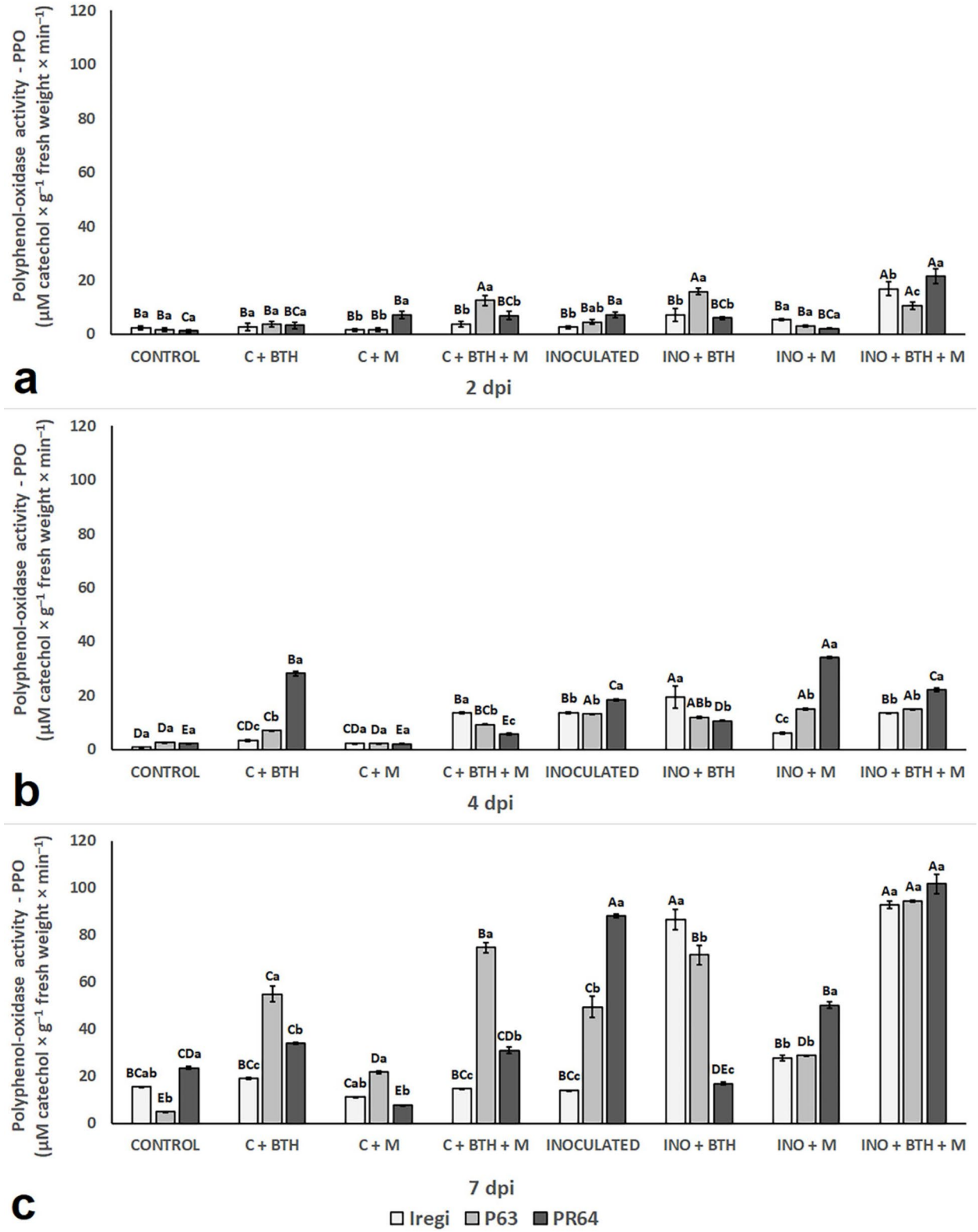

Fig. 2 Polyphenol-oxidase activity (µM catechol x g^{-1} fresh weight x min^{-1}) in three sunflower cultivars with different levels of susceptibility/resistance to *Sclerotinia sclerotiorum* and ± treated with benzothiadiazole (BTH), arbuscular mycorrhizal fungi (AMF) or both combined: Iregi (susceptible), P63 and PR64 (partially resistant). Treatments were as follows: INOCULATED (inoculated and non-treated plants), INO + BTH (BTH-treated and inoculated plants), INO + M (mycorrhiza-mediated and inoculated plants), and INO + BTH + M (BTH-treated, mycorrhiza-mediated and inoculated plants). Different uppercase letters above columns indicate differences amongst cultivars, while lowercase letters indicate treatments with significant differences at $p < 0.05$ followong Tukey's post hoc test

Iregi cultivars showed the highest increase in PPO activity, followed by the P63 hybrid (21.34, 16.79 and 10.61 µM catechol x g^{-1} fresh weight x min^{-1}, respectively) (Fig. 2A).

From 4 dpi onwards the pathogen promoted an increase in PPO activity for all cultivars, with treatments showing a significant increase for the enzyme in inoculated plants, especially the resistant hybrids (P63 and PR64). A significant increase was observed in healthy plants of the C + BTH + M treatment (13.60, 9.24 and 5.75 µM catechol x g^{-1} fresh weight x min^{-1}) for Iregi, P63 and PR64, respectively. However, BTH alone promoted the highest increase in PPO activity of control plants at 4 dpi in non-infected plants of the PR64 sunflower genotype (28.20 µM catechol x g^{-1} fresh weight x min^{-1}) (Fig. 2B). The highest PPO activity at 4 dpi was found in the INO + M treatment, for the PR64 genotype (34.06 µM catechol x g^{-1} fresh weight x min^{-1}). Interestingly, when applied in combination a similar activity of PPO was recorded for all cultivars compared with only inoculated and non-treated plants (Fig. 2B).

PPO activity was highest at 7 dpi, and inoculated plants showed a much more evident increase for this enzyme activity than the non-inoculated and treated plants (Fig. 2C). Both, pathogen inoculation and treatment promoted the highest increased PPO activity for the P63 genotype in the INO + BTH and INO + BTH + M treatments (71.54 and 94.39 µM catechol x g^{-1} fresh weight x min^{-1}, respectively). For inoculated and non-treated plants, PR64 presented the highest increase in PPO activity (88.07 µM catechol x g^{-1} fresh weight x min^{-1}) at 7 dpi (Fig. 2C). BTH alone also promoted an increase in PPO activity in inoculated plants of the susceptible sunflower genotype (Iregi), being higher (86.63 µM catechol x g^{-1} fresh weight x min^{-1}) than the two hybrids (P63 and PR64) 71.54 and 16.90 µM catechol x g^{-1} fresh weight x min^{-1}, respectively. However, it was only in the INO + BTH + M treatment that the highest PPO activity was recorded for all cultivars (92.76, 94.39 and 101.74 µM catechol x g^{-1} fresh weight x min^{-1}, for Iregi, P63 and PR64, respectively).

Overall, PPO activity increased over time (2, 4 and 7 dpi) for all cultivars and treatments, in treated and non-treated plants (Fig. 2). However, this increase was more evident in inoculated and treated plants, especially on plants of the INO + BTH or INO + BTH + M treatments at 7 dpi (Fig. 2C). For the susceptible genotype (Iregi), inoculation with the pathogen in combination with BTH treatments promoted some increase in PPO activity (86.63 and 92.76 µM catechol x g^{-1} fresh weight x min^{-1} for the INO + BTH and INO + BTH + M treatments, respectively) at 7 dpi (Fig. 2C). However, in the hybrids, BTH alone promoted an increase in PPO activity of the P63 genotype in healthy and inoculated plants (54.86 and 71.54 µM catechol x g^{-1} fresh weight x min^{-1}, respectively), and in the PR64 genotype, BTH alone increased PPO activity in healthy plants (33.97 but had no significant effect in inoculated plants (16.90 µM catechol x g^{-1} fresh weight x min^{-1}) for PPO activity under plant-pathogen interaction. This can be observed in plants of the INO + BTH treatment (Fig. 7C). BTH alone significantly elevated PPO enzyme activity in healthy plants of the PR64 genotype (C + BTH treatment), while mycorrhiza alone did not (C + M), compared to the control plants at all dpi measurements (Fig. 2). Arbuscular mycorrhizal fungi colonization, on the other hand, was more effective in increasing the activity of this enzyme for the PR64 genotype in inoculated plants of the INO + M and INO + BTH + M treatments (50.22 and 101.74 µM catechol x g^{-1} fresh weight x min^{-1}, respectively). For the P63 genotype, PPO activity was the highest in plants of the INO + BTH + M treatment (94.39 µM catechol x g^{-1} fresh weight x min^{-1}). Once again, the highest increase in PPO activity was only observed at the INO + BTH + M treatment for all three cultivars, (92.76, 94.39 and 101.74 µM catechol x g^{-1} fresh weight x min^{-1}, for Iregi, P63 and PR64, respectively) (Fig. 2C). Therefore, infection with the pathogen alone increased PPO activity in the different cultivars, but the highest activity was found when both treatments were applied to infected plants (INO + BTH + M). Similarly, to the previous genotype, BTH treatment of PR64 alone significantly elevated PPO enzyme activity in healthy plants, while mycorrhiza alone did not, compared to the control plants. However, the highest activity of PPO was measured when both BTH and AMF were applied before plants were infected (Fig. 2).

Similarly to PPO, POX activity increased over time for all cultivars, with its highest increase being observed in resistant plants at 4 dpi for inoculated plants of the P63 genotype (Fig. 3B), and at 7 dpi for plants of the PR64 genotype (Fig. 3C). Different from PPO, however, POX activity was more influenced by pathogen infection, and BTH had significant effects only for the resistant cultivars (P63 and PR64) of healthy plants at 7 dpi. AMF alone did not promote an increase in POX activity for any of the cultivars, and the increase observed in heathy plants of the C + BTH + M treatment is likely to have been caused by BTH. This was also observed in plants of the INO + BTH + M treatment, where POX activity increased for all cultivars, most likely due to pathogen infection and both treatments applied together. Differences amongst treatments at 2 dpi in healthy plants were only found in the C + BTH and C + BTH + M treatments, where the P63 genotype had the highest POX activity in the C + BTH treatment (0.56 µM catechol x g^{-1} fresh weight x min^{-1}), followed by PR64 in the C + BTH + M (0.45 µM catechol x g^{-1} fresh weight x min^{-1}). This demonstrates that healthy plants only had POX activity enhanced when treated with BTH or BTH + M at this measurement (Fig. 3A). Infection alone did not increase POX activity at 2 dpi for any of

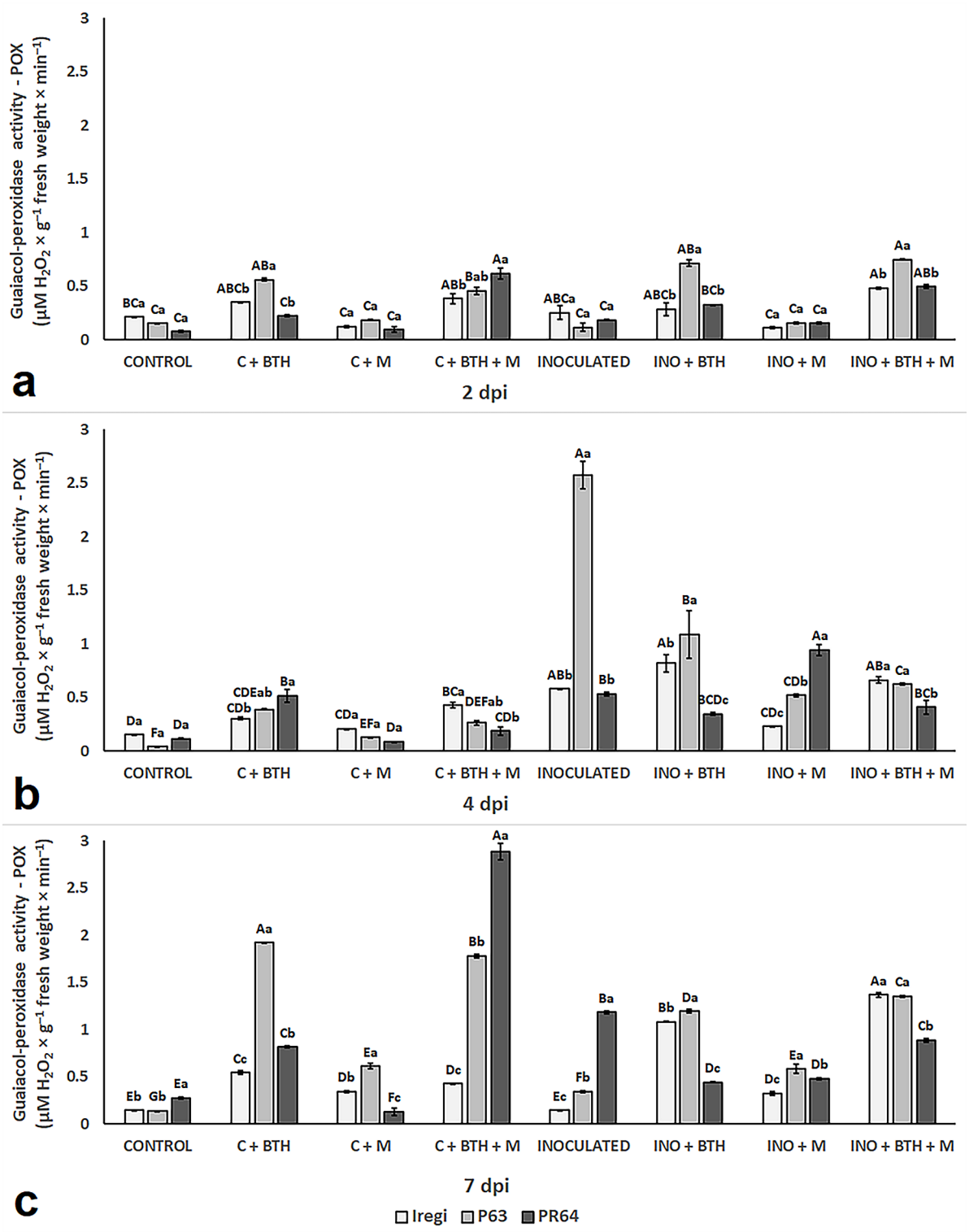

Fig. 3 Guaiacol-peroxidase activity (POX; μM H_2O_2 x g^{-1} fresh weight x min^{-1}) in three sunflower cultivars with different levels of susceptibility/resistance to *Sclerotinia sclerotiorum* and ± treated with benzothiadiazole (BTH), arbuscular mycorrhizal fungi (AMF) or both combined: Iregi (susceptible), P63 and PR64 (partially resistant). Treatments were as follows: INOCULATED (inoculated and non-treated plants), INO + BTH (BTH-treated and inoculated plants), INO + M (mycorrhiza-mediated and inoculated plants), and INO + BTH + M (BTH-treated, mycorrhiza-mediated and inoculated plants). Different uppercase letters above columns indicate differences amongst cultivars, while lowercase letters indicate treatments with significant differences at $p < 0.05$ following Tukey's post-hoc test

the cultivars, and the same was observed for AMF treated plants under pathogen infection. BTH treatment increased POX activity in all three cultivars, but the highest increase at 2 dpi was observed in plants of the P63 genotype in both INO + BTH and INO + BTH + M treatments (0.71 and 0.75 µM catechol x g^{-1} fresh weight x min^{-1}). The combination of both treatments in inoculated plants promoted an increase in POX activity for all three cultivars at 2 dpi for Iregi, P63 and PR64 (0.48, 0.75 and 0.50 µM catechol x g^{-1} fresh weight x min^{-1}, respectively) (Fig. 3A).

Infection alone promoted the highest activity in POX at 4 dpi (Fig. 3B). This was observed for the P63 genotype (2.57 µM catechol x g^{-1} fresh weight x min^{-1}). Significant differences were also observed in healthy and treated plants. For instance, BTH increased POX activity in healthy and treated plants of PR64 (0.39 µM catechol x g^{-1} fresh weight x min^{-1}) in the C + BTH treatment (Fig. 3B). An increase in POX activity was also observed in healthy plants of the C + BTH + M treatment, for Iregi, P63 and PR64 (0.42, 0.26, and 0.19 µM catechol x g^{-1} fresh weight x min^{-1}, respectively) at 4 dpi (Fig. 3B). Under plant-pathogen interaction, BTH and AMF promoted some increase in POX activity. However, for the P63 genotype, this increase was less pronounced than in the case of infection alone at 4 dpi (Fig. 3B). INO + BTH and INO + BTH + M increased POX activity for Iregi (0.82 and 0.66 µM catechol x g^{-1} fresh weight x min^{-1}, respectively), while INO + M was more effective in increasing POX activity for PR64 under infection (0.94 µM catechol x g^{-1} fresh weight x min^{-1}).

The highest increase observed in POX activity was evident in the last measurement (7 dpi) (Fig. 3C). This increase was observed in both healthy and treated plants as well as inoculated and treated plants. BTH alone increased POX activity in healthy plants at 7 dpi and this increase was more pronounced for the P63 genotype (Fig. 3C). Healthy and M-treated plants showed some increase in POX activity, but the highest increase for this enzyme at 7 dpi was also observed when both treatments were applied in combination in healthy plants (C + BTH + M) for the resistant cultivars P63 (1.78 µM catechol x g^{-1} fresh weight x min^{-1}) and PR64 (2.88 µM catechol x g^{-1} fresh weight x min^{-1}). In contrast to what was observed at 4 dpi, inoculation alone only increased POX activity in the resistant cultivars, however, it was much less pronounced than in the C + BTH + M treatment (Fig. 3C). Interestingly, BTH was able to increase POX activity in the susceptible genotype (Iregi) to the same level of the resistant P63 genotype in the INO + BTH and INI + BTH + M treatments (Fig. 3C). Treatment with AMF alone was not so effective in increasing POX activity for all cultivars under infection at 7 dpi, and INO + BTH + M treatment showed a higher increase compared to both treatments applied alone in infected plants. Overall, BTH, AMF and BTH + M treatments were able to increase POX activity in healthy plants for all cultivars, when compared to control plants. However, under plant-pathogen interaction, treatments reduced POX activity for the PR64 genotype, and promoted and increased POX activity for the Iregi and P63 cultivars, compared to infection alone (INOCULATED treatment) (Fig. 3C).

Expressions of some pathogen-related genes in various sunflower and Sclerotinia interactions

To find out if any correlation exists between the resistance/tolerance of sunflower to white rot and the expression of some genes, resistance related gene activities of CAT, Ha-GST, Ha-PDF, and PR-5 were measured. In general, no increase was observed for any of the cultivars for CAT transcript accumulation in healthy plants at 0 or 7 dpi. Even though some differences were observed for this gene expression at 0 dpi, these significant differences are due to genotype, and not the treatments. Control plants of the resistant cultivars (P63 and PR64) presented a higher CAT transcript accumulation than the susceptible genotype (Iregi), particularly P63 (Fig. 4A). This was also observed in healthy plants of the same genotype treated with BTH, where P63 had the highest CAT transcript accumulation in healthy plants amongst cultivars (Fig. 4A). P63 also presented a higher CAT expression than Iregi for the INOCULATED and INO + BTH treatments at 0 dpi (Fig. 4A). At 7 dpi, no significant differences were observed in healthy plants of all cultivars. Inoculation alone with the pathogen, however, promoted an increase in CAT transcript accumulation for all three cultivars at 7 dpi, with PR64 having the highest increase in the INOCULATED treatment (Fig. 4B). All treatments, in combination or not, also promoted an increase in CAT expression for the three cultivars. Interestingly, CAT expression was increased in the susceptible genotype (Iregi) to the same levels of the resistant cultivars (P63 and PR64) at the INO + BTH, INO + M and INO + BTH + M treatments at 7 dpi (Fig. 4B).

The glutathione-S-transferase (GST) gene has been considered an important factor in disease resistance. Significant differences were found between treatments and also between cultivars for this gene expression. Interestingly, P63 was the only genotype that was not affected by treatment or inoculation at 0 dpi (Fig. 5A). In healthy plants, PR64 had the lowest GST transcript accumulation amongst cultivars, followed by Iregi, while P63 had the highest expression of this gene in the control treatment at 0 dpi (Fig. 5A). BTH and AMF alone were able to increase GST expression for Iregi and PR64 in healthy plants. However, the highest increase in non-inoculated plants for both cultivars was noticed when both treatments were applied in combination at the C + BTH + M treatment (Fig. 5A). Inoculation alone, on the

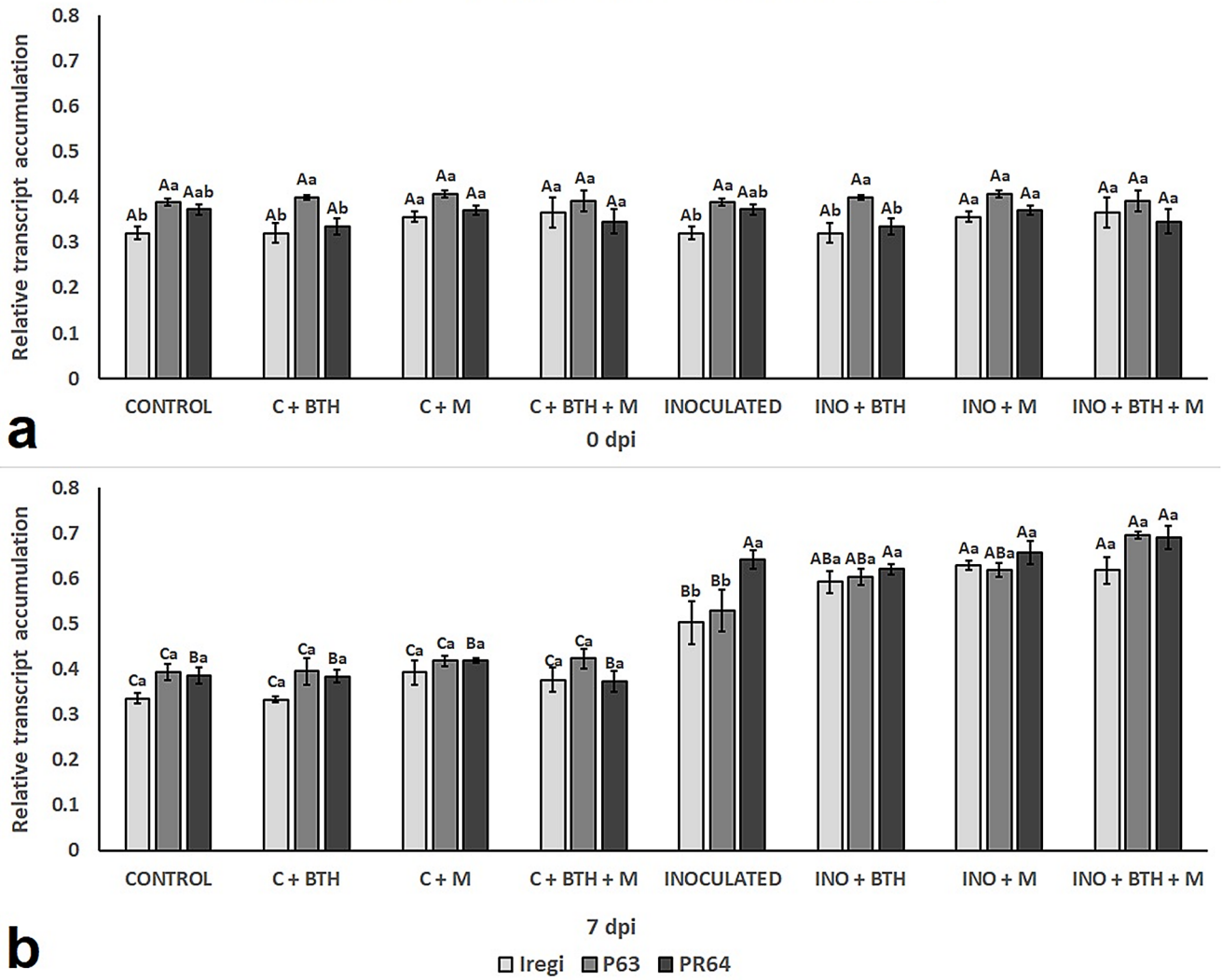

Fig. 4 Sunflower catalase (CAT) transcript accumulation in three sunflower cultivars with different levels of susceptibility/resistance to *Sclerotinia sclerotiorum* and ± treated with benzothiadiazole (BTH), arbuscular mycorrhizal fungi (AMF) or both combined: Iregi (susceptible), P63 and PR64 (partially resistant). Treatments were as follows: INOCULATED (inoculated and non-treated plants), INO + BTH (BTH-treated and inoculated plants), INO + M (mycorrhiza-mediated and inoculated plants), and INO + BTH + M (BTH-treated, mycorrhiza-mediated and inoculated plants). Each value represents the means of the quantity of the signals (± SD) ($n = 3$). Each value represents the mean of signal intensities ± SD ($n = 3$). Different uppercase letters above columns indicate differences amongst cultivars, while lowercase letters indicate treatments with significant differences at $p < 0.05$ following Tukey's post hoc test

other hand, negatively affected the expression of this gene for Iregi and PR64, promoting a decrease in GST transcript accumulation for these two cultivars in the INOCULATED treatment on the day of infection (Fig. 5A). BTH and AMF treatments were able to increase GST expression in infected plants, for both of these cultivars (Iregi and PR64). However, once again, the highest increase was noticed when both treatments were applied in combination in the INO + BTH + M treatment at 0 dpi (Fig. 5A). GST expression did not appear to increase over time in healthy and treated plants (Fig. 5B), demonstrating that treatments alone in non-inoculated plants did not increase this gene expression. However, both infection and treatments combined played a significant role in increasing GST expression 7 dpi for all 3 cultivars. There was no significant difference between cultivars for GST expression under infection alone (INOCULATED treatment) and the same was observed for the INO + BTH treatment (Fig. 5B). Iregi had the highest increase in GST expression in INO + BTH and INO + BTH + M treatments, while plants of the INO + M treatment for this genotype presented a GST expression similar to plants of infection alone (INOCULATED treatment). For the PR64 resistant genotype, the opposite was observed, where BTH alone did not promote any significant increase compared to infection alone (INO + BTH), whereas plants of the INO + M treatment had a significant increase in GST expression for the

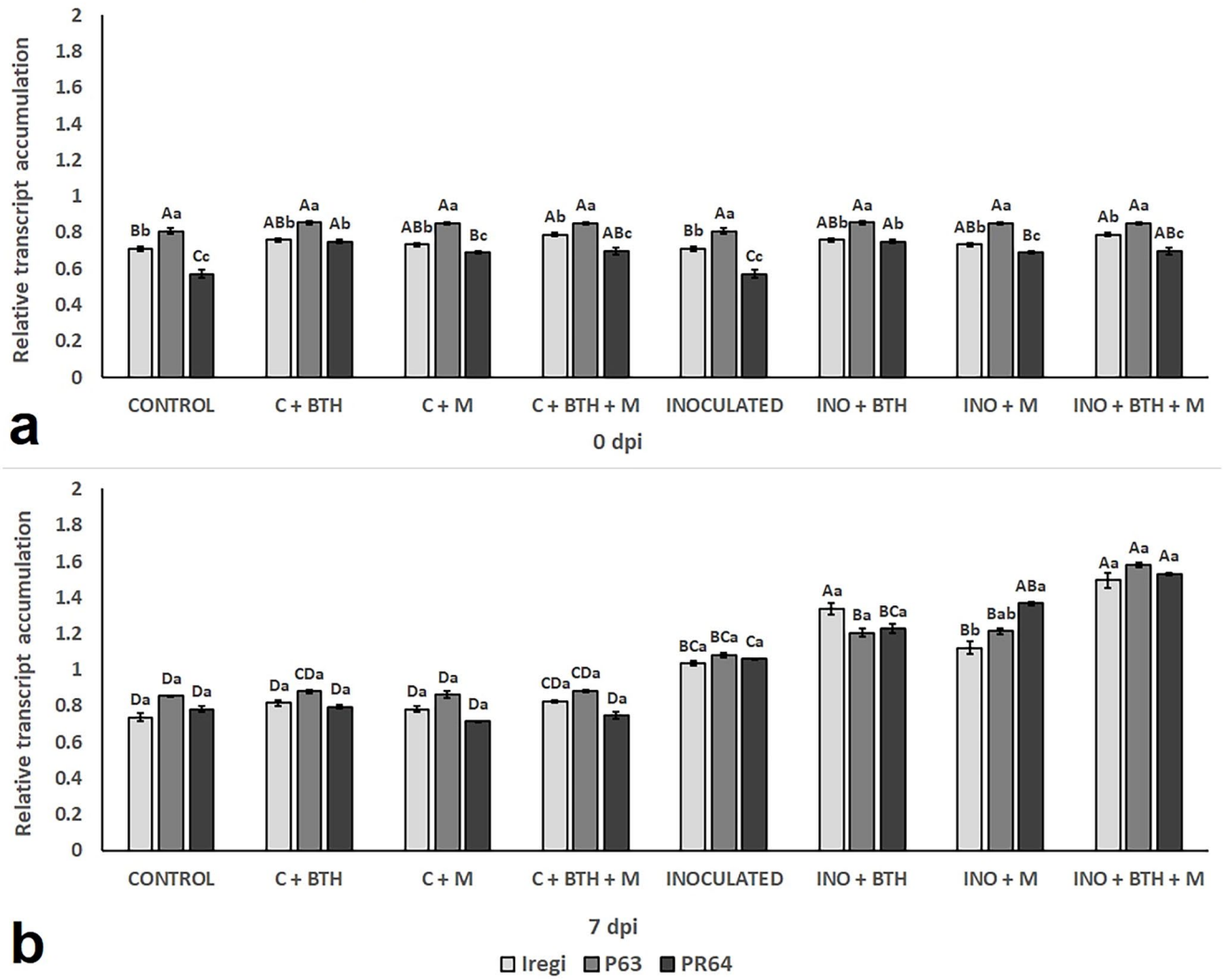

Fig. 5 Sunflower glutathione S-transferase (Ha-GST) transcript accumulation in in three sunflower cultivars with different levels of susceptibility/resistance to *Sclerotinia sclerotiorum* and ± treated with benzothiadiazole (BTH), arbuscular mycorrhizal fungi (AMF) or both combined: Iregi (susceptible), P63 and PR64 (partially resistant). Treatments were as follows: INOCULATED (inoculated and non-treated plants), INO + BTH (BTH-treated and inoculated plants), INO + M (mycorrhiza-mediated and inoculated plants), and INO + BTH + M (BTH-treated, mycorrhiza-mediated and inoculated plants). Each value represents the means of the quantity of the signals (± SD) ($n = 3$). Each value represents the mean of signal intensities ± SD ($n = 3$). Different uppercase letters above columns indicate differences amongst cultivars, while lowercase letters indicate treatments with significant differences at $p < 0.05$ following Tukey's post hoc test

PR64 genotype. Overall, for all three cultivars, the highest increase in GST expression was observed in plants of the INO + BTH + M treatment at 7dpi, where even the susceptible genotype had a GST expression compared to the resistant ones with this treatment (Fig. 5B).

The sunflower defensin (Ha-PDF) gene expression can be measured only in infected plants. In contrast to GST gene, which activated equally in response to infection, accumulation of Ha-PDF was significantly higher in the tolerant P63 and PR64 cultivars compared to the susceptible Iregi in inoculated and non-treated plant. BTH and BTH + M treatments increased Ha-PDF expression in Iregi, while gene expression in P63 was not affected by treatment. PR64 showed an increase in Ha-PDF expression after treatment with AMF, but it was only with the INO + BTH + M treatment that gene expression was the highest for this genotype. In addition, it was only with this treatment that no significant differences were found between cultivars, since both treatments combined increased Ha-PDF expression in the susceptible and infected plants to the same levels as observed in the resistant cultivars (Fig. 6).

Similarly to defensin, Ha-PR5 gene was only detected in infected plants (Fig. 7). For this gene, the resistant (P63 and PR64) cultivars presented a much higher expression than

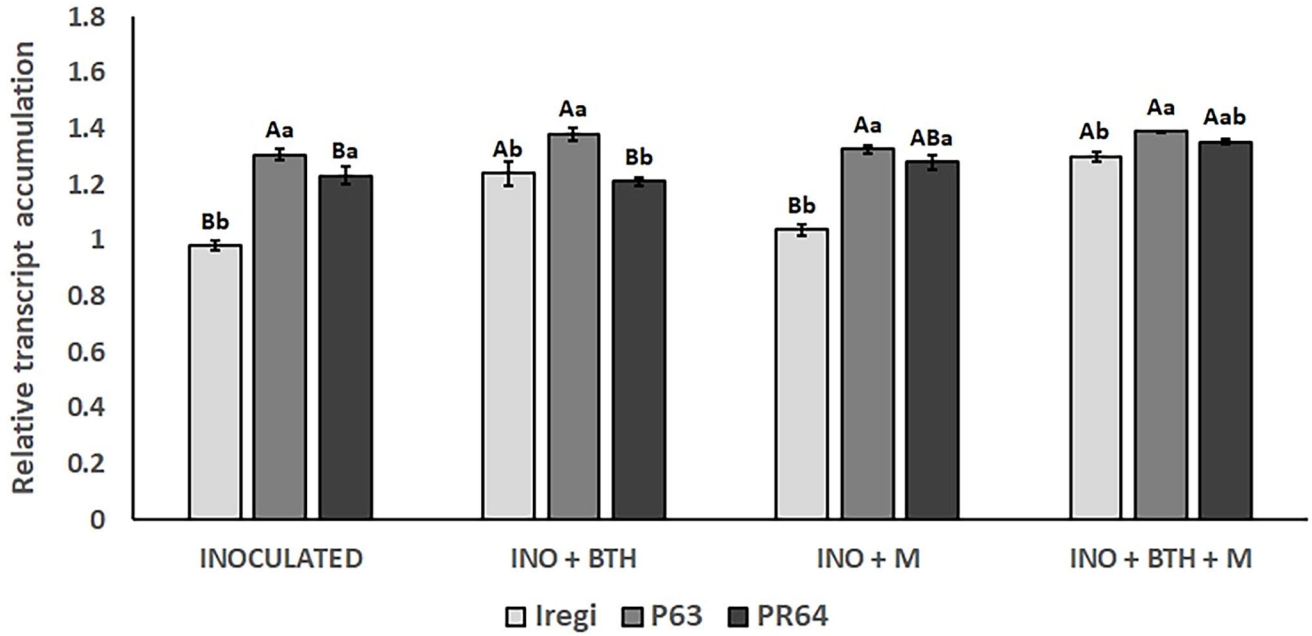

Fig. 6 Sunflower defensin (Ha-PDF) transcript accumulation (7 dpi) in three sunflower cultivars with different levels of susceptibility/resistance to *Sclerotinia sclerotiorum* and ± treated with benzothiadiazole (BTH), arbuscular mycorrhizal fungi (AMF) or both combined: Iregi (susceptible), P63 and PR64 (partially resistant). Treatments were as follows: INOCULATED (inoculated and non-treated plants), INO + BTH (BTH-treated and inoculated plants), INO + M (mycorrhiza-mediated and inoculated plants), and INO + BTH + M (BTH-treated, mycorrhiza-mediated and inoculated plants). Each value represents the means of signal intensities ± SD ($n = 3$). Different uppercase letters above columns indicate differences amongst cultivars, while lowercase letters indicate treatments with significant differences at $p < 0.05$ following Tukey's post hoc test

the susceptible Iregi in all treatments. P63 was the genotype with the highest Ha-PR5 expression, followed by PR64. While treatment with BTH alone did not cause significant changes in Ha-PR5 gene expression in any of the sunflower cultivars, mycorrhiza treatment elevated Ha-PR5 expression for Iregi and PR64. In the case of the susceptible genotype (Iregi) the highest expression of Ha-PR5 was observed in the INO + M treatment, while for PR64 the highest expression was observed in the INO + M and INO + BTH + M treatments.

Discussion

Systemic acquired resistance against *Sclerotinia sclerotiorum* through the use of two different resistance inducers: benzothiadiazole (BTH) and arbuscular mycorrhiza fungi (M) was investigated in relation to biochemical and genetic changes in susceptible and partially resistant sunflower cultivars by measuring antioxidant enzyme activity and pathogenesis-related gene expression (Bán et al. 2017). As an indicator of pathogen colonization in sunflower plants, pathogen transcript accumulation was measured in inoculated plants. As reported in previous research with *Plasmopara halstedii* plant-pathogen interactions (Körösi et al. 2011), the susceptible genotype (Iregi) showed a much more pronounced transcript accumulation in comparison to the resistant cultivars (P63 and PR64), which correlates well with the degree of resistance of the cultivars. The tolerant/resistant plants (P63 and PR64) were shown to be much less affected by the pathogen, accumulating less pathogen transcripts than the susceptible (Iregi) plants.

Since treatments with either of the resistance inducers (BTH or M) did not significantly decrease pathogen transcript accumulation in either susceptible or resistant plants, it is clear that resistant plants were less affected by the pathogen due to their genetic resistance. This is in contrast to previous microscopic observations, where BTH provided some protection to sunflower plants against downy mildew (Körösi et al. 2011), and the same was observed for BTH and AMF against white rot (Bán et al. 2017). However, that does not mean that the inducers did not play a significant role in protecting plants from pathogen colonization, as the host genome may affect the protective effects provided by AMF and a certain level of mycorrhiza root colonization is essential to provide protection against pathogens. The response to BTH and AMF can also be affected by different factors including plant nutrition (Cameron et al. 2013; Hong Duc et al. 2017). For instance, the same sunflower cultivars used in this study responded distinctly to mycorrhizal

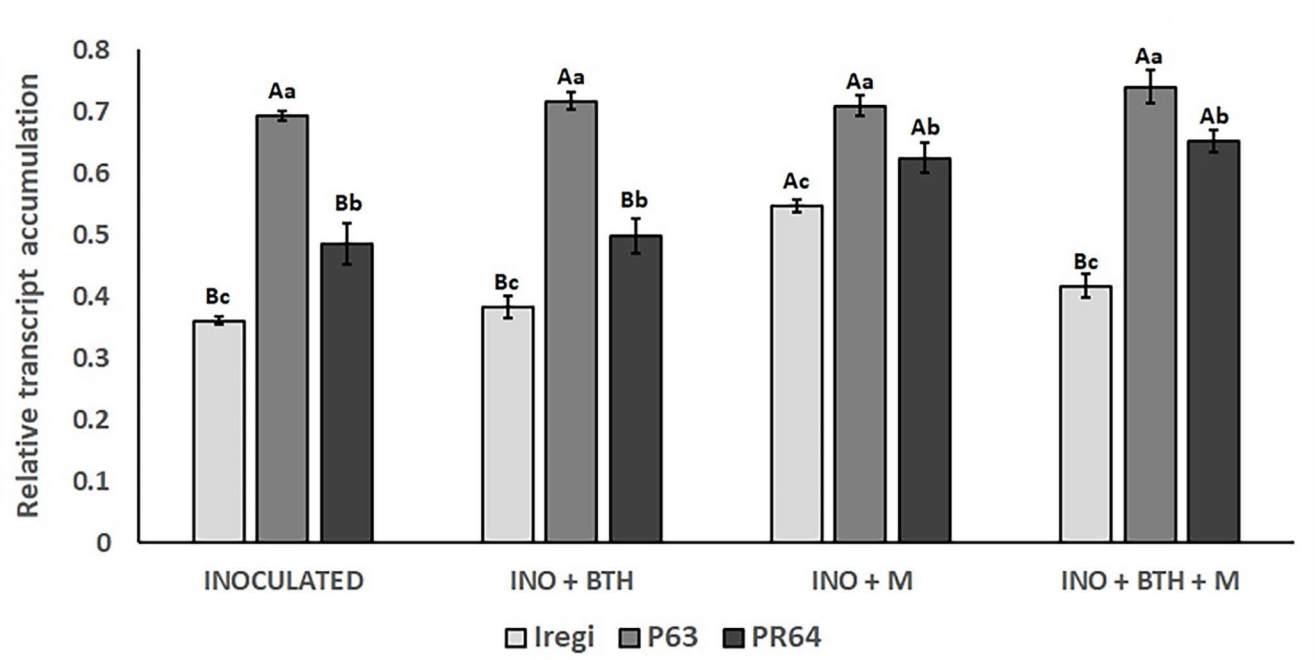

Fig. 7 Sunflower PR-5 (Ha-PR5) transcript accumulation (7 dpi) in three sunflower cultivars with different levels of susceptibility/resistance to *Sclerotinia sclerotiorum* and ± treated with benzothiadiazole (BTH), arbuscular mycorrhizal fungi (AMF) or both combined: Iregi (susceptible), P63 and PR64 (partially resistant). Treatments were as follows: INOCULATED (inoculated and non-treated plants), INO + BTH (BTH-treated and inoculated plants), INO + M (mycorrhiza-mediated and inoculated plants), and INO + BTH + M (BTH-treated, mycorrhiza-mediated and inoculated plants). Each value represents the means of signal intensities ± SD ($n = 3$). Different uppercase letters above columns indicate differences amongst cultivars, while lowercase letters indicate treatments with significant differences at $p < 0.05$ following Tukey's post hoc test

colonization in previous experiments (Bán et al. 2017). It is suggested that the difference in response is due to a weak intensity of mycorrhization and arbuscular abundance was most likely correlated to the short duration of the experiments (4 weeks). The frequency of mycorrhization (F %) for the two resistant sunflower hybrids (PR64 and P63) increased significantly under the BTH treatment, indicating that the BTH seed treatment resulted in a synergistic effect on mycorrhiza root colonization in sunflower plants (Bán et al. 2017). When AMF is used for plant disease control, factors such as the type of plant disease, the relationship between AMF and host plants, the amount and time of AMF inoculation, and environmental factors can affect the symbiosis and its control effects on the pathogens (Weng et al. 2022). Furthermore, the above experiments emphasize the difference between tolerance/ resistance to disease symptoms and to pathogen development.

In the field, plants are affected by numerous factors besides the plant-pathogen interactions. For instance, it is known that pathogens with different lifecycles try to colonize plants using different strategies. This leads to a cross-talk between the different pathways they induce inside the plant, enabling plants to respond in different ways when defending themselves from pathogens (Peluffo et al. 2010). The same can happen when using resistance inducers. Therefore, it is essential to examine cross-talks between synergism (additive effect) and antagonism pathways when testing the combined effects of two different inducers of resistance in plants (Llorens et al. 2017; Sido and Hassan 2023). Although many metabolic profiling studies of host–pathogen relationships have been performed focusing on interactions with biotrophs and symbiotic microorganisms, those involving necrotrophic pathogens are still limited (Peluffo et al. 2010; Nehela et al. 2023).

The protective effects of BTH (Gorlach et al. 1996) or AMF (Cameron et al. 2013; Weng et al. 2022), have been previously reported in a number of plant-pathogen interactions. However, much is still unknown about the combined effect of these inducers in sunflower plants against *Sclerotinia sclerotiorum*. Our results suggest that both inducers can have some effect in inducing resistance, in single or combined applications. However, sunflower plant responses to *Sclerotinia sclerotiorum* are host-dependent (Na et al. 2018). A recent study on the transcriptional changes occurring in sunflower upon infection with *Sclerotinia sclerotiorum* suggested genotype-specific defence responses, which is in line with the diversity of resistance levels observed under field conditions (Fass et al. 2020). A combined

analysis of transcriptomics and QTL mapping, by Fass et al. (2020) provided a promising approach to identify relevant genes and genomic regions associated with quantitative disease resistance (QDR). The involvement of ncRNAs was also reported, stating that they add an unexpected complexity to the discovery of the genetic determinants of white rot (SHR) resistance in sunflower plants (Fass et al. 2020).

Histological examinations of host responses revealed that BTH and AMF significantly decreased pathogenic hyphae development in plants of two cultivars (Iregi and P63), with localized and systemic induction of resistance being observed in plants which were treated with these inducers against *Sclerotinia sclerotiorum* through a higher accumulation of phenolic compounds in association with changes in cell wall composition, which play a significant role in plant responses to *Sclerotinia sclerotiorum* infection (Bán et al. 2017; Na et al. 2018). The accumulation of reactive oxygen species (ROS) has also been found to be associated with induced resistance in BTH-treated sunflower plants against *Plasmopara halstedii* (Körösi et al. 2011). In addition, similar responses were observed with the accumulation of fluorescent compounds, which are suggested to be involved in the induction of a localized and systemic resistance induced by arbuscular mycorrhiza fungus against different pathogens (Weng et al. 2022).

An integrated approach using alternative and complementary control methods is crucial for the effective protection of sunflower plants against *Sclerotinia sclerotiorum* (Albert et al. 2022). This includes induced resistance, which can play a significant role in controlling the major sunflower pathogen (Llorens et al. 2017). Based on the first report and earlier positive results against downy mildew caused by *Plasmopara halstedii* (Körösi et al. 2011) and white rot caused by *Sclerotinia sclerotiorum* (Bán et al. 2017), the combined application of these inducers results in some degree of control for both pathogens, and various studies have demonstrated that the systemic acquired resistance (SAR) can be promoted with the use of inducers which stimulate the enzymatic and non-enzymatic antioxidant system (Llorens et al. 2017; Sido and Hassan 2023; Khoshru et al. 2023). In this way, antioxidant enzyme activity (PPO and POX), as well as pathogenesis-related genes expression were measured in order to better understand biochemical and genetic responses of sunflower plants that were either healthy or affected by *Sclerotinia sclerotiorum*, in accordance with previous findings (Monazzah et al. 2018). Therefore, our results, followed by further research, could provide additional information on the potential use of these inducers as biocontrol agents in future disease management of sunflower.

In this study, it was observed that inoculation with the pathogen itself increased the antioxidant response of plants through a higher activity of both PPO and POX enzymes, when compared to the control plants. This was expected since under plant-pathogen interactions the antioxidant system is activated to protect plants (Kaur et al. 2022; Sido and Hassan 2023; Khoshru et al. 2023). However, it is important to mention that BTH alone, or in combination with AMF also provided an increase in the activity of PPO in healthy plants. Although AMF alone only seemed to have a positive effect in infected plants (INO + M treatment). However, the combined use of both inducers under pathogen infection (INO + BTH + M treatment) promoted the highest increase in PPO activity for all three cultivars.

In this study, three sunflower cultivars with different levels of susceptibility/ resistance to *Sclerotinia sclerotiorum* were compared to provide further information on induced resistance to white rot. From our results, especially at 7 dpi, we can see that BTH alone or BTH + M promoted an increase in activity in both enzymes (PPO and POX) in healthy plants of the resistant cultivars (P63 and PR64). This increase was more pronounced at 7 dpi in POX activity in the PR64 genotype. At this stage, only the partially resistant and non-treated plants (INOCULATED treatment) showed an increased activity in both enzymes when infected with the pathogen, compared to the control plants. This can be explained by the fact that the resistant plants (P63 and PR64) had a faster reaction against the pathogen where both enzymes (PPO and POX) were activated earlier in comparison to the susceptible genotype (Iregi) as previously reported (Davar et al. 2013; Monazzah et al. 2018; Kaur et al. 2022). Once again, the treatment which resulted in the highest activity for both enzymes in inoculated plants and for all three cultivars was the combination of both inducers (INO + BTH + M treatment). This could indicate a synergism between BTH application and mycorrhization, as demonstrated by Bán et al. (2017). Since these two enzymes play roles in oxidation and condensation of lignin precursors, the induction of their activities is effective in construction and solidification of the cell wall against pathogen penetration (Monazzah et al. 2018).

Furthermore, our results also showed that the activity of both enzymes decreased in plants treated with AMF alone at 7 dpi. A possible explanation is that these enzymes are already activated by hypersensitive cell death, and the AMF root colonization treatment suppressed the pathogen attack by reducing fungal growth and host tissue necrosis, reducing PPO and POX activity(Cameron et al. 2013; Weng et al. 2022). This can be linked to the fact that these enzymes may be correlated with the extent of host cell necrosis and not with the degree of resistance, especially POX which is sensitive to ROS accumulation in plant tissue (necrosis) (Hong Duc et al. 2017; Kaur et al. 2022).

Our results demonstrated that BTH and AMF alone or in combination promoted different responses in the three different sunflower cultivars for the major genes evaluated. CAT accumulation in healthy plants was only influenced by BTH application, whereas plants inoculated and treated with either of the inducers showed similar responses to CAT accumulation. Significant differences were also noted for CAT accumulation in non-treated inoculated plants. Interestingly, Ha-GST accumulation was affected by both inducers, in combination or separately, resulting in different responses amongst the cultivars for infected and treated plants.

In addition, we further clarified the effects of infection by *Sclerotinia sclerotiorum* through the expression of two other key genes involved in plant signalling pathways (Ha-PDF and Ha-PR5) (Patkar and Naqvi 2017). As expected, Ha-PDF accumulation was much higher for the resistant cultivars than for the susceptible plants inoculated with the pathogen. However, the inducers also promoted some changes in plant response for this gene. For instance, our results suggest that BTH alone promoted an increase in Ha-PDF accumulation of susceptible plants, whereas AMF had an antagonist effect in susceptible plants. In fact, the combination of both inducers elevated Ha-PDF accumulation of susceptible plants to the same level as in partially resistant cultivars. In infected plants, Ha-PR5 accumulation was mostly influenced by AMF, with the highest accumulation of this gene in plants of all three cultivars treated only with AMF.

Considering that the expression of Ha-PDF and Ha-PR5 genes in the partially resistant cultivars was significantly higher than in susceptible plants, it is concluded that these genes mostly contribute to defensive responses of sunflower against *Sclerotinia sclerotiorum* (Monazzah et al. 2018), and that both inducers had positive effects in protecting plants from white rot. These results are also in alignment with previous findings reported by Körösi et al. (2011) in *Plasmopara halstedii* and sunflower plant-interactions. However, it is important to note that the combination of both inducers elevated Ha-PDF accumulation of susceptible plants to the same level of partially resistant cultivars, whereas Ha-PR5 accumulation was only influenced by AMF alone.

Conclusions

In conclusion, BTH and AMF were found to be capable of inducing systemic resistance in sunflower against white rot, particularly in combination, proving they had a synergistic effect. Under plant-pathogen interactions with *Sclerotinia sclerotiorum*, PPO and POX enzyme activities increased significantly more in the partially resistant cultivars. This was also observed in the accumulation of mRNAs for CAT, Ha-PDF and Ha-PR5 genes. Although Ha-GST accumulation also increased after infection, no difference was recorded between susceptible and resistant cultivars. Only after treatment with either inducer or both in combination were different plant responses detected for Ha-GST accumulation between cultivars. Therefore, we suggest that the ability of the partially resistant cultivars in balancing antioxidant enzymes might have resulted in an early detection of ROS, with H_2O_2 being used as a substrate for POX, together with PPO. This may have led to cell wall lignification, restricting pathogen penetration. The changes in the expression of major pathogenesis-related genes observed in this study mostly happened when both inducers were used in sunflower plants under infection by *Sclerotinia sclerotiorum*, decreasing disease severity in all cultivars. BTH effects were more noticeable in the sunflower cultivar P63, while AMF promoted significant changes in Iregi, and BTH + M resulted in significant changes in both Iregi and P63. Even though the amount of pathogen detected in plants only varied between cultivars, and did not change when inducers were applied, disease severity was reduced, as reported in our previous studies. In addition, the activation of defence-related genes correlated well with the degree of resistance observed in plants. Therefore, our results provide insights for a better understanding of the mechanisms underlying induced-resistance to sunflower white rot infections.

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Data availability All data generated or analyzed during this study are included in the manuscript and its supplementary information files (if requested).

Declarations

Ethical approval All authors would like to confirm that they have read and agree to the submission of this original manuscript and that this study has not been published or submitted elsewhere for publication.

Competing interests The authors declare no conflicts of interest.

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