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Differential regulation of antioxidant machinery on grass pea partial resistance against powdery mildew and rust pathogens

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ABSTRACT

Powdery mildew (*Erysiphe pisi*, *E. trifolii*) and rust (*Uromyces pisi*) are important diseases affecting grass pea (*Lathyrus sativus*). This study investigates grass pea's histological, enzymatic, and metabolic responses to these pathogens using accessions with contrasting resistance. Partially resistant (PR) accessions exhibited smaller fungal colonies from 48 h after inoculation (HAI) onwards. Enhanced superoxide dismutase (SOD) activity was observed as early as 12 HAI in PR accessions against both powdery mildews, associated with increased ascorbate peroxidase (APX) and catalase (CAT) activity in *E. trifolii* and *E. pisi* infections, respectively. Moreover, phenolic compounds and flavonoids accumulated in *E. trifolii*-infected PR accessions (6–48 HAI). For rust, APX activity rose at 48 HAI in PR accessions.

These findings suggest that partial resistance (PR) in grass pea is characterized by restricted pathogen invasion and a dynamic regulation of reactive oxygen species (ROS)-scavenging enzymes, with responses varying across pathosystems. This highlights the importance of pathogen-specific selection strategies to minimize the risk of resistance breakdown and promote durable disease resistance in breeding programs.

1. Introduction

Grass pea (*Lathyrus sativus*) is an annual legume cultivated predominantly in the rainfed regions of South Asia and Sub-Saharan Africa [1,2]. Known for its resilience to a wide range of abiotic stress factors (e.g. salinity, drought, nutrient deficiency, heat, flooding), this crop provides a significant source of calories in regions more affected by extreme environmental events [3]. Despite its resilience to adverse agro-ecological conditions, in particular environmental conditions, the co-occurrence of abiotic and biotic stress events can significantly impact grass pea yield [1].

Rust and powdery mildews are among the major air-borne biotrophic fungal diseases affecting grass pea production. Under suitable conditions, powdery mildew infection has been reported to cause up to 50 % loss in the yield of legumes such as pea and soybean [4,5]. The symptoms induced by rust disease can result in a decline in grain quality and a reduction in overall plant yield between 20 and 80 %, depending on the crop and the environmental conditions [6]. Powdery mildew in grass pea is commonly reported as *Erysiphe pisi* (the causal agent of pea powdery mildew) [7,8]. More recently, studies have demonstrated that *E. trifolii* (causal agent of clover powdery mildew) can also successfully infect grass pea, with similar disease symptoms, making it difficult to differentiate between the two pathogens. However, Martins and collaborators (2023) revealed that *E. trifolii* could constitute a more severe biotic threat to grass pea by inducing higher susceptibility levels than *E. pisi*. Indeed, *E. trifolii* is able to overcome the only widely deployed *er1* gene-mediated resistance in peas and has been detected across different continents [9]. A similar situation might occur in grass pea given pea phylogenetically proximity [10]. As for the occurrence of rust disease, studies have shown that grass pea is mainly infected by *Uromyces pisi* [7,

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11].

Several germplasm collections of grass pea and the related *L. cicera* have been evaluated under controlled conditions to identify resistance against powdery mildew and rust [7,10–14]. These efforts have uncovered promising levels of partial resistance. Subsequent genome-wide association studies (GWAS) and linkage mapping approaches have shown that this resistance is controlled by multiple loci [7,10,12,15]. While the genetic basis of resistance in grass pea has been described, the molecular mechanisms underlying these loci remain largely unexplored. A detailed characterization of grass pea-pathogen interactions at histological, enzymatic, and metabolic levels is essential for identifying the active resistance mechanisms. This knowledge will facilitate the selection of accessions with the most effective defense mechanisms, supporting targeted breeding strategies for durable disease resistance.

Histological observations of pathogen-infected plant tissues provide important insights into the pathogen life cycle and the plant resistance mechanisms, guiding effective breeding strategies based on plant-pathogen interactions at different stages. For instance, hypersensitive reactions (HR), characterized by localized cell death, can be detected histologically even when not macroscopically visible. Additionally, structural defenses such as papillae formation and cell-wall appositions, which partially hinder pathogen penetration, are only observable through histological analysis [10,16].

Legume species employ various resistance mechanisms to prevent pathogen infection during the powdery mildew and rust infection processes. Pre-haustorial defenses, which occur before host cell penetration, involve the formation of localized cell wall reinforcements, such as from the accumulation of callose, lignin, phenolics, or reactive oxygen species, at sites of attempted penetration [4,17–19]. Once the haustorium forms, a post-haustorial hypersensitive response (HR) can be triggered. Histological observations show that this HR involves localized cell death of invaded host cells, effectively cutting off the pathogen's access to the host's nutrient supply [20].

Previous histological studies on *Lathyrus* spp. - *U. pisi* pathosystems revealed that resistance was mainly associated with restriction of haustorium formation and reduction in the number of haustoria per colony, representing a case of pre-haustorial resistance [13,14]. As for the interaction of *Lathyrus* spp. with powdery mildew pathogens, to the best of our knowledge, no previous studies have provided a detailed characterization and comparison of histological events in cultivars with contrasting phenotypic response to either *E. pisi* or *E. trifolii*.

During early infection, plants rapidly produce reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-), which activate pre- and post-haustorial defense mechanisms [21–24]. ROS also act as signaling molecules, regulating resistance-related gene expression and triggering additional defense responses [24]. However, excessive ROS accumulation can cause oxidative damage, including lipid peroxidation and cell membrane degradation [21]. To maintain ROS balance, plants rely on ROS-scavenging enzymes and non-enzymatic antioxidants like phenolic compounds and flavonoids, which help detoxify ROS and mitigate stress [24,25]. Additionally, soluble sugars play a dual role in plant defense: they provide energy for immune responses and function as signaling molecules interacting with hormonal pathways. Sugars also enhance the early oxidative burst, promote cell wall lignification, stimulate flavonoid synthesis, and induce pathogenesis-related proteins [26,27].

Catalase, ascorbate peroxidase (H_2O_2-scavenging enzymes), and superoxide dismutase (O_2^--scavenging enzyme) play a crucial role in detoxifying harmful free radicals while supporting ROS-induce defense responses against pathogens. The regulation of ROS-scavenging activity in grass pea under biotrophic infection remains largely unexplored. In pea, a closely related species, increased ROS-scavenging enzyme activity has been linked to increased resistance against *E. pisi* [22,23]. Resistant pea genotypes also exhibited lower malondialdehyde (MDA) levels, an indicator of reduced lipid peroxidation and oxidative damage. Similarly, resistant wheat and barley cultivars infected with yellow rust (*Puccinia striiformis*) showed increased antioxidant enzyme activity, along with decreased ROS and MDA levels. These findings highlight the crucial role of the antioxidative system in mitigating oxidative stress and enhancing pathogen resistance [28].

As for grass pea interaction with powdery mildew and rust pathogens, little is known about the activity of the described enzymatic and metabolic parameters. A better understanding of the complementation between histological and antioxidant defense mechanisms against *E. pisi, E. trifolii,* or *U. pisi* could provide additional criteria for a more informed and accurate selection of durable multi-resistant grass pea cultivars. To address this, we performed a time-course comparison of grass pea accessions with contrasting phenotypes to *E. pisi, E. trifolii,* and *U. pisi*, where their microscopic response to pathogen invasion and the activity of catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and the accumulation of several defense-related metabolites (MDA, phenolic compounds, soluble sugars, and flavonoids) were characterize, to clarify which are the similarities and differences among these three air born biotrophic pathogen-grass pea interactions.

2. Material and methods

2.1. Histological analysis of grass pea - Erysiphe pisi and Erysiphe trifolii interaction

2.1.1. Erysiphe pisi and Erysiphe trifolii inoculation

The selection of the contrasting partially resistant and susceptible grass pea accessions was based on disease severity scores (percentage of leaf area covered by pathogen's mycelium) obtained from a previous inoculation experiment on a grass pea germplasm collection, infected with *E. trifolii* or *E. pisi* [10].

These were the partially resistant accessions PI221467_A and BGE23931, the susceptible accessions PI426892 and PI426882 in response to *E. pisi*; the partially resistant accessions PI195603 and PI358601, and the susceptible accessions PI667247 and PI391431, to *E. trifolii*.

Inoculations with *E. pisi* and *E. trifolii* were performed using the well-established detached leaves methodology, as described by Martins and collaborators (2023). Prior to inoculations, seeds from grass pea accessions were surfaced sterilized as described by Sampaio et al. (2021b). Following this, seedlings were transferred to 0.5 L pots (one plant/pot) containing 1:1 sand and peat mixture and maintained in controlled conditions (12h light 22 °C/12h dark 20 °C, 60 % relative humidity, and 200 μmol/m^2/s of illumination) [7]. Three leaflets (one from each of 3 plants), per accession, per time-point, per inoculation experiment, were detached from two-week-old grass pea plants and placed abaxial side down on Petri dishes containing agar (4 g/l) supplemented with benzimidazole (62.5 mg/l). The used *E. pisi* isolate Ep-CO-01 and *E. trifolii* isolate CO-11B were maintained on pea cv. 'Messire' seedlings in separate air-filtered growth chambers. Inoculation was performed using a settling tower, by dispersing conidia directly from previously infected pea cv. 'Messire' leaves into the settling tower with the help of pressurized air. Inoculation experiments with either powdery mildew pathogen were conducted on consecutive days to prevent isolate cross-contamination. Glass slides were placed inside the settling tower and used to confirm inoculum density throughout the inoculation process, maintained to approximately 5 conidia/mm2. Petri dishes with inoculated leaves were covered and placed in a growth chamber (20 °C, 12h light/12h dark photoperiod).

2.1.2. Histological analysis of grass pea interaction with Erysiphe pisi and Erysiphe trifolii

Leaf samples were collected for histological observations at different time points, 6 h after inoculation (HAI), 12, 24, 48, and 72 HAI, and processed as described by Fondevilla and collaborators [29], with minor modifications. Briefly, grass pea leaflets were placed abaxial side down on filter paper moistened with a 1:3 (v/v) mixture of glacial acetic acid:

absolute ethanol for chlorophyll removal and tissue fixation. When completely bleached, leaflets were transferred to filter paper moistened with tap water for 1 h to soften the tissues. Following this step, leaflets were transferred to filter paper moistened with a 1:1:1 (v/v) mixture of lactic acid:glycerol:water for storage until microscopic observation. For examination of fungal structures, leaf segments were stained with a drop of Tryptan blue in lactoglycerol (0.1 % w/v). For every leaf segment, the percentage of spore germination was calculated by scoring 150 conidia. Additionally, sporelings were classified according to whether: germinated sporelings developed an appressorium and established colony revealed by the emergence of secondary hyphae. The number of hyphal branches per colony was counted as an estimation of colony development.

2.2. Histological analysis of grass pea – Uromyces pisi interaction

2.2.1. Uromyces pisi inoculation

A total of three partially resistant grass pea accessions (PI283566, PI165528, and PI577138_A) and two susceptible accessions (PI283574, and PI221467_B) to *U. pisi* infection were selected from previous work [7] for histological observations. The selection of the contrasting partially resistant and susceptible grass pea accessions was based on disease severity scores (percentage of leaf area covered by rust's pustules) obtained from a previous inoculation experiment on a grass pea germplasm collection, infected with *U. pisi* [7].

Prior to pathogen inoculation, grass pea accessions were maintained in controlled conditions (12h light 22 °C/12h dark 20 °C, 60 % relative humidity, and 200 μmol/m^2/s of illumination) [7]. Inoculations with *U. pisi* were conducted on 20-days-old whole grass pea plants (three independent plants as three biological replicates) by dusting 2 mg of rust spores/plant diluted with pure talk (1:10, w:w) as described in Vaz Patto and Rubiales [11]. Inoculated plants were incubated for 24 h at 20 °C and 100 % humidity in complete darkness. After incubation, spore germination was checked under the microscope to ensure the inoculation was successful and plants were transferred to a growth chamber (20 °C, 12 h light/12 h dark photoperiod).

2.2.2. Histological analysis of grass pea interaction with Uromyces pisi

Three leaflets per accession (one from each of the three independent plants) were collected at different time points (12, 24, 48 HAI) and processed for histological observations as described by Sillero and Rubiales [30]. Briefly, leaf samples collected at 12 and 24 HAI were used for observations of early stages of infection and therefore, care was taken not to displace spores and germtubes from the leaf surface. For this, leaves were laid abaxial side down on a Petri dish with filter paper dipped in a fixative 1:1 mixture solution (ethanol: glacial acetic acid, v/v) and then transferred to filter paper immersed in water for 1 h to soften the tissues. Finally, leaf samples were maintained in a lactoglycerol solution (1:1:1 lactic acid:glycerol:water, v/v/v) for storage until microscopic observation. Fungal structures on the leaf surface were stained with a drop of Tryptan blue in lactoglycerol (0.1 % w/v). A total of 150 urediospores were grouped into different categories: germinated urediospores (considered when the emerging germ tube was at least as long as the spore diameter), with germ tubes successfully locating a stoma. Regarding the urediospore germ tubes reaching a stoma, a distinction was made whether an appressorium was developed above the contacted stoma or not. Regarding the urediospore germ tubes not reaching a stoma, a distinction was made if the germ tube developed a misplaced appressorium (away from the stoma) or if the germ tube overgrowth at least one stoma without forming an appressorium.

Samples collected at 48 HAI were used to assess later stages of pathogen development, happening inside substomatal vesicles and intercellular spaces, and therefore processed differently. Leaf samples were first fixed by immersion in tubes filled with acetic:ethanol (1:3, v/v) and then stained by boiling in 0.05 % Tryptan blue in lactophenol: ethanol (1:2, v/v) for 10 min and cleared in a nearly saturated aqueous chloral hydrate solution (5:2, w/v) to remove Tryptan blue from the chloroplast membrane. Per leaflet, 10 infection units were studied. The number of hyphal branches and haustoria were counted. The occurrence of host cell necrosis was recorded, as observed by the uptake of Tryptan blue by the host cells.

2.3. Enzymatic analysis of grass pea - Erysiphe pisi, Erysiphe trifolii, and Uromyces pisi interactions

The grass pea accessions with contrasting disease responses to *E. pisi, E. trifolii,* and *U. pisi*, previously described, were used also for the assessment of antioxidative enzyme activity (catalase, ascorbate peroxidase, and superoxide dismutase). Five plants per accession (five biological replicates), per time-point, per inoculation experiment, were inoculated separately for each pathogen.

For the characterization of the antioxidative enzyme activity upon infection with either powdery mildew pathogen, inoculation assays were performed as previously described (material and methods, section 4.1.) with slight modifications. Whole plants were independently inoculated with *E. pisi* or *E. trifolii* on a settling tower, providing an inoculum density of 40–45 conidia/mm2. Leaves were collected from non-inoculated control plants and inoculated ones at 6, 12, and 24 HAI, immediately placed in liquid nitrogen, and stored at −80 °C.

Regarding *U. pisi* experiments, inoculations were performed as previously described (material and methods, section 3.2.). Leaves were collected on non-inoculated control plants and inoculated ones at 12, 24, and 48 HAI, immediately immersed in liquid nitrogen, and stored at −80 °C.

Approximately 100 mg of leaf tissue (with 15 mg of polyvinylpyrrolidone in the maceration tube) were grounded to a fine powder with a TissueLyzer (30 cycles, 30 s each). The macerated tissue sample was homogenized in 50 mM potassium phosphate buffer (pH 7.8), containing 0.1 mM EDTA, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). Homogenized samples were centrifuged at 14000 g for 20 min at 4 °C. After centrifugation, the supernatant was collected, stored at low temperatures (4 °C), and used as an enzymatic extract for further spectrophotometer measurements to determine the antioxidative enzymatic activity and protein quantification according to manufacturer instructions.

2.3.1. Estimation of catalase activity

Catalase enzymatic activity was estimated following Mohapatra and collaborators (2016). Enzymatic assays were prepared in triplicates in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 10 mM hydrogen peroxide (H_2O_2), and 50 μl enzymatic extract, which was added last. The reaction mixture was poured into a 1 ml quartz cuvette and mixed before absorbance measurements. A blank sample was also prepared, replacing the enzymatic extract with potassium phosphate buffer (pH 7.8). Changes in absorbance were measured at 240 nm at 15-s (s) intervals for 135 s.

2.3.2. Estimation of ascorbate peroxidase activity

Ascorbate peroxidase activity was estimated following the method of Elavarthi and collaborators [31]. Enzymatic activity assays were prepared in triplicates in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 0.25 mM ascorbate, 50 μl enzymatic extract, and 0.5 mM H_2O_2, which was added last. The reaction mixture was poured into a 1 ml quartz cuvette. A blank sample was prepared replacing the sodium ascorbate and H_2O_2 with potassium phosphate buffer (pH 7). Moreover, correction for H_2O_2-induced ascorbate oxidation in the absence of enzymatic extract was assessed by replacing the enzymatic extract with potassium phosphate buffer (pH 7). Changes in absorbance were measured at 290 nm at 15 s intervals for 135 s.

2.3.3. Estimation of superoxide dismutase activity

Superoxide dismutase enzymatic activity was estimated following

Mohapatra and collaborators (2016). For estimation of enzymatic activity, a 940 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.025 % (v/v) Triton X-100, 13 mM L-methionine, 55 μM nitroblue tetrazolium chloride (NBT) and 50 μl enzymatic extract were poured into a plastic cuvette. Test samples containing enzymatic extract were prepared in triplicate for spectrophotometer measurements. The enzymatic reaction was initiated by adding 10 μl of 0.1 mM riboflavin and placing the samples under an 8 W LED light source for 10 min, on a standard electrical shaker. Two blank samples were taken as control by replacing the enzymatic extract with potassium phosphate buffer (pH 7.8) and exposing them to the same light source. Similarly, two cuvette samples were taken as blank control and wrapped in aluminum foil (non-irradiated) to prevent riboflavin reduction by light exposure. Absorbance was measured in the test samples against the dark blank samples at 560 nm. One enzymatic unit (EU) of SOD activity was defined as the amount of enzyme that caused 50 % inhibition of NBT reduction per mg of protein.

2.4. Defense-related metabolites quantification

Grass pea accessions with contrasting phenotypic responses to *E. pisi, E. trifolii,* and *U. pisi* were infected with the different pathogens, leaves were collected on non-inoculated plants and on inoculated ones at different time-points and stored at −80 °C. Leaves were collected from *E. pisi*-infected accessions at 6, 12, 24, 48, and 72 HAI, from *E. trifolii* infected accessions at 6, 12, 24, and 48 HAI, and from *U. pisi*-infected accessions at 12, 24, and 48 HAI. In all cases, leaves were also collected from non-inoculated control plants. For the quantification of metabolism markers, plant material was ground in a liquid nitrogen-cooled mortar, weighed (50–70 mg fresh weight), homogenized in 80 % (v/v) ethanol, and incubated for 10 min in an ultrasonic bath. Malondialdehyde (MDA), total phenolic compounds (TPC), total flavonoids (TFL), and total soluble sugars (TSS) were quantified following the colorimetric measurements described in López-Hidalgo and collaborators [32]. Three biological replicates, per time-point, and pathogen were quantified.

2.5. Statistical analysis

Analysis of variance (ANOVA) of the histological parameters, ROS-scavenging enzymes activity, and defense-related metabolites contents was performed using Genstat software 21st edition (VSN, International, 2021). Residuals were inspected to evaluate distribution normality (quantile-quantile plot, QQ), outliers, and homogeneity of variance (residuals vs. fitted values). When needed, histological and enzymatic data were subjected to angular ($y = \arcsine\sqrt{x/100}$) or BOX-COX (ABOXCOX Genstat procedure) [33] transformation, respectively. Differences in histology, enzyme activity, and metabolite contents were analyzed separately for each accession and time point. Multiple comparisons of means were performed using the Tukey test (p-value ≤ 0.05).

3. Results

3.1. Histological characterization of grass pea - powdery mildew and rust pathogens interaction

3.1.1. Erysiphe trifolii -infected grass pea leaves

Histological observations from *E. trifolii*-infected grass pea leaves revealed no clear distinction between partially resistant and susceptible accessions in the early pre-penetration stages of pathogen development (rate of spore germination and germinated spores that form an appressorium) (Table 1, Table 2).

Nevertheless, at 48 HAI, both PR accessions, PI195603 and PI358601, showed a significantly lower portion of sporelings that developed an appressorium and went on to develop secondary hyphae and colonies (Table 2). Similarly, by 72 HAI, colonies formed on the mentioned PR accessions developed less branched colonies (on average with only 2 hyphal branches per colony) than the susceptible accessions (over 6 hyphal branches per colony). No microscopic cell necrosis was observed in any of the studied grass pea accessions in response to *E. trifolii* infection (Fig. 1).

Table 1
Mean values for developmental stages of *Erysiphe trifolii* on infected leaves of selected grass pea accessions at 6 h after inoculation (HAI) and 12 HAI[a].

Accessions	Germination rate (%)	Germinated sporelings forming appressorium (%)
	6 HAI	
PI195603 (DS = 5 %)	57.3 ± 0.7[a]	58.8 ± 5.1[a]
PI358601 (DS = 5 %)	55.6 ± 1.2[a]	53.4 ± 5.3[a]
PI667247 (DS = 55 %)	53.6 ± 3.6[a]	46.0 ± 3.3[a]
PI391431 (DS = 70 %)	54.0 ± 2.0[a]	42.2 ± 3.0[a]
	12 HAI	
PI195603 (DS = 5 %)	69.8 ± 5.3[a]	87.4 ± 1.5[a]
PI358601 (DS = 5 %)	68.9 ± 1.2[a]	91.6 ± 3.2[a]
PI667247 (DS = 55 %)	70.4 ± 4.0[a]	78.2 ± 2.3[a]
PI391431 (DS = 70 %)	72.7 ± 6.0[a]	89.5 ± 3.5[a]

[a] Values with letters in common, in each column, per time-point were not significantly different ($P \leq 0.05$, Tukey test). DS: Disease Severity (% leave area covered by mycelia). The data shown is the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. DS values were retrieved from Martins and collaborators (2023).

3.1.2. Erysiphe pisi-infected grass pea leaves

Table 3 shows that for the majority of grass pea accessions, most *E. pisi* conidia germinated within the first 6 h of infection. The exception to this was observed for the susceptible accession, PI426892, with a significantly reduced germination rate compared with the remaining accessions. However, by 12 HAI, no differences in sporeling germination rate were detected anymore among accessions.

Although the vast majority of the germinated spores went on to develop an appressorium, significant differences were observed among accessions. At 24 HAI, the percentage of sporelings successfully forming an appressorium was lower in the partially resistant accession (BGE23931) (Table 4). At the same time of infection, the susceptible accession PI426892 had the highest proportion of germinated sporelings establishing a colony. However, too few sporelings among the different accessions were observed to form colonies, at 24 HAI, to give reliable data. At later stages of infection (72 HAI), we observed a significant reduction in the number of hyphal branches per colony in the partially resistant accession (BGE23931), compared to susceptible accessions PI426892 and PI426882.

3.1.3. Uromyces pisi-infected grass pea leaves

Within the first stages of *U. pisi* infection (12 and 24 HAI), spore germination and germ tube orientation towards stoma were not significantly different among partially resistant and susceptible grass pea accessions (Fig. 2, Table 5).

At later stages of the infection process (48 HAI), differences in the success of the infection unit's development, following stomata penetration, became more evident among resistant and susceptible grass pea accessions (Table 6). Rust colonies on leaves of the three partially resistant accessions showed significantly lower intercellular growth, compared to the susceptible accessions. Also, the number of haustoria per colony in the partially resistant PI283566 and PI577138_A was significantly lower than in the susceptible PI221467_B.

Table 2
Mean values for developmental stages of *Erysiphe trifolii* on infected leaves of selected grass pea accessions at 24 h after inoculation (HAI), 48 HAI, and 72 HAI[a].

Accession	Germinated sporelings forming appressorium (%)	Proportion of germinated sporelings establishing a colony (%)	Number of hyphal branches
		24 HAI	
PI195603 (DS = 5 %)	83.0 ± 0.1[a]	6.5 ± 2.6[a]	1.0 ± 0.0[a]
PI358601 (DS = 5 %)	83.0 ± 1.3[a]	3.4 ± 0.9[a]	1.0 ± 0.0[a]
PI667247 (DS = 55 %)	92.4 ± 2.4[a]	6.9 ± 1.1[a]	1.7 ± 0.2[b]
PI391431 (DS = 70 %)	86.7 ± 5.3[a]	4.2 ± 2.0[a]	1.3 ± 0.3[ab]
		48 HAI	
PI195603 (DS = 5 %)	99.7 ± 0.3[a]	12.1 ± 1.4[a]	1.3 ± 0.2[a]
PI358601 (DS = 5 %)	99.4 ± 0.6[a]	9.2 ± 2.5[a]	1.7 ± 0.1[ab]
PI667247 (DS = 55 %)	98.4 ± 1.1[a]	33.9 ± 1.2[b]	1.2 ± 0.0[a]
PI391431 (DS = 70 %)	100.0 ± 0.0[a]	37.9 ± 7.0[b]	3.1 ± 0.4[b]
		72 HAI	
PI195603 (DS = 5 %)	100.0 ± 0.0[a]	9.9 ± 1.7[a]	2.0 ± 0.5[a]
PI358601 (DS = 5 %)	97.2 ± 2.9[a]	8.0 ± 3.3[a]	2.5 ± 0.7[a]
PI667247 (DS = 55 %)	99.3 ± 0.4[a]	33.4 ± 10.1[ab]	9.4 ± 2.5[b]
PI391431 (DS = 70 %)	98.4 ± 1.6[a]	62.5 ± 9.1[b]	6.5 ± 0.9[ab]

[a] Values with letters in common, in each column, per time-point were not significantly different ($P \leq 0.05$, Tukey test). DS: Disease Severity (% leave area covered by mycelia). The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. DS values were retrieved from Martins and collaborators (2023).

3.2. Kinetics of ROS-scavenging enzyme activity in grass pea accessions upon infection with powdery mildew and rust pathogens

ROS-scavenging enzyme activity in several grass pea accessions in response to *E. trifolii* or *E. pisi* (non-inoculated, 6, 12, and 24 HAI) and *U. pisi* (non-inoculated, 12, 24, 48 HAI) are shown in Fig. 3, S1, 4, S3, 5 and S4. ANOVA (Table S1) indicated significant ($P \leq 0.05$) interaction among accessions and the enzyme's activity (CAT, APX, and SOD) assessed during the *E. trifolii* and *U. pisi* infection. As for *E. pisi* infection, significant interaction among accessions was observed for CAT enzyme activity.

3.2.1. Erysiphe trifolii-infected grass pea leaves

Upon infection with *E. trifolii*, the partially resistant accession PI195603 had a significantly higher APX-scavenging activity from 6 HAI onwards, as compared with the more susceptible grass pea accession. As for CAT activity, no clear difference was detected among partially resistant and susceptible grass pea accessions infected with *E. trifolii* (Fig. 3).

A significantly higher SOD activity was observed on non-inoculated control leaves of the partially resistant accessions compared to non-inoculated leaves of the susceptible PI391431 accession (Fig. 3). Although such differences dissipated after inoculation at 6 HAI, from 12 HAI onwards, the partially resistant grass pea accessions (PI195603 and PI358601) showed again significantly higher SOD activity than the remaining susceptible accessions.

A high correlation between SOD and CAT activity (0.84) was detected among the grass pea accessions infected with *E. trifolii*. A moderate correlation was recorded among the remaining enzymes studied (0.63 for APX and SOD activities correlation and 0.57 for APX and CAT) (Fig. S2).

3.2.2. Erysiphe pisi-infected grass pea leaves

In response to *E. pisi* infection, although at control non-inoculated conditions the most susceptible accession had a higher APX activity compared with the partially resistant accessions PI221467_A and BGE23931, this situation was reversed as observed at 24 HAI. Both partially resistant accessions (PI221467_A and BGE23931) recorded a significantly higher (2-fold) ROS-scavenging activity of APX in relation to the susceptible accessions at 24 HAI (Fig. 4).

Regarding SOD and CAT, from 12 HAI onwards, the enzymatic activity of partially resistant accession PI221467_A was significantly higher than the one from the most susceptible grass pea accession PI426882 (Fig. 4). For the mentioned partially resistant accession (PI221467_A), a continuous increase in SOD and CAT activity was observed throughout the infection process (Fig. S3).

Correlation coefficient analysis revealed a moderate and positive correlation among the studied enzyme's activity during *E. pisi* infection. The highest correlation was recorded among APX and CAT (0.68), and CAT and SOD (0.67) (Fig. S2).

3.2.3. Uromyces pisi-infected grass pea leaves

In the *U. pisi*-infected grass pea accessions, a significant reduction in APX activity was observed at 24 HAI for the grass pea accessions, except for the susceptible PI221467_B (Fig. S4). By 48 HAI, the ROS-scavenging activity of APX in the most susceptible accessions (PI221467_B) remained significantly lower (1.5-fold) compared with the remaining grass pea accessions (Fig. 5).

Regarding CAT, susceptible accessions had the highest enzymatic activity as compared with the more resistant accessions in non-inoculated conditions and at 12 HAI, respectively. At later stages of infection, such differences among accessions were dissipated (Fig. 5).

No clear patterns among grass pea accessions were observed when comparing SOD activity throughout the time points assessed (Fig. 5). Moreover, a moderate correlation coefficient was observed among enzymes' activity, the highest detected between CAT and APX activity (0.66) (Fig. S2).

3.3. Quantification of malondialdehyde, total phenolic compounds, flavonoids, and soluble sugars in grass pea accessions upon infection with powdery mildew and rust pathogens

Significant differences detected on the accumulation of malondialdehyde (MDA), total soluble sugars (TSS), and secondary metabolites (total phenolic compounds, TPC, and flavonoids, TFL) among contrasting grass pea accessions were particularly evident in response to *E. trifolii*. Accumulation of the analyzed compounds in response to *E. trifolii* (non-inoculated control, inoculated at 12, 24, 48 HAI), *E. pisi* (non-inoculated control, inoculated at 6, 12, 24, 48 and 72 HAI) and *U. pisi* (non-inoculated control, inoculated at 12, 24 and 48 HAI) are shown in Figs. 6–8, respectively.

3.3.1. Erysiphe trifolii-infected grass pea leaves

A similar pattern of accumulation of the majority of the studied metabolites (except for TSS), was observed in the partially resistant accession PI358601, during the infection with *E. trifolii*, with an initial decrease by 6 HAI compared to the non-inoculated control, followed by a recovery of the initial content (by 24 HAI). Regarding TSS, differences among resistance contrasting grass pea accessions were only observed at

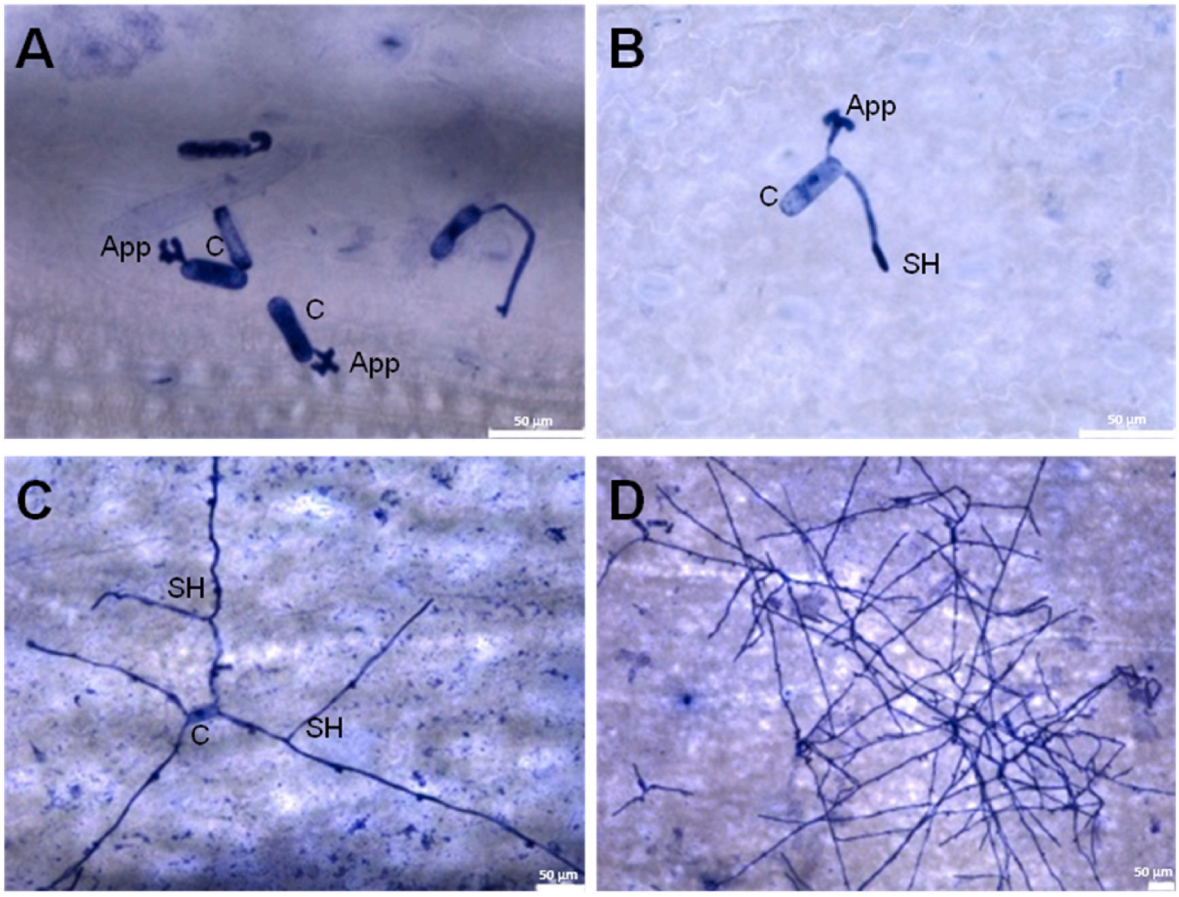

Fig. 1. Light micrographs showing examples of *Erysiphe pisi* (A, C) and *Erysiphe trifolii* (B, D) development on infected leaves of *Lathyrus sativus* accessions. A) Germinated conidia with an appressorium at the tip of the appressorial germ tube at 12 h after inoculation (HAI). B) Emergence of small secondary hyphae from the conidia (12 HAI). C) Germling that penetrated an epidermal cell and development of long secondary hyphae indicative of a functional haustorium (24 HAI). D) Successfully established colony (72 HAI). C, conidium; App, appressorium; SH, secondary hyphae.

Table 3
Mean values for developmental stages of *Erysiphe pisi* on infected leaves of selected grass pea accessions at 6 h after inoculation (HAI) and 12 HAI[a].

Accessions	Germination rate (%)	Germinated sporeling forming appressorium (%)
	6 HAI	
PI221467_A (DS = 5 %)	78.7 ± 3.7[b]	70.2 ± 1.4[b]
BGE23931 (DS = 5 %)	61.6 ± 3.2[b]	56.8 ± 2.2[a]
PI426892 (DS = 15 %)	37.6 ± 3.8[a]	72.7 ± 2.5[b]
PI426882 (DS = 20 %)	73.3 ± 4.7[b]	75.7 ± 2.0[b]
	12 HAI	
PI221467_A (DS = 5 %)	78.7 ± 4.2[a]	91.2 ± 4.2[b]
BGE23931 (DS = 5 %)	66.9 ± 1.5[a]	76 ± 2.8[a]
PI426892 (DS = 15 %)	78.2 ± 4.3[a]	87.3 ± 2.2[ab]
PI426882 (DS = 20 %)	79.1 ± 2.5[a]	87.8 ± 2.6[ab]

[a] Values with letters in common, in each column, per time-point were not significantly different ($P \leq 0.05$, Tukey test). The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Disease severity (DS) values were retrieved from Martins and collaborators (2023).

non-inoculated conditions, with the partially resistant PI358601 depicting significantly higher TSS contents than the susceptible accession PI391431, which dissipated throughout the infection process (Fig. 6).

As for MDA in *E. trifolii* infected leaves, contents were significantly higher in the partially resistant PI358601 at 12 HAI, followed by a decrease and a recovery of the original content at 48 HAI, again significantly higher, when compared with the susceptible accession PI391431 (Fig. 6). Similarly, the accumulation of total phenolic compounds in the partially resistant PI358601 was significantly higher in non-inoculated conditions (as well as in the partially resistant PI95603), when compared with the susceptible accessions (PI391431 and PI667247). Such differences were maintained for the partially resistant PI358601 at early (6 HAI) and later (24 and 48 HAI) stages of infection. Similarly, the partially resistant accession, PI358601, was found to accumulate a higher total flavonoid content at early (6 HAI) and later (48 HAI) stages of infection, when compared with the susceptible PI391431.

3.3.2. *Erysiphe pisi*-infected grass pea leaves

In *E. pisi*-infected leaves, the total soluble sugar content was significantly higher, at 24 HAI, in the partially resistant accession BGE23931, when compared with the susceptible accessions (PI426892 and PI426882) (Fig. 7A). No consistent differences were detected for the MDA content among resistance-contrasting accessions (Fig. 7B). Regarding the remaining metabolites assessed (TPC and TFL), there were no significant differences among resistance contrasting accessions upon infection with *E. pisi* (Fig. 7C and D).

Table 4
Mean values for developmental stages of *Erysiphe pisi* on infected leaves of selected grass pea accessions at later stages of infection, 24 h after inoculation (HAI), 48 HAI, and 72 HAI[a].

Accession	Germinated sporeling forming appressorium (%)	Proportion of germinated sporelings establishing a colony (%)	Number of hyphal branches/ colony
		24 HAI	
PI221467_A (DS = 5 %)	98.1 ± 0.3[b]	1.7 ± 1.7[a]	1.2 ± 0.1[a]
BGE23931 **(DS = 5 %)**	90.5 ± 2.0[a]	1.2 ± 1.2[a]	0.3 ± 0.3[a]
PI426892 (DS = 15 %)	96.2 ± 0.4[b]	11.0 ± 2.8[b]	1.2 ± 0.1[a]
PI426882 (DS = 20 %)	97.3 ± 1.6[b]	0.6 ± 0.5[a]	0.5 ± 0.5[a]
		48 HAI	
PI221467_A (DS = 5 %)	97.0 ± 1.1[a]	35.9 ± 2.3[a]	4.5 ± 0.6[b]
BGE23931 **(DS = 5 %)**	99.5 ± 0.5[a]	11.9 ± 2.0[a]	2.0 ± 0.3[a]
PI426892 (DS = 15 %)	85.1 ± 8.8[a]	36.9 ± 9.4[a]	4.2 ± 0.2[ab]
PI426882 (DS = 20 %)	91.9 ± 1.8[a]	13.4 ± 9.6[a]	3.4 ± 0.7[ab]
		72 HAI	
PI221467_A (DS = 5 %)	94.0 ± 1.2[ab]	15.9 ± 1.1[ab]	4.3 ± 1.1[ab]
BGE23931 **(DS = 5 %)**	98.8 ± 19.6[b]	28.1 ± 0.1[ab]	1.6 ± 0.1[a]
PI426892 (DS = 15 %)	95.4 ± 1.9[ab]	29.5 ± 12.2[b]	12.8 ± 1.9[b]
PI426882 (DS = 20 %)	87.9 ± 4.2[a]	5.3 ± 1.2[a]	8.7 ± 3.7[b]

[a] Values with letters in common, in each column, per time-point were not significantly different ($P \leq 0.05$, Tukey test). The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Disease severity (DS) values were retrieved from Martins and collaborators (2023). None of the studied grass pea accessions exhibited cell necrosis in host cells associated with the pathogen's colonies, indicating a fully biotrophic relationship with *E. pisi* (Fig. 1).

3.3.3. *Uromyces pisi-infected grass pea leaves*

In *U. pisi*-infected leaves, no consistent differences were observed between resistance contrasting accessions, in the contents of the studied metabolites (Fig. 8).

4. Discussion

Airborne biotrophic fungal diseases, like powdery mildew and rust, that affect grass pea production, have high evolutionary potential and are more likely to overcome resistance controlled by single resistance genes (R genes) that provide near-complete host resistance. Grass pea sources of potentially more durable partial resistance have been identified, characterized by incomplete resistance not associated with a hypersensitive response. However, the underlying antioxidant and histological defense mechanisms of this resistance remain poorly understood, hindering the development of more sustainable, multi-resistant varieties.

The time-course comparison of PR and susceptible grass pea accessions to *E. pisi, E. trifolii,* and *U. pisi*, revealed similarities in antioxidant machinery regulation, with SOD and APX activity triggered at different rates depending on the pathogens-grass pea interaction. Indeed, APX activity increased early (6HAI) in response to *E. trifolii* infection, whereas this activation occurred later for the remaining pathogens. A common SOD activity increase was observed at 12 HAI in PR accessions for both powdery mildews. In the *E. trifolii*-grass pea pathosystem, additional defense responses included increased accumulation of flavonoids and phenolic compounds in PR accessions. Histological observations indicated that defense mechanisms activated at later infection stages were more effective in restricting powdery mildew and rust development in host leaf tissue.

The timing of increased APX activity in PR accessions varied by pathosystems suggesting this enzyme's role in disease response. In the PR accession (PI195603) infected with *E. trifolii*, APX activity was significantly higher from 6 HAI onwards compared to susceptible accessions. In contrast, APX activity increased later in PR accessions infected with *E. pisi* (24 HAI) and with *U. pisi* (48 HAI). Indeed, for the *U. pisi* pathosystem interaction, a sharp increase in APX activity was observed at 48 HAI across all accessions, except the susceptible PI221467_B, highlighting its potential role in resistance. Similar results were obtained by Ribeiro et al [34], with increasing enzyme activity of APX playing a key role in common bean resistance against *Colletotrichum lindemuthianum*.

In the susceptible accession PI221467_B, significantly larger fungal colonies (measured by haustoria number and intercellular growth) were associated with lower APX activity compared to the remaining accessions. SOD activity increased in response to powdery mildew infection from 12 HAI onwards. Previous studies showed that SOD activity was significantly induced in *E. pisi*-infected resistant pea genotypes carrying the well-characterized resistance genes *er1* and *er2* [22,23]. SOD facilitates the conversion of 0_2^- anions to H_2O_2, which is more stable, less reactive with organic molecules, and capable of diffusing across cell membranes to act at distant sites from its production [35].

In the partially resistant accession PI221467_A, catalase activity was significantly higher in the *E. pisi*-inoculated leaves at 12 and 24 HAI compared to the susceptible accession. Similar trends have been reported in resistant pea genotypes infected with *E. pisi* highlighting the role of H_2O_2-scavenging enzymes in mitigating ROS toxicity [23]. In a different pathosystem, *Vigna radiata-Cercospora canescens*, CAT-induced activity was also observed as the earliest and most illustrative trait distinguishing the susceptible and resistance interactions [36].

The data here presented for powdery mildew pathogens suggests that SOD activity increased significantly in partially resistant accessions from 12 HAI onwards. Additionally, catalase activity was induced in response to *E. pisi* in partially resistant accessions while APX activity was enhanced in response to both powdery mildews, contributing to ROS regulation through H_2O_2 degradation.

Regulating ROS-scavenging enzyme activity during pathogen infection is essential to balance ROS accumulation and support ROS-mediated defense reactions [24]. Uncontrolled ROS accumulation can cause lipid peroxidation and damage to proteins, nucleic acids, and other vital cellular components [24]. Malondialdehyde (MDA) content is commonly used as a marker of oxidative stress resulting from lipid peroxidation in pathogen-infected plant tissues [37]. In *E. trifolii*-infected plants, the inability to induce CAT activity to control H_2O_2 accumulation may have led to significantly increased MDA content in leaf tissue, particularly in the PR accession PI358061, at 12 and 48 HAI.

Increased accumulation of flavonoids (at 48 HAI) and phenolic compounds (at 6, 24, and 48 HAI) in the partially resistant accession PI358601 suggests that defense mechanisms against *E. trifolii* may involve the phenylpropanoid pathway [38]. In *E. trifolii* infected partially resistant accessions, these mechanisms likely restricted pathogen development in leaf tissues at later stages of infection (48 HAI onwards), as confirmed by histological observations. Previous studies have highlighted the multifaceted action of flavonoids and phenolic compounds in mediating defense mechanisms against plant pathogens. These compounds contribute to plant defense by serving as precursors for protective metabolites and reinforcing physical barriers against pathogen invasion [35,38]. The antifungal activity of flavonoids is involved in inhibition of spore development and hyphal growth, pathogenic enzyme inhibition [39]. Additionally, both metabolites function as ROS scavengers, providing an additional layer of protection against

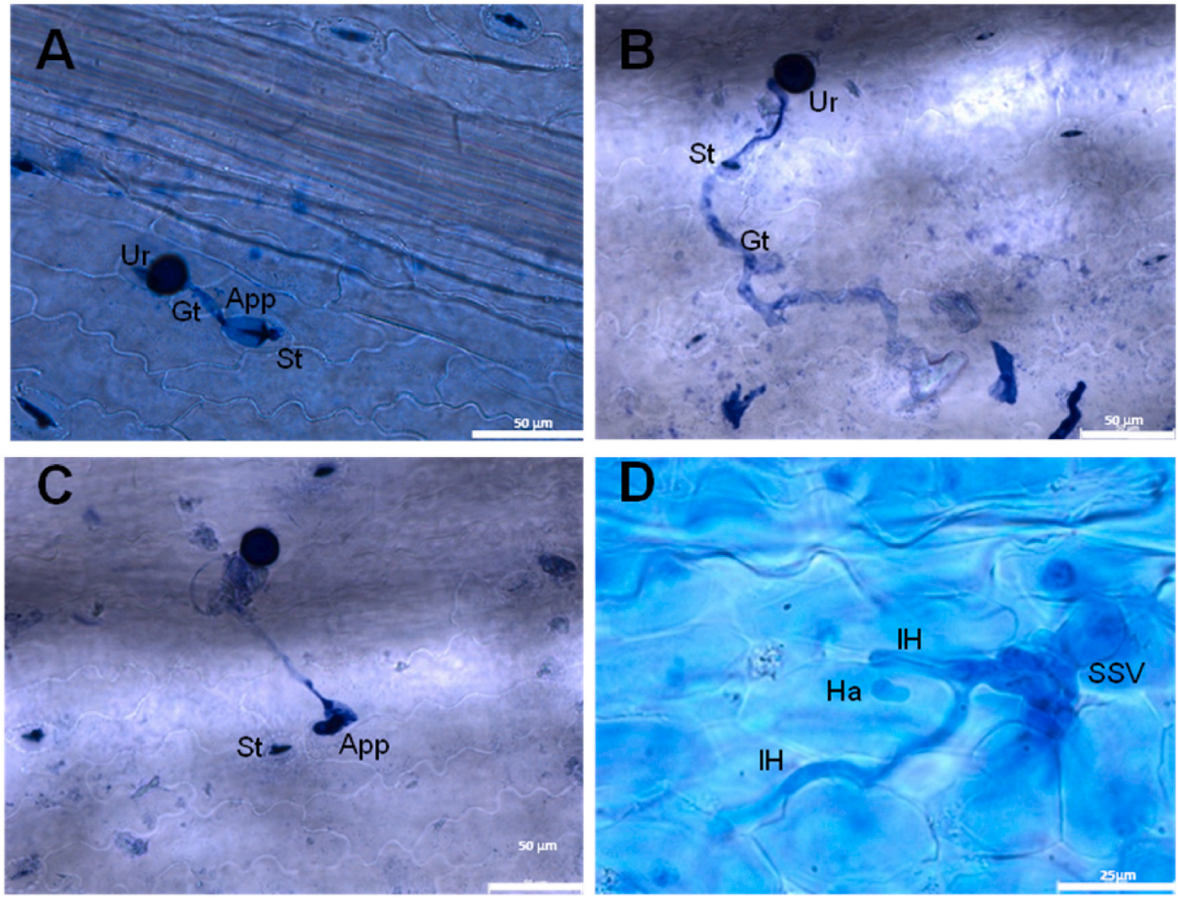

Fig. 2. Interaction between *Uromyces pisi* and leaves of *Lathyrus sativus* accessions. A) Germinated urediospore forming an appressorium over a stoma at 12 h after inoculation (HAI). B) Germinated urediospore overgrowing a stoma without forming an appressorium (24 HAI). C) Germinated urediospore developing a misplaced appressorium (24 HAI). D) Stoma penetrated by *U. pisi* forming a substomatal vesicle in the substomatal cavity and emergence of intercellular hyphae (48 HAI). App, appressorium; Gt, germ tube; Ha, haustorium; IH intercellular hyphae, SSV, substomatal vesicle; St, stoma; Ur, urediospore.

Table 5
Mean values for developmental stages of *Uromyces pisi* on infected leaves of selected grass pea accessions at 12 h after inoculation (HAI) and 24 HAI[a].

Accessions	Germination rate (%)	Germ tubes reaching stoma (%)	Germ tubes reaching stoma and forming an appressorium (%)	Germ tubes not reaching a stoma (%)	Germ tubes forming a misplaced appressorium (away from the stoma) (%)	Germ tubes overgrowing at least one stoma without forming an appressorium (%)
			12 HAI			
PI283566 (DS = 10 %)	71.7 ± 6.2^{ab}	33.5 ± 10.4^{a}	87.2 ± 4.1^{a}	62.1 ± 8.8^{a}	43.6 ± 10.7^{a}	0.8 ± 0.8^{a}
PI165528 (DS = 20 %)	85 ± 2.0^{ab}	56.0 ± 6.9^{a}	89.4 ± 3.2^{a}	47.6 ± 3.1^{a}	49.8 ± 9.2^{a}	1.6 ± 0.8^{a}
PI577138_A (DS = 22 %)	62.3 ± 3.3^{a}	35.1 ± 5.3^{a}	86.9 ± 1.1^{a}	59.2 ± 3.5^{a}	44.1 ± 2.0^{a}	3.2 ± 0.7^{a}
PI283574 (DS = 30 %)	68.5 ± 13.0^{ab}	23.4 ± 11.4^{a}	91.3 ± 8.7^{a}	69.8 ± 10.8^{a}	35.4 ± 10.4^{a}	1.04 ± 1.0^{a}
PI221467_B (DS = 40 %)	88.5 ± 2.4^{b}	42.9 ± 10.1^{a}	89.9 ± 1.8^{a}	55.1 ± 5.8^{a}	43.8 ± 0.6^{a}	4.1 ± 0.9^{a}
			24 HAI			
PI283566 (DS = 10 %)	79.8 ± 6.0^{a}	44.3 ± 1.1^{a}	89.6 ± 5.6^{a}	55.7 ± 1.1^{abc}	48.1 ± 1.5^{bc}	8.2 ± 0.1^{b}
PI165528 (DS = 20 %)	77.0 ± 3.6^{a}	46.4 ± 8.2^{a}	93.0 ± 1.04^{a}	61.7 ± 1.9^{bc}	61.0 ± 0.8 ^{d}	11.1 ± 2.1^{bc}
PI577138_A (DS = 22 %)	82.7 ± 2.8^{a}	61.2 ± 6.4^{a}	95.8 ± 1.6^{a}	45.1 ± 2.0^{a}	42.6 ± 0.9^{b}	1.8 ± 0.5^{a}
PI283574 (DS = 30 %)	88.1 ± 5.3^{a}	53.0 ± 12.2^{a}	85.9 ± 1.6^{a}	64.8 ± 5.5^{c}	54.4 ± 3.4^{cd}	14.6 ± 2.1^{c}
PI221467_B (DS = 40 %)	75.5 ± 4.4^{a}	53.3 ± 5.2^{a}	91.8 ± 2.9^{a}	51.6 ± 2.4^{ab}	29.5 ± 2.0^{a}	8.8 ± 0.8^{b}

[a] Values with letters in common, in each column, per time-point were not significantly different ($P \leq 0.05$, Tukey test). The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Disease severity (DS) values were retrieved from Martins and collaborators (2022).

Table 6
Mean values for the number of hyphal branches and haustorium per infection unit on *Uromyces pisi*-infected leaves of selected grass pea accessions at 48 h after inoculation (HAI).

Accessions	Number of intercellular hyphae per infection unit	Number of haustoria per infection unit
PI283566 (DS = 10 %)	10.1 ± 0.2^{b}	0.5 ± 0.1^{a}
PI165528 (DS = 20 %)	6.5 ± 0.5^{a}	1.8 ± 0.4^{ab}
PI577138_A (DS = 22 %)	5.6 ± 0.1^{a}	0.8 ± 0.2^{a}
PI283574 (DS = 30 %)	11.4 ± 0.1^{c}	1.3 ± 0.1^{ab}
PI221467_B (DS = 40 %)	11.7 ± 0.3^{c}	2.3 ± 0.4^{b}

*Values with letters in common, in each column, were not significantly different ($P \leq 0.05$, Tukey test). The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Disease severity (DS) values were retrieved from Martins and collaborators (2022).

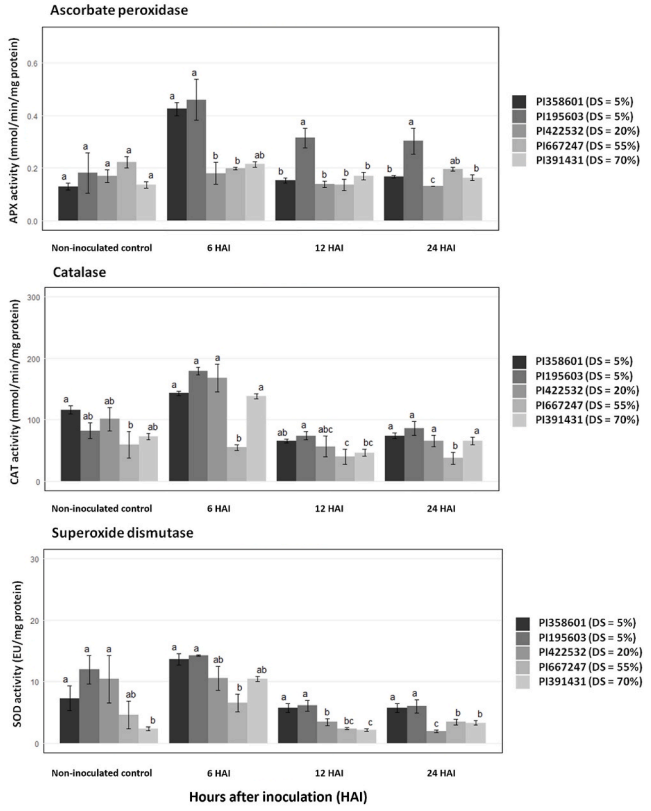

Fig. 3. Mean comparison of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) activity in *Erysiphe trifolii*-infected leaves of selected grass pea accessions in non-inoculated conditions and at different time-points of infection (6, 12, 24 h after inoculation). The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Different letters represent significant differences ($P \leq 0.05$, Tukey test) among grass pea accessions for the same time point of infection. Disease severity (DS) values were retrieved from Martins and collaborators (2023).

oxidative damage [40].

Appressoria of *E. trifolii* sporelings were less effective at penetrating the partially resistant accessions, resulting in significantly lower colony formation. From 24 to 48 HAI, colony frequency increased 1.8 and 2.7-fold in the partially resistant accessions PI195603 and PI358601,

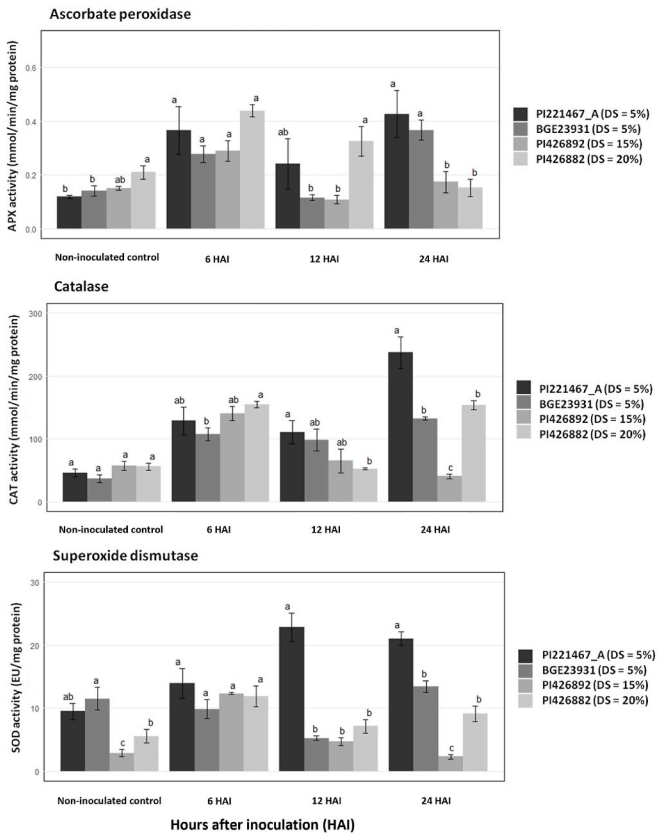

Fig. 4. Mean comparison of ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) activity in *Erysiphe pisi*-infected leaves of selected grass pea accessions in non-inoculated conditions and at different time-points of infection (6, 12 and 24 h after inoculation). The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Different letters represent significant differences ($P \leq 0.05$, Tukey test) among grass pea accessions for the same time-point of infection. Disease severity (DS) values were retrieved from Martins and collaborators (2023).

respectively, whereas susceptible accessions PI667247 and PI391431 exhibited a 4.7 and 8.8-fold increase, respectively. Additionally, partially resistant accessions effectively restricted epiphytic growth, as indicated by the lowest number of hyphal branches observed per colony in accession PI195603.

Histological analysis at later infection stages confirmed these findings, showing that partially resistant accession BGE23931 significantly limited the number of hyphal branches. While the number of hyphal branches remained stable from 48 to 72 HAI in the PR accession BGE23931, susceptible accessions PI426892 and PI426882, experienced a 3- and 2.5-fold increase, highlighting their reduced ability to control pathogen development.

In *U. pisi*-infected leaf tissue, differences between partially resistant and susceptible grass pea accessions became more pronounced after stoma penetration, particularly in intercellular growth and haustorium formation. Once an infection unit successfully established its first haustorium, partially resistant accessions appeared to activate mechanisms to limit further penetration attempts, restricting colony expansion. At 48 HAI, *U. pisi* partially resistant PI283566 and PI165528 accessions exhibited significantly smaller colonies, characterized by fewer intercellular hyphae and haustoria, compared to the most susceptible accession PI221467_B.No signs of a hypersensitive response were detected in any of the grass pea-pathogen interactions studied.

Histological analysis suggests that defense mechanisms acting at later stages of infection play a key role in partial resistance against rust

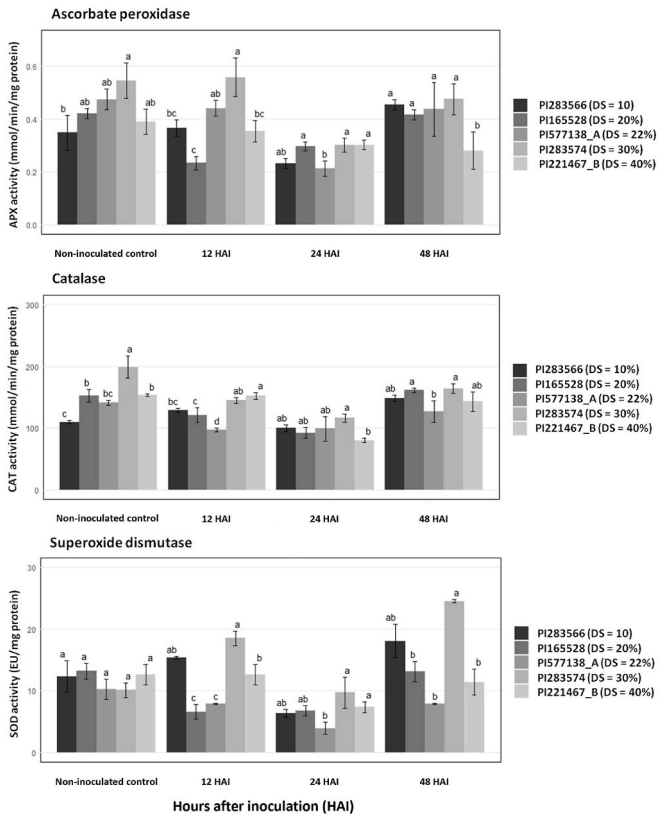

Fig. 5. Mean comparison of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) activity in *Uromyces pisi*-infected leaves of selected grass pea accessions in non-inoculated conditions and at different time-points of infection (12, 24 and 48 h after inoculation). The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Different letters represent significant differences ($P \leq 0.05$, Tukey test) among grass pea accessions for the same time point of infection. Disease severity (DS) values were retrieved from Martins and collaborators (2022).

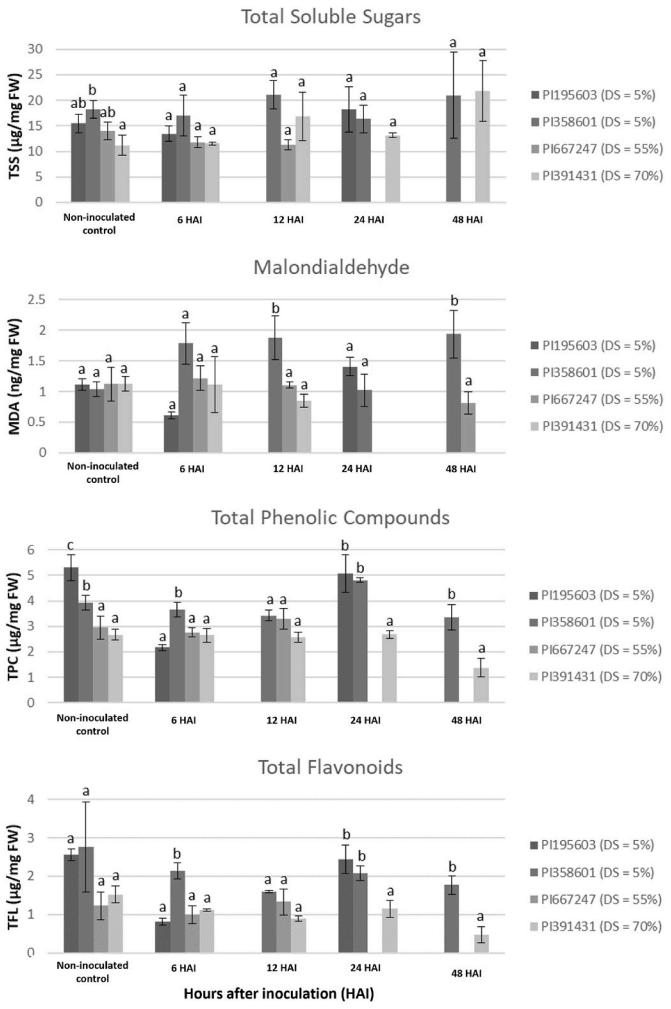

Fig. 6. Mean comparison of total soluble sugars (TSS), malondialdehyde (MDA), total phenolic compounds (TPC) and total flavonoids (TFL) in *Erysiphe trifolii*-infected leaves of selected grass pea accessions at 6, 12, 24 and 48 h after inoculation (HAI) compared to non-inoculated conditions. The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Missing data in a few accessions, for some metabolites, at the later time-points, due to insufficient amount of biological material to obtain reliable data by the quantification method. Different letters represent significant differences ($P \leq 0.05$, Duncan test) among grass pea accessions for the same time point of infection. Disease severity (DS) values were retrieved from Martins and collaborators (2023).

and powdery mildew pathogens in grass pea. These findings support the broader understanding that early infection events, such as fungal development on the leaf surface, have minimal impact on reducing disease severity, as observed in other pathosystems [14,30]. Similarly, in the closely related *L. cicera* and pea infected with *U. pisi*, differences in spore germination or directional growth between resistant and susceptible accessions are rarely reported [14,41]. Only in a few exceptional cases have variations in stomatal recognition for appressorial formation been identified for either rust or powdery mildew [42]. These differences have been attributed to factors such as host stomatal morphology [43], differential metabolites excretion on the leaf surface [44], or the presence of epicuticular waxes covering the stomatal apparatus [45].

The restricted colony development of *U. pisi, E. trifolii,* and *E. pisi* in partially resistant accessions may be attributed to a more effective limitation of host cell penetration, preventing haustorium formation and subsequently hindering pathogen progression. In other pathosystems, resistance is often mediated by cell wall appositions that act as a physical barrier to pathogen entry [18,46]. For example, In the *er1*-mediated resistance in pea against *E. pisi,* protein cross-linking rather than callose deposition, has been shown to prevent haustoria formation [17].

This study provides insights into molecular pathways involved in grass pea's partial resistance to powdery mildew and rust. The early activation of SOD and subsequent increase in APX and CAT activity in response to *E. trifolii* and *E. pisi* infections, respectively, suggests a tightly regulated antioxidant defense system that mitigates pathogen-induced cellular damage, thereby contributing to resistance. Additionally, the accumulation of phenolic compounds and flavonoids in partially resistant grass pea accessions infected with *E. trifolii* suggests the activation of secondary metabolic pathways, likely governed by genes involved in phenylpropanoid biosynthesis. The combined action of these pathogen-specific enzymatic and metabolic mechanisms underscores the need for further transcriptomic and functional studies to identify key resistance-associated genes.

The characterization of the mechanisms and compounds related to defense responses in grass pea against powdery mildew and rust will not only increase our understanding of the pathosystems but also ease efforts of improvement through resistance breeding. Biochemical markers, including increased superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), phenolic compounds, and flavonoids contents, which were in the present study associated with partial resistance (PR) in grass pea, could serve as proxies for PR in marker-assisted selection (MAS) [36,47,48]. However, field-based phenotyping trials should be conducted to validate the stability of these biochemical resistant traits across a variety of environmental conditions. Their integration into

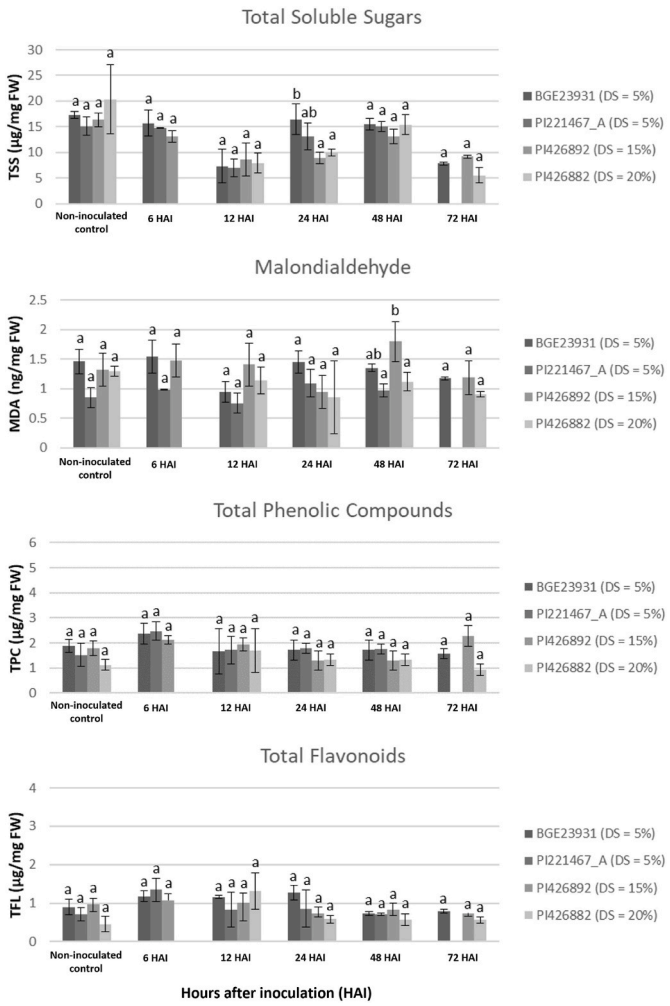

Fig. 7. Mean comparison of total soluble sugars (TSS), malondialdehyde (MDA), total phenolic compounds (TPC) and total flavonoids (TFL) in *Erysiphe pisi*-infected leaves of selected grass pea accessions at 6, 12, 24, 48 and 72 h after inoculation compared to non-inoculated conditions. The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Missing data in a few accessions, for some metabolites, at some time points, due to insufficient amount of biological material to obtain reliable data by the quantification method. Different letters represent significant differences ($P \leq 0.05$, Duncan test) among grass pea accessions for the same time point of infection. Disease severity (DS) values were retrieved from Martins and collaborators (2023).

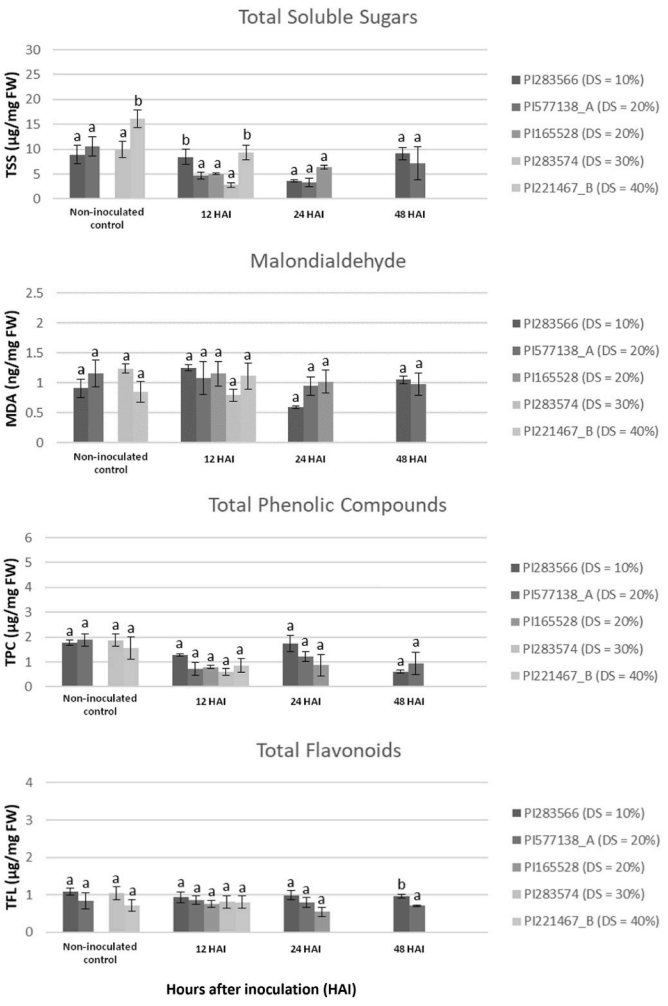

Fig. 8. Mean comparison of total soluble sugars (TSS), malondialdehyde (MDA), total phenolic compounds (TPC) and total flavonoids (TFL) in *Uromyces pisi*-infected leaves of selected grass pea accessions at 12, 24 and 48 h after inoculation, compared to non-inoculated conditions. The data shown are the original untransformed values for a clearer biological meaning, but statistical tests were performed on transformed data when needed. Missing data in a few accessions, for some metabolites, at some time points, due to insufficient amount of biological material to obtain reliable data by the quantification method. Different letters represent significant differences ($P \leq 0.05$, Duncan test) among grass pea accessions for the same time point of infection. Disease severity (DS) values were retrieved from Martins and collaborators (2022).

early screening for resistant genotypes would be feasible if rapid and cost-effective quantification methods, such as spectrophotometric or chromatography-based techniques, are developed for large-scale population screening. Alternatively, their utility in MAS would be enhanced once the respective associated molecular markers or candidate genes are identified in grass pea.

Overall, the results here presented suggest that resistance in grass pea to powdery mildew and rust pathogens could be the result of multiple factors taking place at different steps of pathogen infection, including delayed pathogen development, regulation of antioxidative enzyme activities, and controlled accumulation of defense-related metabolites. The complementary histological and biochemical approaches contributed to a better understanding of the complexity of grass pea's defense mechanisms, still largely unknown to date. Moving forward, the integration of these traits and the pyramiding of the different mechanisms here observed in new grass pea varieties could constitute an efficient strategy to achieve broad-spectrum and durable resistance.

5. Conclusion

This study provides new insights into the defense mechanisms underlying partial resistance in grass pea against biotrophic pathogens. The results indicate that partial resistance is linked to a coordinated regulation of antioxidative enzyme activities (CAT, SOD, and APX) and accumulation of phenolic compounds and flavonoids. These biochemical responses were pathogen-specific, with timing variations suggesting distinct roles in modulating reactive oxygen species (ROS) homeostasis and defense signaling. The observed controlled modulation of antioxidative machinery suggests the potential for using elicitors or biostimulants in field conditions as chemical priming agents of plant immunity to activate the same biochemical mechanisms. The integration of these treatments into agronomic practices should be further explored as a strategy to potentially enhance plant resistance to pathogens and reduce the need for fungicides. Histological analyses further confirmed that partially resistant accessions effectively restricted pathogen development at later stages of infection. Identifying biochemical

markers associated with partial resistance highlights their potential for breeding programs to screen and select resistant varieties. Future transcriptomic and functional studies will be essential to uncovering the molecular basis of these responses, the candidate genes, and the respective molecular markers to assist MAS. Integrating a multilayered resistance mechanism into breeding efforts may offer a sustainable approach to achieving broad-spectrum and durable resistance in grass peas.

CRediT authorship contribution statement

Davide Coelho Martins: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Francisco A. Mendes:** Writing – review & editing, Methodology, Investigation. **Susana de Sousa Araújo:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Diego Rubiales:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Maria Carlota Vaz Patto:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2025.102717.

Data availability

Data will be made available on request.

References

[1] G.P. Dixit, A.K. Parihar, A. Bohra, N.P. Singh, Achievements and prospects of grass pea (*Lathurys sativus* L.) improvement for sustainable food production, Crop J. 4 (2016) 407–416, https://doi.org/10.1016/j.cj.2016.06.008.
[2] F. Lambein, S. Travella, Y.H. Kuo, M. Van Montagu, M. Heijde, Grass pea (*Lathyrus sativus* L.): orphan crop, nutraceutical or just plain food? Planta 250 (2019) 821–838, https://doi.org/10.1007/s00425-018-03084-0.
[3] E.C.P. Goncalves, A. Orlando, M.A.P. Cruz Centarion, Genetics of resistance to powdery mildew (*Microsphaera diffusa*) in Brazilian soybean populations, Genet. Mol. Biol. 25 (3) (2022) 339–342.
[4] D. Martins, S.d.S. Araújo, D. Rubiales, M.C. Vaz Patto, Legume crops and biotrophic pathogen interactions: a continuous cross-Talk of a multilayered array of defense mechanisms, Plants 9 (11) (2020) 1460, https://doi.org/10.3390/plants9111460.
[5] S. Fondevilla, D. Rubiales, Powdery mildew control in pea. A review, Agron. Sustain. Dev. 32 (2012) 401–409, https://doi.org/10.1007/s13593-011-0033-1.
[6] S. Osuna-Caballero, N. Rispail, E. Barilli, D. Rubiale, Management and breeding for rust resistance in legumes, J. Plant Pathol. (2024), https://doi.org/10.1007/s42161-024-01679-z.
[7] D. Martins, D. Rubiales, M.C. Vaz Patto, Association mapping of *Lathyrus sativus* disease response to *Uromyces pisi* reveals novel loci underlying partial resistance, Front. Plant Sci. 13 (2022) 1–13, https://doi.org/10.3389/fpls.2022.842545.
[8] M.C. Vaz Patto, M. Fernández-Aparicio, A. Moral, D. Rubiales, Characterization of resistance to powdery mildew (*Erysiphe pisi*) in a germplasm collection of *Lathyrus sativus*, Plant Breed. 125 (2006) 308–310, https://doi.org/10.1111/j.1439-0523.2006.01220.x.
[9] S. Fondevilla, C. Chattopadhyay, N. Khare, D. Rubiales, *Erysiphe trifolii* is able to overcome *er1* and *Er3*, but not *er2*, resistance genes in pea, Eur J Plant Pathol 136 (2013) 557–563. https://doi.org/10.1007/s10658-013-0187-6.
[10] D.C. Martins, C. Santos, A.M. Sampaio, D. Rubiales, M.C. Vaz Patto, *Lathyrus sativus* resistance against existing and emerging powdery mildews (*Erysiphe pisi* and *Erysiphe trifolii*): a case of commonalities or total discrepancy? Phytopathology 13 (5) (2023) 866–872, https://doi.org/10.1094/PHYTO-06-22-0227-FI.
[11] M.C. Vaz Patto, D. Rubiales, Identification and characterization of partial resistance to rust in a germplasm collection of *Lathyrus sativus* L, Plant Breed. 128 (2009) 495–500, https://doi.org/10.1111/j.1439-0523.2008.01601.x.
[12] C. Santos, D. Martins, D. Rubiales, M.C. Vaz Patto, Partial resistance against *Erysiphe pisi* and *E. trifolii* under different genetic control in *Lathyrus cicera*: outcomes from a linkage mapping approach, Plant Dis. 104 (11) (2020) 2875–2884, https://doi.org/10.1094/PDIS-03-20-0513-RE.
[13] M.C. Vaz Patto, M. Fernández-Aparicio, A. Moral, D. Rubiales, Resistance reaction to powdery mildew (*Erysiphe pisi*) in a germplasm collection of *Lathyrus cicera* from Iberian origin, Genet. Resour. Crop Evol. 54 (2007) 1517–1521, https://doi.org/10.1007/s10722-006-9142-0.
[14] M.C. Vaz Patto, M. Fernández-Aparicio, A. Moral, D. Rubiales, Pre- and posthaustorial resistance to rusts in *Lathyrus cicera* L, Euphytica 165 (2009) 27–34, https://doi.org/10.1007/s10681-008-9737-y.
[15] C. Santos, D.C. Martins, M.J. González-Bernal, D. Rubiales, M.C. Vaz Patto, Integrating phenotypic and gene expression linkage mapping to dissect rust resistance in chickling pea, Front. Plant Sci. 13 (2022) 837613, https://doi.org/10.3389/fpls.2022.837613.
[16] B.A. McDonald, C. Linde, Pathogen population genetics, evolutionary potential, and durable resistance, Annu. Rev. Phytopathol. 40 (2002) 349–379, https://doi.org/10.1146/annurev.phyto.40.120501.101443.
[17] R. Iglesias-García, D. Rubiales, S. Fondevilla, Penetration resistance to *Erysiphe pisi* in pea mediated by *er1* gene is associated with protein cross-linking but not with callose apposition or hypersensitive response, Euphytica 201 (2015) 381–387, https://doi.org/10.1007/s10681-014-1221-2.
[18] C. Kushwaha, R. Chand, A.K. Singh, R. Rai, C.P. Srivastava, B.D. Singh, et al., Lignification and early abortive fungal colonies as indicators of partial resistance to rust in pea, Trop. Plant Pathol 41 (2016) 91–97, https://doi.org/10.1007/s40858-016-0071-y.
[19] H. Thordal-Christensen, Z. Zhang, Y. Wei, D.B. Collinge, Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction, Plant J. 11 (1997) 1187–1194, https://doi.org/10.1046/j.1365-313X.1997.11061187.x.
[20] R.T. Voegele, M. Hahn, K. Mendgen, The Uredinales: cytology, biochemistry, and molecular biology, in: The Mycota, Springer Berlin Heidelberg, Berlin, Heidelberg, 2009, pp. 69–98, https://doi.org/10.1007/978-3-540-87407-2_4.
[21] M.A. Torres, ROS in biotic interactions, Physiol. Plantarum 138 (2010) 414–429, https://doi.org/10.1111/j.1399-3054.2009.01326.x.
[22] C. Mohapatra, R. Chand, S. Navathe, S. Sharma, Histo-chemical and biochemical analysis reveals association of er1 mediated powdery mildew resistance and redox balance in pea, Plant Physiol. Biochem. 106 (2016) 54–63, https://doi.org/10.1016/j.plaphy.2016.04.035.
[23] S. Bhosle, N. Marathe, R. Makandar, The *er2* gene resistance against powdery mildew infection is associated with enhanced antioxidative protection and defense gene expression, Physiol. Mol. Plant Pathol. 106 (2019) 253–262, https://doi.org/10.1016/j.pmpp.2019.02.008.
[24] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (2002) 405–410, https://doi.org/10.1016/S1360-1385(02)02312-9.
[25] K. Kulbat, The role of phenolic compounds in plant resistance, Biotechnol. Food Sci. 80 (2) (2016) 97–108.
[26] P. Jeandet, M. Formela-Luboinska, M. Labudda, I. Morkunas, The role of sugars in plant responses to stress and their regulatory function during development, Int. J. Mol. Sci. 23 (9) (2022) 5161, https://doi.org/10.3390/ijms23095161.
[27] I. Morkunas, L. Ratajczak, The role of sugar signaling in plant defense responses against fungal pathogens, Acta Physiol. Plant. 36 (2014) 1607–1619, https://doi.org/10.1007/s11738-014-1559-z.
[28] P. Singla, R.D. Bhardwaj, S. Kaur, J. Kaur, Stripe rust induced defence mechanisms in the leaves of contrasting barley genotypes (*Hordeum vulgare* L.) at the seedling stage, Protoplasma 257 (2020) 169–181, https://doi.org/10.1007/s00709-019-01428-5.
[29] S. Fondevilla, T.L.W. Carver, M.T. Moreno, D. Rubiales, Macroscopic and histological characterization of genes *er1* and *er2* for powdery mildew resistance in pea, Eur. J. Plant Pathol. 115 (2006) 309–321, https://doi.org/10.1007/s10658-006-9015-6.
[30] J.C. Sillero, D. Rubiales, Histological characterization of resistance to *Uromyces viciae-fabae* in faba bean, Phytopathology 92 (2002) 294–299, https://doi.org/10.1094/PHYTO.2002.92.3.294.
[31] S. Elavarthi, B. Martin, Spectrophotometric assays for antioxidant enzymes in plants, in: R. Sunkar (Ed.), Plant Stress Tolerance. Meth. In Mol. Biol, vol. 639, Humana Press, 2010.
[32] C. López-Hidalgo, M. Meijón, L. Lamelas, L. Valledor, The rainbow protocol: a sequential method for quantifying pigments, sugars, free amino acids, phenolics,

flavonoids and MDA from a small amount of sample, Plant Cell Environ. 44 (6) (2021) 1977–1986, https://doi.org/10.1111/pce.14007.
[33] G.E.P. Box, D.R. Cox, An analysis of transformation, J. Roy. Stat. Soc. 26 (1964) 211–252.
[34] C.F. Ribeiro, M.C. de Borba, A.C. Geller, E.S. Pontes, F.R. Kulcheski, M.B. de Freitas, M.J. Stadnik, Enhanced oxidative enzymes activity and lipid peroxidation are associated with hypersensitive response and atypical lesions in resistant bean plants infected with *Colletotrichum lindemuthianum*, Physiol. Mol. Plant Pathol. 127 (2023) 102099.
[35] M. Hasanuzzaman, M.H.M.B. Bhuyan, F. Zulfiqar, A. Raza, S.M. Mohsin, J. Al Mahmud, et al., Reactive oxygen species and antioxidant defense in plants under abiotic stress: revisiting the crucial role of a universal defense regulator, Antioxidants 9 (2020) 1–52, https://doi.org/10.3390/antiox9080681.
[36] P. Choudhary, R. Chand, Biochemical dynamics during plant pathogen interaction and its potential as disease screening index in *Cercospora canescens*–mung bean interaction, Indian Phytopathol. 75 (2022) 395–403, https://doi.org/10.1007/s42360-021-00453-w.
[37] M. Morales, S. Munné-Bosch, Malondialdehyde: facts and artifacts, Plant Physiol. 180 (3) (2019) 1246–1250, https://doi.org/10.1104/pp.19.00405.
[38] Y. Tak, M. Kumar, Phenolics: a key defence secondary metabolite to counter biotic stress, Plant Phenolics Sustain. Agric. 1 (2020) 309–329.
[39] J.R. Patil, K.J. Mhatre, K. Yadav, et al., Flavonoids in plant-environment interactions and stress responses, Discov. Plants 1 (2024) 68, https://doi.org/10.1007/s44372-024-00063-6.
[40] C.H. Foyer, G. Noctor, Redox homeostasis and signaling in a higher-CO2 world, Annu. Rev. Plant Biol. 71 (2020) 157–182, https://doi.org/10.1146/annurev-arplant-050718-095955. Apr 29.
[41] S. Osuna-Caballero, N. Rispail, E. Barilli, D. Rubiales, Identification and characterization of novel sources of resistance to rust caused by *Uromyces pisi* in *Pisum* spp, Plants 11 (17) (2022) 2268.
[42] X. Ke, J. Wang, X. Xu, Y. Guo, Y. Zuo, L. Yin, Histological and molecular responses of *Vigna angularis* to *Uromyces vignae* infection, BMC Plant Biol. 22 (1) (2022) 489.
[43] D. Rubiales, M.C. Ramírez, T.L.W. Carver, R.E. Niks, Abnormal germling development by brown rust and powdery mildew on *cer* barley mutants, Hereditas 135 (2001) 271–276, https://doi.org/10.1111/j.1601-5223.2001.t01-1-00271.x.
[44] E. Prats, M.J. Llamas, J. Jorrin, D. Rubiales, Constitutive coumarin accumulation on sunflower leaf surface prevents rust germ tube growth and appressorium differentiation, Crop Sci. 47 (2007) 1119–1124, https://doi.org/10.2135/cropsci2006.07.0482.
[45] M.C. Vaz Patto, R.E. Niks, Leaf wax layer may prevent appressorium differentiation but does not influence orientation of the leaf rust fungus *Puccinia hordei* on *Hordeum chilense* leaves, Eur. J. Plant Pathol. 107 (2001) 795–803, https://doi.org/10.1023/A:1012410330287.
[46] A.S. Yadav, S.S. Vaish, A.K. Singh, R. Chand, Lignification based screening of pea (*Pisum sativum* L.) germplasm for resistance to rust (*Uromyces viciae–fabae*), J. Plant Pathol. 106 (4) (2024) 1559–1570.
[47] L. Jiang, P. Wu, L. Yang, C. Liu, P. Guo, H. Wang, S. Wang, F. Xu, Q. Zhuang, X. Tong, et al., Transcriptomics and metabolomics reveal the induction of flavonoid biosynthesis pathway in the interaction of *Stylosanthes-Colletotrichum gloeosporioides*, Genomics 113 (2021) 2702–2716.
[48] S. Kaur, M.K. Samota, M. Choudhary, M. Choudhary, A.K. Pandey, A. Sharma, J. Thakur, How do plants defend themselves against pathogens-Biochemical mechanisms and genetic interventions, Physiol. Mol. Biol. Plants 28 (2) (2022) 485–504, https://doi.org/10.1007/s12298-022-01146-y. Feb.