Abstract: Chocolate spot, which is caused by the necrotrophic fungus *Botrytis fabae*, is a major foliar disease occurring worldwide and dramatically reducing crop yields in faba bean (*Vicia faba*). Although chemical control of this disease is an option, it has serious economic and environmental drawbacks that make resistant cultivars a more sensible choice. The molecular mechanisms underlying the defense against *B. fabae* are poorly understood. In this work, we studied the leaf proteome in two faba bean genotypes that respond differently to *B. fabae* in order to expand the available knowledge on such mechanisms. For this purpose, we used two-dimensional gel electrophoresis (2DE) in combination with Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF/TOF). Univariate statistical analysis of the gels revealed 194 differential protein spots, 102 of which were identified by mass spectrometry. Most of the spots belonged to proteins in the energy and primary metabolism, degradation, redox or response to stress functional groups. The MS results were validated with assays of protease activity in gels. Overall, they suggest that the two genotypes may respond to *B. fabae* with a different PSII protein repair cycle mechanism in the chloroplast. The differences in resistance to *B. fabae* may be the result of a metabolic imbalance in the susceptible genotype and of a more efficient chloroplast detoxification system in the resistant genotype at the early stages of infection.

Keywords: *Botrytis fabae*; faba bean; resistance; proteomic analysis; photosystem II repair cycle

1. Introduction

By virtue of its high nutritional value, faba bean (*Vicia faba* L.) is an important food legume for human consumption and livestock feeding [1]. In fact, it is regarded as an excellent protein crop on the basis of its ability to provide nitrogen inputs into temperate agricultural systems, and also because of its increased yield potential and nitrogen-fixing capacity relative to other grain legumes [2,3]. Faba bean is the fourth most widely grown cool season grain legume (pulse) globally after pea (*Pisum sativum*), chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*) (FAOSTAT 2019; https://www.fao.org, accessed on 15 September 2021). However, its yield is greatly affected by some environmental conditions, including biotic and abiotic stresses [3,4].

The necrotrophic fungus *Botrytis fabae* Sard. (teleomorph: *Botryotinia fabae* Lu & Wu) causes chocolate spot, which is one of the most destructive diseases for faba bean plants worldwide [5,6]. Infected plants exhibit chocolate-colored lesions on aboveground parts and, especially, on leaves. The disease, which starts in bean crops where inoculum is present in residues from previous years or in contaminated seeds [7], may be especially aggressive under high humidity and temperature conditions. Such conditions can lead to extensive necrosis of plant tissues and severe damage, and can also favor the spread of the pathogen to other plants [8]. Prolonged favorable conditions for *B. fabae* growth can result in considerable economic crop losses through reduced grain yields and quality [9]. The
severity of chocolate spot epidemics can be mitigated with integrated disease management strategies, such as the use of clean seeds, crop rotations, lower planting densities, application of fungicides and selection of more resistant plant varieties [6,10]. Although some germplasm accessions have shown moderate to high levels of resistance [11], resistant cultivars adapted to different cultivation areas are scarcely available.

As a necrotrophic pathogen, *B. fabae* must kill and decompose host cells in order to feed on them. The fungus can infect plants via a variety of mechanisms mediated by lytic enzymes, toxins, stress-induced reactive oxygen species (ROS), necrosis-secreted proteins and a wide variety of secondary metabolites [12–16]. On the other hand, plants can stop the progression of the fungus by using constitutive or infection-induced mechanisms. Such mechanisms can be of the physical (cuticle and cell wall) [17,18] or chemical type (phytoanticipins and phytoalexins) [19], but can also involve induction of pathogenesis-related proteins or defensins, or accumulation of antimicrobial compounds [20,21].

Plants are known to accumulate ROS in response to necrotrophic fungi [22–27] and faba bean cultivars have been found to respond to *B. fabae* with differential ROS accumulation, lipid peroxidation and enzymatic ROS scavenging activity [28]. Recently, enhanced functionality in photosystem II (PSII), probably resulting from ROS accumulation in response to short-time exposure to *B. cinerea*, was reported in tomato plants [27]. However, little is known about the specific molecular mechanisms by which plants respond to *B. fabae*. A transcription factor (TF) analysis of the response of *M. truncatula* to *B. fabae* and *B. cinerea* [29] revealed some TFs to be involved in differential responses and others to be responsible for resistance to the two pathogens.

To our knowledge, few omics studies have focused on plant responses to *Botrytis*. As confirmed by using mutants at the transcriptomic level, *Arabidopsis thaliana* and tomato possess some genes whose expression is related to *B. cinerea* resistance. Such defense-related genes include some encoding PR protein 1 (PR1), β-1,3-glucanase and subtilisin-like protease, and other proteins involved in secondary metabolite synthesis (reviewed in [15]), but still others are involved in responses to abiotic stresses, such as signaling hormone pathways, which affect photosynthesis, and protein synthesis and transport [15,30,31]. Even fewer proteomics studies have addressed plant–*Botrytis* interactions [32,33] and most have focused on the pathogen *B. cinerea* (reviewed in [15]). Thus, Marra et al. used 2DE coupled to Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF) analysis [32] to examine the interaction of beans with *B. cinerea* and *Trichoderma*. They found pathogenesis-related proteins and other disease-related factors, such as potential resistance genes, to be seemingly associated with interactions with both the pathogen and *Trichoderma*. In addition, a shotgun proteomic study of *B. cinerea*-infected tomato fruit at different ripening stages identified a substantial number of proteins responsible for pathogenicity (mainly PR and disease resistance proteins, proteases and peroxidases), as well as others protecting the fruit from the oxidative stress response by the host [33].

In the absence of a reference genome assembly for *Vicia faba* owing to its enormous size (13 Gbp) and complexity (e.g., abundance of transposable elements), high-throughput methods, such as transcriptome analysis, have proved efficient for enriching genomic resources (reviewed in [1]). However, only limited DNA sequence data from reported transcriptome datasets have been made available on public databases [34]. Using high-throughput omic technology can no doubt help expand existing knowledge of the plant–pathogen interaction and provide a basis for developing improved crop breeding programs. The main aim of this work was to go deeper into the knowledge of the molecular mechanisms underlying the defense against *B. fabae* in faba bean. For this purpose, we studied the leaf proteome in two faba bean genotypes that respond differently to *B. fabae* by using two-dimensional gel electrophoresis (2DE) in combination with (MALDI-TOF/TOF) mass spectrometry (MS). Some results of the MS analysis were validated by assays of protease activity in gels. Overall, the results suggest that the key to stopping the spread of the pathogen onto leaves is mainly a regulatory ROS production mechanism occurring in the chloroplast.
2. Materials and Methods

2.1. Plant Material and Sample Collection

Two faba bean genotypes known to exhibit a contrasting response to *B. fabae* were used, namely: Baraca as susceptible genotype and BPL710 as resistant genotype [10,35]. Seeds of the two genotypes were grown in 1 L pots filled with a (1:1) sand–peat mixture under controlled conditions: (20 ± 2) °C, 12 h dark/12 h light photoperiod and a photon flux density of 150 μmol m⁻² s⁻¹.

A total 27 plants per genotype were used, with leaves being sampled from 12 inoculated plants and 12 uninoculated (control) plants of each. Three other plants were inoculated and were used to score disease symptoms. Plants were inoculated according to Villegas-Fernandez et al. [35], with *B. fabae* local monosporic isolate (Bf-CO-05) being grown on Petri dishes containing V8 medium and spores suspended at a 4.5 × 10⁵ spores/mL concentration in a glucose/water solution (1.2% w/v). Three-week-old plants were then sprayed with the suspension at a rate of 1.5 mL/plant and incubated in a growth chamber at a relative humidity above 95% in the dark. By contrast, control (uninoculated) plants were sprayed with a glucose solution containing no spores. Disease symptoms were evaluated after restoring the photoperiod 24 h later but keeping the relative humidity above 90%. Evaluations were done two and six days after inoculation (dai). Disease severity (DS) was calculated by visual estimation of the proportion of plant surface covered with chocolate spots, with estimates being corrected by increasing the weight of the aggressive lesions by 50% with the formula DS = (% nonaggressive lesions) + 1.5 × (% aggressive lesions). The results thus obtained were analyzed statistically with the software Statistix 8 (Analytical Software, Tallahassee, FL, USA). Data were subjected to the arcsin √x transformation in order to offset evaluation bias and to increase normality in their distribution prior to analysis of variance (ANOVA).

Faba leaves for proteomic analyses were collected at two different times while both control and inoculated plants were still under incubation in the dark [35]. Sampling was done 6 h post-inoculation (hpi)—an early time at which no symptoms were apparent—and then 12 hpi—when the earliest symptoms of chocolate spots became macroscopically visible. All of the leaves from six individual plants (three biological replicates from two plants each) per condition (treatment and sampling time) were collected, frozen in liquid nitrogen and stored at −80 °C until protein extraction.

2.2. Protein Extraction and Gel Electrophoresis

Faba leaves samples (ca. 0.5 g fresh weight) from three independent replicates per treatment, sampling time and genotype were crushed with liquid nitrogen in a precooled mortar to obtain a fine powder. Proteins were extracted into TCA–phenol [36] and the resulting pellets were resuspended in a solubilization buffer containing 7 M urea (Merck, Kenilworth, NJ, USA), 2 M thiourea (Sigma–Aldrich, St. Louis, MI, USA), 2% (w/v) CHAPS (Sigma–Aldrich), 2% (w/v) Bio-Lyte 3–10 carrier ampholytes (BioRad, Hercules, CA, USA), 2% (w/v) DTT (Sigma–Aldrich) and Bromophenol Blue traces (Sigma–Aldrich). Protein concentrations were determined with the Bradford assay (BioRad) and proteins were then separated in 2D electrophoresis gels.

For 2DE analysis, 18-cm IPG DryStrips (Amersham Biosciences, Amersham, UK) were used with nonlinear pH gradients over the range 3–10. Strips were rehydrated passively for 6 h and then actively at 50 V for a further 6 h with 300 μL of sample buffer containing an amount of 400 μg of protein. Strips were loaded onto a PROTEAN IEF System (BioRad), focused at 20 °C with an increasing linear voltage and equilibrated according to Castillejo et al. [37]. They were then transferred onto vertical slabs of 10% SDS polyacrylamide gels. Electrophoresis runs were done at 30 V at 15 °C for 30 min, and then at 60 V for about 14 h until the dye front reached the bottom of the gel. The gels were loaded with broad-range molecular markers (Bio-Rad) and, after electrophoresis, stained with Coomassie Brilliant Blue G-250 [38].
2.3. Image Acquisition and Statistical Analysis

Gels were scanned with the Molecular imager FX ProPlus Multi-imager system (BioRad) and the images thus obtained (Supplementary Figure S1) were analyzed with the software PDQuest Advanced v. 8.0.1 (BioRad), using 10 times the background signal as the presence threshold for spots. The quantitative data gathered from the spots in each gel (viz., normalized spot volumes given as individual spot intensity/normalization factor) were used to designate differences when comparing gel images. A multivariate statistical analysis of the entire data set was performed by using the web-based software tool NIA array [39]. Those spots showing significant differences ($p \leq 0.05$) in intensity, exhibiting a minimum change of ±2 and being consistently present among replicates were selected for further MS/MS analysis.

2.4. Protein Identification by Mass Spectrometry (MALDI-TOF/TOF)

Differential gel spots were excised for digestion with trypsin [40] and peptide fragments from digested proteins were analyzed by mass spectrometry. For that purpose, peptides were crystallized in an α-cyano-4-hydroxycinnamic acid matrix and subjected to MALDI-TOF/TOF analysis over the $m/z$ range 800–4000 by using a 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) at an accelerating voltage of 20 kV. Spectra were internally calibrated against peptides from trypsin autolysis (M + H$^+$ = 842.509, M + H$^+$ = 2211.104) and the five most abundant peptide ions in each spectrum were used for fragmentation analysis to elucidate peptide sequences. A combined peptide mass fingerprinting (PMF)/tandem mass spectrometry (MS + MSMS) search was performed by using the software GPS Explorer™ 5 (Applied Biosystems) over the nonredundant NCBI nr database restricted to Viridiplantae taxonomy in combination with the MASCOT search engine (Matrix Science, London; http://www.matrixscience.com accessed on 15 September 2019). The following parameters were allowed: a minimum of two peptides matches and a single trypsin miscleavage, and peptide modifications by carbamidomethylcysteine and methionine oxidation. The maximum tolerance for peptide mass matching was limited to 20 ppm. The score level and a minimum of four peptides per protein were chosen as PMF confidence parameters. Proteins were characterized in functional terms against the NCBI nr database (https://www.ncbi.nlm.nih.gov/guide/proteins/, accessed on 15 October 2021). In addition, BLAST analysis (tblastn) was performed for all the identified proteins using the reference transcriptome Vicia faba RefTrans v2 (2017), with 37,378 sequences deposited in Pulse Crops Database (https://www.pulsedb.org/, accessed on 15 October 2021). Only matches with an expectation (E) value of $\leq 1 \times 10^{-6}$ were considered. Mass spectrometry analyses were conducted at the Proteomics Facility of the Central Research Support Service (SCAI) of the University of Córdoba (Spain).

2.5. Zymography

Proteins from leaves (200 mg of frozen powdered tissue) were extracted with a mixture of 200 mM TrisHCl at pH 7.4, 3% (w/v) insoluble polyvinylpolypyrrolidone (PVPP), 10% (v/v) glycerol, 5 mM diethiothreitol (DTT) and 0.25% (v/v) Triton X-100. Samples were allowed to stand on ice for at least 10 min and were then centrifuged at 16,000 $\times$ g at 4 °C for 30 min, with the proteins present in the supernatant then being quantified with the Bradford assay [41].

SDS-PAGE slabs containing 0.1% gelatin and 9% acrylamide were analyzed according to Heussen and Dowdle [42]. Thus, samples containing 100 $\mu$g of protein were diluted with a nondenaturing Laemmli buffer [62.5 mM TrisHCl, 10% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue] and loaded onto 1 mm thick gel slabs for electrophoresis at 50 V at 4 °C for 30 min, with the voltage being raised to 80 V until the front reached the end of the gel. The gels were loaded with Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). After electrophoresis, gels were incubated in 2.5% (v/v) Triton X-100 at room temperature under constant agitation for 30 min to remove SDS. They were then washed with distilled water three times to remove Triton X-100 and incubated in a proteolysis
buffer (100 mM citrate buffer, Na₂HPO₄/citric acid pH 6.8, 4 mM DTT and 10 mM cysteine) under constant agitation at 35 °C overnight. Proteolysis was stopped by transferring the gels to a solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250 [43]. Finally, the gels were destained in a solution containing 40% methanol and 10% acetic acid until clear bands formed over a dark blue background.

3. Results

3.1. Disease Assessment

As can be seen in Figure 1, twelve hpi symptoms of *B. fabae* infection were already visible in the susceptible genotype (Baraca), but not in the resistant genotype (BPL710). Analyses of variance of the DS results revealed that the Baraca genotype was strongly affected both 2 and 6 dpi (average DS 23.9 and 52.5, respectively). On the other hand, the BPL710 genotype was highly resistant (ANOVA \( p \leq 0.05 \)) in both samplings (average DS 11.6 and 14.6, respectively) (Figure 1; Supplementary Figure S2).

![Figure 1. Contrasting response to *B. fabae* infection of three-week-old faba bean plants of Baraca (susceptible genotype) and BPL710 (resistant genotype) 12 h after inoculation.](image)

3.2. Two-Dimensional Gel Electrophoresis and MSMS Analysis

Image analysis with the software PDQuest allowed, on average, 224 individual protein spots to be detected (Figure 2a; Supplementary Table S1). In addition, a hierarchical clustering analysis clearly separated the genotypes into two clusters (Figure 2b), thus confirming the reproducibility of the experiment. Principal component analysis (PCA) allowed 194 differential protein spots from the entire dataset to be identified by comparing genotypes (susceptible and resistant) and treatments (uninoculated and inoculated) in both samplings (6 and 12 hpi) (Figure 2c). The first two principal components (PCs) jointly explained 70% of the total variability in the data, PC1 separating genotypes. The PCs for individual genotypes explained 74% and 86% of variability in the susceptible and resistant genotype, respectively (Figure 2d,e). In both cases, samples clustered by sampling times.
susceptible and resistant genotype, respectively (Figure 2d,e). In both cases, samples clustered by sampling times.

Spots were classified as variable if they met the following criteria: (a) being consistently present or absent in the three replicates under each set of experimental conditions; (b) exhibiting at least a two-fold change in abundance ratio; and (c) exhibiting statistically significant differences ($p \leq 0.05$) between genotypes or treatments. A total of 129 protein spots were thus selected for MALDI-TOF/TOF analysis.

Figure 2. Typical Coomassie Brilliant Blue 2DE gel results for the susceptible genotype. (a) Dendrogram showing hierarchical clustering of experimental conditions. (b) Two-dimensional biplots showing associations between all experimental conditions in both genotypes (c), or independent genotypes (d,e), as generated by principal component analysis (PCA). Dendrogram and PCA data were obtained from average values under each set of experimental conditions: Susceptible (S) and resistant (R) genotypes; control (C) and *B. fabae* inoculated (I); 6 hpi (1) and 12 hpi (2).

3.3. Protein Identification and Abundance Pattern Analysis

A protein search against the *Viridiplantae* index in the nonredundant NCBI database was performed and a total of 102 proteins were thus identified with high confidence (Table 1; Supplementary Figure S3), 70% of which matched legume species. Most of the proteins met the confidence identification criteria [viz., a score higher than 70 and at least four peptides per protein except for three spot proteins (viz., SSP 2802, 5002 and 11, which should be considered with caution)]. The proteins thus identified belonged to the main functional groups of energy and primary metabolism [photosynthesis (30) and other energy metabolism (6), carbohydrate (12) and amino acid metabolism (9)], followed by proteins of degradation (10), redox and response to stress groups (8) (Figure 3a).
Table 1. Proteins identified by MALDI-TOF MS analysis.

| SSP  | Protein Name (% Identity to *V. faba* Transcriptome Entry) | NCBI Accession | Score | Species | PM × Coverage % | Mr/pI Experimental (Theoretical) | Functional Category | More/Less Abundance Change Ratio (FDR) |
|------|------------------------------------------------------------|----------------|-------|---------|----------------|-------------------------------|-------------------|-----------------------------------|
| 3504 | Enolase (93.3% *Vf* _0033189)                             | gi|42521309 | 261    | Glycine max | 10/28                        | 57.2/5.7 (48.0/5.3) | Carbohydrate met. | 3.0 6.3 1.0 1.0 0.0 0.0 |
| 6302 | Isocitrate dehydrogenase [NADP], chloroplastic (88.1% *Vf* _0028303) | gi|2497259 | 153    | Medicago sativa | 17/40                        | 44.9/6.2 (48.7/6.2) | Carbohydrate met. | 0.4 9.0 0.8 0.6 1.2 0.6 |
| 2204 | Fructose-1,6-bisphosphatase (93.2% *Vf* _0028268)         | gi|5305145  | 109    | Pisum sativum | 4/14                         | 40.0/5.5 (36.3/6.3) | Carbohydrate met. | 16.4 6.7 1.7 0.2 0.4 0.9 |
| 6203 | Fructose-bisphosphate aldolase, cytoplasmic isozyme 1 (77.9% *Vf* _0002012) | gi|1168408  | 352    | Pisum sativum | 15/54                        | 39.4/6.5 (38.7/6.4) | Carbohydrate met. | 1.1 3.8 1.5 1.5 0.3 0.1 |
| 4104 | Fructose-bisphosphate aldolase 2, chloroplastic (80.6% *Vf* _0002012) | gi|461501   | 183    | Pisum sativum | 10/37                        | 38.4/5.8 (38.0/5.5) | Carbohydrate met. | 0.6 1.2 27.6 0.2 0.5 1.6 |
| 5103 | Fructose-bisphosphate aldolase 2, chloroplastic (80.6% *Vf* _0002012) | gi|461501   | 474    | Pisum sativum | 17/51                        | 36.8/6.3 (38.0/5.5) | Carbohydrate met. | 3.8 1.9 27.7 0.0 0.3 1.5 |
| 105  | N-glyceraldehyde-2-phosphotransferase-like (84.2% *Vf* _0037968) | gi|8885622  | 7/22   | Arabidopsis thaliana | 33.4/5.2 (32.0/5.1) | Carbohydrate met. | 6.4 0.8 0.5 1.0 0.2 0.0 |
| 1007 | Triose-phosphate isomerase (75.1% *Vf* _0012679)          | gi|15226479 | 205    | Arabidopsis thaliana | 9/25                         | 29.5/5.6 (33.6/7.7) | Carbohydrate met. | 3.6 2.3 1.1 1.2 0.5 0.2 |
| 2002 | Triose-phosphate isomerase (75.1% *Vf* _0012679)          | gi|15226479 | 292    | Arabidopsis thaliana | 11/29                        | 29.1/5.8 (33.6/7.7) | Carbohydrate met. | 10.4 5.1 1.9 1.1 0.5 0.2 |
| 3004 | Triose-phosphate isomerase (89.1% *Vf* _0024793)          | gi|15728985 | 165    | Phaseolus vulgaris | 7/31                         | 21.9/5.6 (27.4/5.9) | Carbohydrate met. | 2.2 4.7 1.0 0.4 0.0 1.6 |
| 2705 | Phosphoglucomutase, cytoplasmic (97.9% *Vf* _0002214)      | gi|12585296 | 109    | Pisum sativum | 10/8                         | 74.3/5.6 (63.5/5.5) | Carbohydrate met. | 9.5 0.0 1.0 1.0 0.0 0.0 |
| 3702 | Phosphoglucomutase, cytoplasmic (97.9% *Vf* _0002214)      | gi|12585296 | 222    | Pisum sativum | 10/23                        | 70.8/5.7 (63.5/5.5) | Carbohydrate met. | 6.3 19.0 0.4 0.9 2.3 8.8 |
| 603  | Beta-amyrase (88.5% *Vf* _0019893)                         | gi|3913031  | 149    | Medicago sativa | 7/17                         | 61.3/5.0 (56.5/5.3) | Major CHO met.  | 3.1 8.2 1.0 0.5 1.0 1.6 |
| 4808 | Methionine synthase/Cobalamin-independent synthase family protein (94.5% *Vf* _0043524) | gi|219522337| 104    | Cicer arietinum | 12/19                        | 92.8/5.9 (84.6/6.0) | Amino acid met. | 6.9 190.5 1.5 0.7 3.4 20.1 |
| 5805 | Methionine synthase/Cobalamin-independent synthase family protein (94.5% *Vf* _0043524) | gi|219522337| 312    | Cicer arietinum | 15/26                        | 93.2/6.0 (84.6/6.0) | Amino acid met. | 20.8 50.3 0.5 0.7 5.2 4.0 |
| 5808 | Methionine synthase/Cobalamin-independent synthase family protein (94.5% *Vf* _0043524) | gi|219522337| 344    | Cicer arietinum | 20/31                        | 92.2/6.1 (84.6/6.0) | Amino acid met. | 3.2 25.4 0.3 2.3 3.9 1.0 |
| 2402 | Alanine aminotransferase (82.2% *Vf* _0008500)             | gi|29569153 | 140    | Oryza sativa | 8/17                         | 54.1/5.6 (54.0/8.0) | Amino acid met. | 8.1 17.6 0.7 2.9 2.4 0.8 |
| 3402 | Alanine aminotransferase 2 (82.2% *Vf* _0008500)           | gi|29569153 | 104    | Oryza sativa | 4/9                          | 54.1/5.7 (54.0/8.0) | Amino acid met. | 63.4 0.0 1.3 1.4 51.7 11.9 |
| 1401 | 5-enolpyruvylshikimate 3-phosphate synthase (79.5% *Vf* _0024699) | gi|155740769| 73     | Camptotheca acuminata | 8/14                         | 52.1/5.2 (56.1/8.2) | Amino acid met. | 0.0 1.0 1.0 1.0 0.0 1.0 |
| 3613 | Ketcid acid reductoisomerase chloroplastic (97.3% *Vf* _0004275) | gi|1625542  | 560    | Pisum sativum | 23/43                        | 61.5/5.7 (63.2/6.6) | Amino acid met. | 12.0 0.5 0.7 0.4 2.2 0.4 |
| 7501 | Serine hydroxymethyltransferase 2 (91.7% *Vf* _0037980)    | gi|222142531| 203    | Glycine max | 13/29                        | 57.1/6.9 (55.0/8.2) | Amino acid met. | 2.1 1.7 1.6 1.0 0.3 0.0 |


| SSP | Protein Name (% Identity to V. faba Transcriptome Entry) | NCBI Accession | Score | Species | PM / Coverage % | Mr / PI Experimental (Theoretical) | Functional Category | More/Less Abundance Change Ratio (FDR) |
|-----|---------------------------------------------------------|----------------|-------|---------|----------------|-----------------------------------|---------------------|-----------------------------------------|
| 1104 | Putative lactoylglutathione lyase (**78.0%** Vf_0028431) | gi|115810219 | 183 | Arabidopsis thaliana | 8/26 | 33.4/5.3 (32.0/5.1) | Amino acid met. | 3.7 | 1.2 | 0.6 | 0.4 | 0.5 | 0.4 |
| 7602 | Chain A, Dihydrolipoamide Dehydrogenase (**99.6%** Vf_0005095) Pyruvate dehydrogenase E1 | gi|9953321 | 337 | Pisum sativum | 22/62 | 57.4/6.4 (50.0/6.1) | Lipid metabolism | 51.2 | 25.1 | 0.9 | 0.8 | 7.7 | 1.9 |
| 2102 | component subunit beta, mitochondrial (**96.1%** Vf_0005095) | gi|1709454 | 171 | Pisum sativum | 6/18 | 39.3/5.5 (39.0/5.9) | Lipid metabolism | 0.7 | 6.7 | 1.0 | 1.0 | 0.0 | 0.0 |
| 3705 | Zeaxanthin epoxidase, chloroplastic (**72.1%** Vf_0003026) | gi|5902706 | 92 | Solanum lycopersicum | 5/9 | 74.3/5.8 (73.6/6.2) | Hormone met. | 1.0 | 1.0 | 0.6 | 1.1 | ∞ | ∞ |
| 5601 | Polyphenol oxidase A1, chloroplastic (**98.5%** Vf_0006701) | gi|1172586 | 254 | Vicia faba | 8/8 | 64.9/6.0 (68.9/7.0) | Pigment biosynt. | 0.0 | 1.0 | 1.0 | 1.0 | 0.0 | 1.0 |
| 4607 | Polyphenol oxidase A1, chloroplastic (**98.5%** Vf_0006701) | gi|1172586 | 274 | Vicia faba | 9/12 | 64.4/5.9 (68.9/7.0) | Pigment biosynt. | 1.0 | 1.0 | 0.0 | 1.0 | ∞ | 1.0 |
| 4605 | Polyphenol oxidase A1, chloroplastic (**98.5%** Vf_0006701) | gi|1172586 | 151 | Vicia faba | 11/13 | 61.8/5.8 (68.9/7.0) | Pigment biosynt. | 8.7 | 0.6 | 1.0 | 1.0 | 0.0 | 1.0 |
| 2103 | Coproporphyrinogen-III oxidase, chloroplastic (**70.9%** Vf_0022525) | gi|2493810 | 148 | Nicotiana tabacum | 4/11 | 38.6/5.6 (45.3/7.6) | Co-factor and vitamine met. | 7.7 | 1.2 | 1.0 | 1.0 | 0.0 | 0.0 |
| 2601 | ATP synthase CF1 alpha subunit (**96.7%** Vf_0021629) | gi|219673973 | 546 | Trifolium subterranum | 19/36 | 59.9/5.4 (55.7/5.1) | Energy metabolism | 2.1 | 2.7 | 28.2 | 0.0 | 1.5 | 4.6 |
| 1601 | ATP synthase CF1 alpha subunit (**95.3%** Vf_0021629) | gi|139387459 | 126 | Phaseolus vulgaris | 9/20 | 57.0/5.3 (55.7/5.2) | Energy metabolism | 0.6 | 1.2 | 1.5 | 1.3 | 22.4 | 2.3 |
| 604 | ATP synthase CF1 alpha subunit (**96.4%** Vf_0021629) | gi|295136979 | 572 | Pisum sativum | 18/48 | 58.9/5.2 (53.2/5.1) | Energy metabolism | 0.7 | 2.7 | 1.0 | 1.6 | 9.0 | 2.3 |
| 505 | ATP synthase CF1 beta subunit (**97.6%** Vf_0021629) | gi|295136979 | 882 | Pisum sativum | 26/65 | 57.9/5.2 (53.2/5.1) | Energy metabolism | 2.0 | 17.9 | 1.2 | 1.9 | 5.8 | 9.5 |
| 1503 | ATP synthase CF1 alpha subunit (**95.4%** Vf_0021629) | gi|295137014 | 573 | Pisum sativum | 21/38 | 56.5/5.3 (54.7/5.7) | Energy metabolism | 4.5 | 3.2 | 1.2 | 0.9 | 3.9 | 2.7 |
| 1501 | ATP synthase CF1 beta subunit (**95.4%** Vf_0021629) | gi|295136979 | 755 | Pisum sativum | 21/51 | 56.2/5.2 (53.2/5.1) | Energy metabolism | 2.4 | 1.5 | 1.3 | 1.2 | 1.5 | 1.2 |
| 9701 | Sulite reductase (**96.4%** Vf_0029426) | gi|119225844 | 129 | Pisum sativum | 17/25 | 75.6/8.4 (77.3/9.1) | S metabolism | ∞ | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 3704 | Transketolase (**95.5%** Vf_0028016) | gi|4586600 | 214 | Cicer arietinum | 3/30 | 82.8/5.8 (17.1/5.8) | Photosynthesis | 11.3 | 7.0 | 1.2 | 1.0 | 35.9 | 3.2 |
| 3706 | Transketolase (**95.5%** Vf_0028016) | gi|4586600 | 91 | Cicer arietinum | 4/44 | 82.5/5.9 (17.1/5.8) | Photosynthesis | 4.0 | 4.0 | 1.0 | 0.5 | 18.0 | 2.8 |
| 4704 | Transketolase (**95.5%** Vf_0028016) | gi|4586600 | 86 | Cicer arietinum | 4/44 | 82.5/5.9 (17.1/5.8) | Photosynthesis | 3.8 | 17.1 | 0.6 | 0.7 | 6.4 | 1.7 |
| 4702 | Transketolase (**95.5%** Vf_0028016) | gi|4586600 | 189 | Cicer arietinum | 5/48 | 81.6/5.8 (17.1/5.8) | Photosynthesis | 3.8 | 3.1 | 1.0 | 1.1 | 8.1 | 1.5 |
| 1604 | RuBisCO large subunit-binding protein subunit beta chloroplastic/chaperonin subunit beta (**96.3%** Vf_0035079) | gi|2506277 | 423 | Pisum sativum | 16/39 | 67.8/5.5 (63.3/5.8) | Photosynthesis | 19.1 | 30.5 | 0.9 | 0.4 | 11.5 | 9.4 |
| 2602 | RuBisCO large subunit-binding protein subunit beta chloroplastic/chaperonin subunit beta (**96.3%** Vf_0035079) | gi|2506277 | 72 | Pisum sativum | 11/27 | 66.8/5.6 (63.3/5.8) | Photosynthesis | 1.8 | 2.4 | 2.2 | 0.5 | 1.9 | 1.5 |
| Protein Name (% Identity to V. faba Transcriptome Entry) | NCBI Accession | Score | Species | PM ± Coverage % | Mr/pI Experimental (Theoretical) | Functional Category | More/Less Abundance Change Ratio (FDR) |
|--------------------------------------------------------|----------------|-------|---------|-----------------|-------------------------------|---------------------|------------------------|
| RuBisCO large subunit-binding protein subunit beta chloroplastic/chaperonin subunit beta (96.3% Vf_0035079) | gi|2506277 | 257 | Pisum sativum | 10/26 65.7/5.5 (63.3/5.8) | Photosynthesis | 0.9 3.7 1.5 0.5 5.9 4.1 |
| RuBisCO large subunit-binding protein subunit alpha chloroplastic/chaperonin-60alpha (98.4% Vf_0031659) | gi|1710807 | 480 | Pisum sativum | 19/36 65.8/5.0 (62.0/5.2) | Photosynthesis | 2.4 5.2 0.7 0.2 1.0 3.3 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (95.9% Vf_0007913) | gi|825737 | 556 | Carya illinoinensis | 20/40 57.1/5.8 (51.6/6.1) | Photosynthesis | 26.5 7.5 0.5 37.7 4.3 2.8 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (96.5% Vf_0007913) | gi|13311331 | 584 | Carya ovate | 23/42 55.8/5.9 (51.4/6.1) | Photosynthesis | 5.1 1.7 6.2 1.4 41.3 3.3 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (96.5% Vf_0007913) | gi|12163407 | 485 | Cressa depresa | 23/44 55.5/5.8 (50.6/6.7) | Photosynthesis | 3.3 4.3 0.1 17.6 38.4 17.3 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (96.4% Vf_0007913) | gi|22554409 | 709 | Caragana camilli-schneideri | 25/45 54.4/6.0 (52.8/6.3) | Photosynthesis | 1.9 1.4 0.8 2.4 41.8 2.0 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase (95.7% Vf_0007913) | gi|74179244 | 619 | Aristolochia arboresa | 23/44 52.5/6.1 (52.0/6.1) | Photosynthesis | 14.6 1.3 0.9 0.7 21.7 7.2 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (95.3% Vf_0007913) | gi|62861204 | 759 | Pararacetum zeylanicus | 25/50 50.6/6.2 (52.0/6.0) | Photosynthesis | 14.1 0.9 0.6 0.9 11.2 3.7 |
| Phosphoglycerate kinase chloroplastic (86.1% Vf_0008651) | gi|129915 | 192 | Triticum aestivum | 9/19 49.6/5.0 (50.6/6.6) | Photosynthesis | 96.2 2.6 1.0 1.0 0.0 0.0 |
| Phosphoglycerate kinase chloroplastic (86.1% Vf_0008651) | gi|129915 | 254 | Triticum aestivum | 7/16 46.1/5.0 (50.6/6.6) | Photosynthesis | 12.8 3.9 6.9 0.8 6.3 4.6 |
| Phosphoglycerate kinase chloroplastic (85.6% Vf_0008651) | gi|2499497 | 571 | Nicotiana tabacum | 15/33 44.5/5.6 (50.3/8.5) | Photosynthesis | 3.2 3.7 2.5 1.5 3.7 1.7 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (95.9% Vf_0007913) | gi|825737 | 372 | Carya illinoinensis | 21/42 56.2/6.7 (51.6/6.1) | Photosynthesis | 3.5 6.8 0.9 1.8 98.3 3.2 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase (95.7% Vf_0007913) | gi|74179244 | 589 | Aristolochia arboresa | 24/42 53.7/6.3 (52.0/6.1) | Photosynthesis | 15.2 1.7 0.8 1.4 1.3 2.5 |
| Ribulose-1,5-bisphosphate carboxylase/oxygenase (91.3% Vf_00032793) | gi|19749359 | 267 | Glycine max | 18/38 48.6/8.7 (51.7/9.1) | Photosynthesis | 1.7 5.0 0.9 0.4 2.1 5.9 |
| Sedoheptulose-1,7-bisphosphatase (81.8% Vf_0007079) | gi|22997543 | 230 | Cucumis sativus | 9/21 42.8/5.2 (42.5/5.9) | Photosynthesis | 2.4 3.2 1.0 0.7 1.6 1.4 |
| Sedoheptulose-1,7-bisphosphatase (81.8% Vf_0007079) | gi|22997543 | 215 | Cucumis sativus | 12/34 42.6/5.6 (42.5/5.9) | Photosynthesis | 5.5 11.2 1.5 3.3 1.7 1.5 |
| Phosphoribulokinase (99.1% Vf_0003052) | gi|1885326 | 350 | Pisum sativum | 14/46 41.4/5.4 (39.2/5.4) | Photosynthesis | 2.4 2.5 4.8 0.9 2.3 2.7 |
## Table 1. Cont.

| SSP a | Protein Name (% Identity to V. faba Transcriptome Entry) b | NCBI Accession | Score c | Species | PM / Coverage % | Mr/pI Experimental (Theoretical) d | More/Less Abundance Change Ratio (FDR) e | Functional Category |
|-------|-----------------------------------------------------------|----------------|---------|---------|----------------|-----------------------------------|------------------------------------------|---------------------|
| 2202  | Phosphoribulokinase (99.1% _0003052) gi|1885326 | 160 | Pisum sativum | 7/27 | 41.1/5.3 (39.2/5.4) | Photosynthesis | 1.2 1.5 1.2 0.8 3.4 2.0 |
| 1201  | Photosystem II stability/assembly factor HCF136, chloroplast precursor (78.8% Vf_003934) gi|25559812 | 237 | Ricinus communis | 8/20 | 41.1/5.3 (43.4/7.1) | Photosynthesis | 1.3 2.2 1.8 2.0 0.5 0.3 |
| 3107  | Aldolase (80.8% Vf_0017749) gi|169039 | 137 | Pisum sativum | 9/27 | 38.5/5.6 (38.0/5.5) | Photosynthesis | 2.3 1.6 5.3 1.2 0.3 1.7 |
| 3105  | Aldolase (80.8% Vf_0017749) gi|169039 | 88 | Pisum sativum | 8/29 | 38.0/5.6 (38.0/5.5) | Photosynthesis | 0.0 0.1 ∞ 0.8 0.0 0.1 |
| 1001  | Chloroplast chlorophyll a/b-binding protein (99.2% Vf_0037012) gi|157786302 | 265 | Pisum sativum | 10/34 | 30.3/5.2 (28.4/5.5) | Photosynthesis | 0.9 0.8 6.8 0.9 0.3 1.4 |
| 4306  | Transaminase mtnE, putative (80.9% Vf_0007069) gi|4105131 | 159 | Ricinus communis | 5/11 | 46.2/5.9 (50.9/6.9) | Photosynthesis | 1.2 4.4 1.0 1.0 0.0 0.0 |

### Notes:
- **SSP**: Source Sequence Proportion
- **Protein Name**: The name of the protein
- **NCBI Accession**: National Center for Biotechnology Information Accession number
- **Score**: The score associated with the protein
- **Species**: The species of the protein
- **PM**: Protein Mass
- **Coverage %**: Percentage of coverage
- **Mr/pI**: Molecular Weight/pI
- **Experimental (Theoretical)**: Experimental pI/Theoretical pI
- **More/Less Abundance Change Ratio (FDR)**: The change ratio with FDR
- **Functional Category**: The functional category of the protein
| SSP a | Protein Name (% Identity to *V. faba* Transcriptome Entry) b | NCBI Accession | Score c | Species | PM / Coverage % | Mr/pI Experimental (Theoretical) d | Functional Category | More/Less Abundance Change Ratio (FDR) e |
|-------|---------------------------------------------------------------|----------------|---------|---------|----------------|-------------------------------------|---------------------|------------------------------------------|
| 3103  | CDSF32 protein (Chloroplast Drought-induced Stress Protein of 32 kDa) (69.4% *Vf*0033087) | gi|2592822 | 133 | Solanum tuberosum | 4/14 | 33.8/6.0 (33.8/8.1) | Stress response | 0.9 0.8 0.3 0.2 0.4 0.7 |
| 1206  | NADPH-dependent alkaline/one oxidoreductase; chloroplastic (85.3% *Vf*0022822) | XP_00332009.1 | 190 | Glycine max | 6/28 | 40.6/5.4 (31.2/9.2) | Redox | 1.0 1.0 1.0 ∞ 1.0 1.0 |
| 11    | Thioredoxin peroxidase (80.1% *Vf*0034677) | gi|21912927 | 108 | Nicotiana tabacum | 3/16 | 21.6/5.2 (30.1/8.2) | Redox | 2.4 0.3 16.3 0.6 0.0 0.7 |
| 5305  | GDP-D-Mannose 3′,5′-Epimerase (90.2% *Vf*0022282) | gi|15241945 | 108 | Arabidopsis thaliana | 8/26 | 49.6/6.0 (43.1/5.8) | Cell wall | 8.0 19.0 1.0 1.0 0.0 0.0 |
| 502   | Hydroxyproline-rich glycoprotein family protein | gi|18411523 | 147 | Arabidopsis thaliana | 9/13 | 60.7/4.8 (49.4/5.2) | Cell wall | 3.8 2.0 0.8 0.7 0.8 0.6 |
| 4404  | Gdp-Mannose-3′, 5′-Epimerase (89.9% *Vf*0022282) | gi|38754656 | 112 | Arabidopsis thaliana | 9/22 | 50.6/5.9 (43.2/5.8) | Cell wall | 0.5 3.7 1.0 1.0 0.0 0.0 |
| 10    | PAP fibrillin (84.1% *Vf*0025094) | gi|87240799 | 268 | Solanum tuberosum | 8/18 | 34.4/4.9 (34.1/4.9) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 1308  | Actin (99.7% *Vf*0005527) | gi|34541966 | 260 | Solanum tuberosum | 8/18 | 34.4/4.9 (34.1/4.9) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 1404  | Actin (99.1% *Vf*0028038) | gi|1496334 | 113 | Solanum tuberosum | 8/18 | 34.4/4.9 (34.1/4.9) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 1304  | Actin (99.7% *Vf*0005527) | gi|34541966 | 431 | Solanum tuberosum | 16/51 | 49.5/5.9 (41.9/5.3) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 1305  | Actin (99.7% *Vf*0005527) | gi|1496334 | 113 | Solanum tuberosum | 8/18 | 34.4/4.9 (34.1/4.9) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 3205  | Elongation factor Tu (97.5% *Vf*0005994) | gi|6015084 | 284 | Pisum sativum | 5/19 | 43.1/5.9 (37.3/5.5) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 3302  | Elongation factor Tu (97.5% *Vf*0005994) | gi|6015084 | 284 | Pisum sativum | 15/35 | 48.1/5.6 (53.1/6.6) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 3309  | Elongation factor Tu (97.5% *Vf*0005994) | gi|6015084 | 284 | Pisum sativum | 5/19 | 43.1/5.9 (37.3/5.5) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 3307  | Elongation factor Tu (97.5% *Vf*0005994) | gi|6015084 | 284 | Pisum sativum | 5/19 | 43.1/5.9 (37.3/5.5) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 1708  | V-type proton ATPase catalytic subunit (99.5% *Vf*0002956) | gi|12585490 | 146 | Citrus unshiu | 16/51 | 49.5/5.9 (41.9/5.3) | Cell organization | 1.4 1.7 1.8 0.5 0.2 1.5 |
| 5002  | Carbonic anhydrase (85.8% *Vf*0002210) | gi|47606728 | 105 | Flaviera bidentis | 3/17 | 30.3/6.2 (35.9/5.8) | Miscellaneous | 1.3 5.8 1.3 0.6 0.5 3.1 |
| 6003  | Carbonic anhydrase (85.8% *Vf*0002210) | gi|270342124 | 153 | Phaseolus vulgaris | 7/34 | 29.0/6.5 (35.9/8.1) | Miscellaneous | 0.9 0.5 3.3 ∞ 0.2 0.0 |

* a Standard spot number assigned to each spot protein (SSP) by PDQuest software (BioRad).
* b Percentages of identity to *V. faba* transcriptome entries [Vicia faba RefTrans V2 (2017) (https://www.pulsedb.org/, accessed on 15 October 2021)] obtained by Blast (tblastn) analysis are displayed in brackets. The coding “v.faba_CSFL_reftransV2_number” has been simplified by “Vf_number” for each transcript.
* c PM: number of peptides matched (from peptide mass fingerprinting) with the homologous protein from the database. Some of these peptides were automatically MSMS fragmented.
* d Experimental mass (Mr, kDa) and pI were calculated with PDQuest software and standard molecular mass markers. Theoretical values were retrieved from the protein database (NCBI).
* e Values are given as normalized volume (calculated with PDQuest software) and represent change ratios in response to *B. fabae* inoculation of each genotype (S: Baraca; R: BPL710), and between uninoculated genotypes (R/S) at both sampling times (1: 6 hpi; 2: 12 hpi).
**Figure 3.** Functional groups of differential proteins. (a) Proteins up- or down-accumulating in response to *B. fabae* inoculation as grouped by functional category. (b) Susceptible (S) and resistant (R) genotype; control (C) and *B. fabae* inoculated (I); 6 hpi (1) and 12 hpi (2).

Figure 3b compares treatments, genotypes and sampling times. The susceptible genotype (S) showed the largest number of changes in proteins in response to inoculation, but mainly in those of the primary and energy metabolism groups (Figure 3b). In fact, a strikingly large number of degradation proteins showed changes in S genotype in response to inoculation that were not observed in the resistant genotype (R). The group of degradation products comprised ten proteases, namely: three cell division proteases ftsH chloroplastic (gi|17865463), two ATP-dependent Clp proteases (gi|461753), two Clp proteases (gi|4105131, gi|461753), one ubiquitin-specific protease 5 (gi|257050978), one zinc dependent protease/FTSH protease 8 (gi|84468324) and one serine-type endopeptidase (gi|270342123). Interestingly, most of the identified proteases were significantly increased in response to inoculation in the susceptible genotype but, as revealed by the heat map (Figure 4a, Table 1), none was in the resistant genotype. A comparison of the uninoculated leaf proteome revealed a much greater number of proteins of the energy metabolism and protein degradation groups in R than in S, mainly in the first sampling (Figures 3b and 4a). In addition, these functional groups were increased in control S plants in the second sampling.

**Figure 4.** (a) Hierarchical clustering of proteases identified by MALDI-TOF analysis based on protein abundance as determined by 2DE. Susceptible genotype (S) and resistant genotype (R); control (C) and *B. fabae* inoculated (I); 6 hpi (1) and 12 hpi (2). (b) Zymogram of proteases in faba leaves separated by SDS-PAGE bearing gelatin under nondenaturing conditions. Arrowheads denote differential bands between genotypes or treatments.
3.4. Protease Gel Activity

The results of the protease gel activity assay confirmed those previously obtained by MS. Protease bands corresponding to high molecular weights (~90 to 120 kDa) were stronger in inoculated plants of the susceptible genotype in both samplings, coinciding with the molecular weight of some proteases identified by MALDI-TOF (viz., Clp proteases, SSPs 1802, 2801, 2804, 3803; and Ubiquitin-specific-processing protease 8, SSP 2802) (Figure 4b; Supplementary Figure S4). Gel activity assay also exposed a strong band at 40 kDa not changing substantially with the specific conditions and potentially corresponded to the serine-type endopeptidase identified by MS analysis (SSP 2101). In any case, the most striking result was the presence of two well-defined protease bands at high molecular weights (140–250 kDa) that were especially strong in the resistant genotype (and, particularly, in the second sampling). The area of the protease activity bands were estimated using ImageJ software (ImageJJS (imjoy.io)), and the data are presented in Supplemental Figure S5. Since activity gels were used under native conditions and 2DE-MS analyses conducted under denaturing conditions, these bands may well correspond to protein complexes not identified with the denaturing gels.

4. Discussion

Botrytis fabae is a necrotrophic plant pathogen causing chocolate spot, which is one of the most devastating diseases for faba bean production worldwide [5,6]. The mechanism by which plants counteract infection by this pathogen is of great agronomic interest. ROS production (especially \( \text{H}_2\text{O}_2 \) induction by Botrytis) is known to occur in a wide variety of plants [23,25–27] and to be one of the earliest plant responses to fungal infection [22,24]. Botrytis fabae reportedly increases lipid peroxidation, and the levels of ROS and antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase), substantially in faba bean [28]. In fact, ROS were found to accumulate rapidly in leaf tissue of a resistant cultivar at early stages of infection, but more markedly and over longer periods at later stages in its susceptible counterpart [28].

4.1. The Role of Chloroplasts as Redox Sensors Eliciting an Acclimatory Response to Stressing Conditions

ROS accumulation at an early stage of infection is triggered by plasma membrane-bound NADPH oxidases and typically occurs in the apoplast [44]. However, chloroplastic and peroxisomal ROS production have been reported to contribute to plant immunity as well [45,46]. Although ROS can also be produced by other organelles (notably peroxisomes and mitochondria), the chloroplast is possibly a major source. Some proteins in the chloroplast are involved in cross-talk with the cytosol and nucleus to govern the outcome of defense signaling [47]. Besides triggering ROS signals, chloroplasts can perceive, mediate or even amplify ROS signals originating in the apoplast [48]. In addition, there is evidence that the role of chloroplastic ROS production in coordinating cell death or modulating defense outputs is highly specific in targeting various types of invading pathogens. Thus, some chloroplastic components may be specific targets for microbial effector molecules, which suggest that chloroplasts communicate through these target molecules to elicit ROS production in the apoplast, presumably to contain spread of the lesion [49].

Chloroplast-derived ROS has been shown to play a role in plant resistance against B. cinerea [27,50]. Thus, histochemical analysis revealed ROS accumulation in tomato leaves 24 h after application of B. cinerea spores. A defense response accompanied by an improvement in photosystem II (PSII), possibly triggered by ROS upon short-time exposure, was observed. However, the relatively increased time of exposure to these molecules made them harmful to PSII functionality [27,51]. In addition, \( \text{H}_2\text{O}_2 \) levels in strawberry leaves were found to correlate positively with disease severity, and to be influenced by both leaf age and light quality [52].

Through photosynthesis, chloroplasts play a central role as redox sensors of environmental conditions by eliciting acclimatory or stress-defense responses [53,54]. In chloro-
plasts, chaperone systems refold proteins after stress, while proteases degrade misfolded and aggregated proteins that cannot be refolded [55]. A study on Arabidopsis thaliana demonstrated a major role of Hsp70 chaperones and Clp proteases in the folding and degradation of misfolded or damaged proteins under variable stress conditions in the chloroplast [55]. Caseinolytic proteases (Clps) function as molecular chaperones and confer thermotolerance to plants. The results of a differential gene expression analysis of Clps in wheat suggest a potential role in cold, salt and biotic stresses, and confirm the previously reported role in thermotolerance [56]. ClpATPases class I (ClpB/HSP100 and ClpC) function in assembly and disassembly with protein complexes, acting together with the HSP70/DnaK chaperone system to remodel denatured protein aggregates [57]. On the other hand, plant ClpC proteins act as stromal molecular chaperones in importing and protecting unfolded newly synthesized proteins, which are responsible for maintaining homeostasis [58–61].

In the present work two ATP-dependent Clp, two ClpC proteases and three Hsp70 (one as Chaperone DnaK) were found to be considerably increased in the susceptible genotype in response to inoculation. This result suggests that the chloroplast may respond to B. fabae inoculation by triggering a mechanism to repair damaged proteins. The previous proteins were highly represented at the constitutive level in resistant uninoculated plants, which may represent a temporary advantage in response to the pathogen.

### 4.2. Homeostatic Control as a Dynamic Regulation Mechanism for Energy and Redox Status in Response to Botrytis fabae

Enhanced photosystem II (PSII) functionality at the early stages of pathogen infection may be responsible for the increased sugar production required to strengthen the response by inducing defense genes [27,62]. However, B. cinerea has been reported to use large amounts of soluble sugars to grow on tomato leaves [63].

Nonphotochemical chlorophyll fluorescence quenching (NPQ) is the key photoprotective process used by plants to dissipate excess light energy as heat and preserve photosynthesis as a result [64–67]. A substantial increase in NPQ was observed in tomato leaflets up to 6 h after application of a B. cinerea spore suspension; the increase, however, was followed by a decrease down to control levels [27]. This outcome suggests an imbalance between energy supply and demand, resulting in increased ROS production similarly as in photoinhibition, causing damage in chloroplast and eventual cell death (necrosis) [68].

On physiological grounds, Clp and FtsH proteases are believed to play major roles in chloroplast protein homeostasis. Thus, FtsH (filamentation temperature sensitive H) proteases are membrane-bound ATP-dependent zinc metalloproteases involved in the biogenesis of thylakoid membranes and quality control in the PSII repair cycle [69]. ROS production and PSII photodamage are linked to the high turnover rate of the D1 reaction center protein, which is degraded and replaced with de novo synthesized protein in the so-called “PSII repair cycle” [70]. FtsH proteases are among the many components mediating coordinated turnover in D1. In addition, there is evidence that programmed inhibition of the PSII repair cycle through specific downregulation of protease activity may provide plants with a mechanism to elicit ROS production and cell death upon infection [71].

In parallel to the recognition of ROS as key signaling molecules, antioxidant enzymes and ROS scavenging, scientists have accepted their potential involvement in fine-tuning defense reactions. In chloroplasts, the antioxidants ascorbate and glutathione contribute chemically to ROS quenching. In addition, H2O2 can be detoxified by ascorbate peroxidases (APX), peroxiredoxine (PRX) or glutathione peroxidase (GPX), reviewed in [72], as confirmed by a study on Gentiana triflora which suggested that PRXQ plays a role in mediating responses against the necrotrophic fungus B. cinerea [73].

The proteomic analysis conducted in this work revealed that three proteins identified as chloroplastic cell division protease FtsH, and a zinc dependent protease/FTSH protease 8, were highly represented in the susceptible genotype in response to B. fabae inoculation. The same proteins were better represented constitutively in the resistant genotype in the first sampling. In addition, a monodehydroascorbate reductase I, an L-ascorbate peroxidase
and a thioredoxin peroxidase were also better represented in both genotypes in response to inoculation in the first sampling. Zymogram analysis confirmed the results of the MS analysis, where differences in the activity bands at the molecular weights of the proteins identified by 2DE-MS were observed. In addition, inoculated and uninoculated plants of the resistant genotype exhibited some activity bands at very high molecular weights (140–250 kDa) that were not clearly observed in the susceptible genotype. This result can be ascribed to differences in the experimental conditions, which were native in the zymograms and denaturing in the 2DE-MS analysis.

Consistent with the results obtained in this work, a recent study on tomato plants revealed H$_2$O$_2$ production and enhanced photosystem II functionality 30 min after B. cinerea inoculation. The effect, which lasted 4 h, was suggestive of a tolerant response; however, increasing the length of exposure led to plant damage [27] by fully inhibiting PSII functionality at the application spot and nearby. This was probably a time-dependent hormetic response, suggesting a positive biological response whose effect might be reversed upon extended exposure [27,67].

5. Conclusions

Based on the proteins identified in this study (Clp and Hsp70, together with FstH proteases and ROS proteins) and their increased levels upon inoculation with B. fabae, a signaling response mechanism based on ROS production in the chloroplast may be elicited by the fungus. This mechanism appears to be harmful to PSII in the susceptible genotype by effect of its being associated with lengthy exposures to high ROS levels. The differential response of the two genotypes can be ascribed to a metabolic imbalance in the susceptible genotype not observed in the resistant genotype and confirming that the latter retains normal metabolism under stress. On the other hand, there is evidence that the two genotypes differ in chloroplast detoxification system, the resistant genotype exhibiting a more efficient PSII repair mechanism at the early stages of infection. Further research is required in any case to ascertain whether the ROS dose or exposure time (hormesis) is associated with the differential V. faba phenotypes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11112247/s1, Figure S1: Coomassie-stained 2DE gels images of all experimental conditions throughout the experiment: susceptible (S) and resistant (R) genotypes; control (C) and B. fabae inoculated (I); 6 hpi (T1) and 12 hpi (T2) and three repetitions (R1-R3), Figure S2: Chocolate spot disease severity (DS) values in the genotypes Baraca and BPL710, 2 and 6 days after B. fabae inoculation, Figure S3: Location of 102 identified protein spots on a virtual 2DE gel. (a) Representative Coomassie stained 2DE gel of the susceptible (left) and the resistant (right) genotypes, (b) Figure S4: Zymographic detection of proteases in faba bean leaves separated by SDS-PAGE bearing gelatin under non-denaturing conditions, control (A) and B. fabae-inoculated (B). Susceptible (S) and resistant genotype (R); 6 hpi (1) and 12 hpi (2). Numbers following dashes designate the particular replicates, Figure S5: Quantification of the area of protease activity bands detected by zymogram analysis corresponding to the molecular weights 40, 70, 90, 100, 140 and 250 kDa, Table S1: Dataset of the 224 protein spots detected by PDQuest image analysis.

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