

The Effect of Hydrolysis and Protein Source on the Efficacy of Protein Hydrolysates as Plant Resistance Inducers against Powdery Mildew

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Abstract

The substitution of synthetic chemical pesticides has become a priority in agriculture and the induction of plant resistance by protein hydrolysates may offer a sustainable alternative. The protein source, method and degree of hydrolysis, as well as the amino acid and peptide composition may affect the efficacy of protein hydrolysates in protecting plants against pathogens. The aim of this work was to clarify the effect of enzymatic and acid hydrolysis on different plant protein sources (soybean, rapeseed and guar protein meal), in terms of efficacy against the powdery mildew of courgette plants (caused by *Podosphaera xanthii*). The enzymatic hydrolysates were obtained by incubating each protein suspension with a measured amount of Alcalase or Flavourzyme at 50°C for 24 h, in order to obtain an enzyme/substrate ratio of 1% and 50%. The chemical hydrolysates were obtained by acid hydrolysis using 6 N H₂SO₄ at 121°C for 15 min (6N A), or at 100°C for 8 h (6N B) respectively. Preventive foliar treatments with guar hydrolysates produced with both enzymatic (Alcalase 50% E/S ratio) and chemical (6N B) hydrolysis significantly reduced disease symptoms compared to the non-hydrolysed protein source. A positive correlation was found between efficacy and the degree of hydrolysis of guar acid hydrolysates, suggesting that the hydrolysis method may enhance the functional properties of the original protein source. In addition, positive correlations were found between the efficacy of guar hydrolysates and concentrations of specific peptides and amino acids. In conclusion, our results showed that the biocontrol effect of plant protein hydrolysates was related to the original protein source and two specific hydrolysis processes improved the functional properties of guar, producing peptide fragments and free amino acids that may be involved in the regulation of innate immune response in plants.

Keywords: Acid hydrolysis; Enzymatic hydrolysis; Biocontrol; *Podosphaera xanthii*; Cucurbitaceae; DAMPs

Introduction

The development of sustainable alternatives to synthetic chemicals in plant protection has become a priority in agriculture, because of increasing concerns about the negative impact of pesticides on human health and the environment [1-3]. As a response, scientists have increased efforts to find natural substances, called elicitors, that could stimulate the innate immune response in plants [4]. Indeed, plants are able to recognise and respond to specific pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), and induce pathways of triggered immunity [5], through the activation of specific surface receptors [6]. In addition, damage to plant cells by pathogens can release endogenous damage-associated molecular patterns (DAMPs) that also act as warning signals [7]. After perceiving these signals, plant cells rapidly activate a sophisticated surveillance system, by increasing the cell cytosolic Ca²⁺ concentration, generating reactive oxygen species and activating mitogen-activated protein kinases (MAPKs). These early signals lead to specific transcriptional and metabolic modulations, such as the expression of genes encoding pathogenesis related (PR) proteins and the synthesis of antimicrobial secondary metabolites [7]. In addition to locally restricted responses, elicitors can induce systemic resistance, which is commonly split into two groups: systemic acquired resistance (SAR), mediated by a salicylic acid-dependent process, and induced systemic resistance (ISR), which is mediated by jasmonic acid- and ethylene-sensitive pathways [8]. The origin of elicitors can be biological or synthetic [9], and they confer broad protection against multiple pathogens [10]. Among other things, protein hydrolysates and peptides from various sources can act as mediators, amplifiers or initial triggers of plant immunity, and increasing attention has been devoted to

investigation of their bioactive role in plant defence [11]. Endogenous peptides generated as degradation products from precursor proteins during infection were demonstrated to act as DAMPs [12], showing a similar mode of action despite their different cellular origin [11]. Artificial protein hydrolysates are mixtures of polypeptides, oligopeptides and free amino acids obtained by hydrolysis of protein contained in agro-industrial by-products of animals (i.e., leather, viscera, feathers, blood and other animal waste) or plant origin (i.e., crop residues or seed), and enzymes and strong acids or alkalis can be alternatively employed in hydrolysis [13,14]. Proteolysis enhances the functional properties of the original protein, allowing activation of the latent biological activities of peptides encrypted in the protein structure [15]. The efficiency of a protein hydrolysate is linked to the type and composition of peptides generated during hydrolysis [16,17], and peptide functionalities depend on molecular size, structure and amino acid sequences [18]. The degree of hydrolysis (DH, the percentage of cleaved peptide bonds) is one of the main parameters used to indicate the extent of protein hydrolysis, and consequently the properties of hydrolysates [19,20]. Although protein

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hydrolysates are commonly applied in small quantities, bioactive amino acids and peptide fragments can be readily absorbed by plants through diffusion processes and easily reach active sites [21]. Foliar applications of protein hydrolysates produce biostimulant effects on crops, especially under stress-inducing environmental conditions [22]. In particular, they could enhance the activity of the antioxidant system and boost plant metabolism, thus increasing root and shoot growth and promoting the productivity and fruit quality of several crops [14,23]. Moreover, peptide fragments can act as inducers of innate plant immunity. For example, casein and soybean hydrolysates have been shown to elicit grapevine defence mechanisms against downy mildew and grey mould by the up-regulation of PR genes [24,25]. Likewise, a protein derivative was effective in controlling courgette powdery mildew and grapevine [26], and activated the expression of defence-related genes in grapevine, suggesting the stimulation of plant resistance mechanisms [27]. In addition, the characteristics and functional properties of hydrolysates are influenced by the method used for hydrolysis and by the choice of the original protein source [28]. Despite significant economic benefits, chemical hydrolysis presents several disadvantages compared to the enzymatic process [14,29], such as the increased salinity of the final product. Moreover, animal-derived hydrolysates have frequently been demonstrated to have negative effects on plant growth [30,31], while plant-derived protein hydrolysates have been shown to act as non-toxic signalling molecules for plant defence, growth and development [32]. Accordingly, plant-derived protein hydrolysates generated by agro-industrial by-products may represent a low-cost organic strategy against crop diseases, considering their potential biocontrol properties and their harmless origin. Furthermore, they could become a sustainable solution to the inconvenience of industrial waste disposal, making their production interesting from environmental and economic points of view [28]. Among others, soybean (*Glycine max L.*, Fabaceae family), rapeseed (*Brassica campestris L.*, Brassicaceae family) and guar (*Cyamopsis tetragonoloba L.*, Fabaceae family) meals are currently used for animal feed. They have high protein concentrations (30-45%) and well-balanced amino acid profiles [33-35], and they are available at competitive prices as compared to other protein sources [36]. More specifically, soybean and rapeseed meals are agro-industrial waste by-products obtained from the solvent extraction of oil [33-35], and guar meal is a by-product of guar-gum extraction, which originates from its seeds, containing a gelling agent [34].

In this study, courgettes (*Cucurbita pepo L.*) and powdery mildew caused by *Podosphaera xanthii* [(Castagne) U. Braun and N. Shishkoff; *Podosphaera fusca* (Castagne) U. Braun and N. Shishkoff] were selected as the study pathosystem, because they are both economically significant and easy to handle in an experimental set-up [37]. In addition, systemic resistance against powdery mildew in cucurbits is easy to induce with several compounds, such as oxalate or phosphate salts [38-40] and oligochitosans or oligopectates [41]. The aim of this work was to compare the effect of acid and enzymatic hydrolysis of soybean, rapeseed and guar meal in terms of biocontrol efficacy against courgette powdery mildew under greenhouse conditions, and to investigate the potential contribution of amino acids and peptide fragments generated during hydrolysis to the activation of plant resistance.

Materials and Methods

Production of protein hydrolysates

The raw materials used as a protein source were industrial soybean and guar meal (45% and 35% protein content, respectively) and rapeseed

pellets (32% protein content), provided by Zebele Srl (Padua, Italy). Each product was milled to a powder using a jug blender (JB 5050, Braun, Kronberg im Taunus, Germany) before hydrolysis. As described in previous studies [18,42-44], the enzymatic hydrolysates were obtained with two commercial proteolytic enzymes, namely Alcalase 2.4 L (Sigma-Aldrich, St. Louis, MO, USA) and Flavourzyme 500 L (Sigma-Aldrich), which are widely used for protein hydrolysis in industrial and research applications [45]. Specifically, Alcalase (a non-specific microbial protease of *Bacillus licheniformis* with endopeptidase activity) has a density of 1.25 g/ml and a specific activity of 2.4 Anson Units (AU) per gram, while Flavourzyme (a protease complex of *Aspergillus oryzae* with endo- and exoprotease activities) has a density of 1.10-1.30 g/ml and a specific activity of 500 Leucine Aminopeptidase Units (LAPU) per gram. For each protein source (soybean, rapeseed and guar), 20 g of powder were mixed with 100 ml of distilled water and pasteurised at 85°C for 5 min. After cooling down to 50°C, the pH was adjusted to the manufacturer's recommended values for each specific protease (pH 8 for Alcalase and pH 6.5-7 for Flavourzyme respectively) with 10 N KOH. Subsequently, each protein suspension was treated with a measured amount of Alcalase or Flavourzyme, in order to obtain an enzyme/substrate ratio (E/S ratio) of 1% and 50% (enzyme unit/protein weight), based on the protein content of the protein source [18]. Digestion was carried out through incubation at 50°C for 24 h in a 500 ml-flask under orbital shaking at 200 rpm. After inactivating the enzymes by heating at 85°C for 5 min, the undigested proteins and insoluble particulates were discarded as a pellet after centrifugation at 3,800 × g for 20 min. Finally, the pH was adjusted to pH 7 with 10 N KOH and hydrolysates were kept at -20°C until analysis. The acid hydrolysates were produced according to the method described by Aaslyng et al. [46], with some modifications. For each protein source (soybean, rapeseed and guar), 20 g of powder were mixed with 100 ml of 6 N sulphuric acid (H₂SO₄), and treated in glass bottles for 15 min at 121°C (6N A) or for 8 h at 100°C (6N B), respectively. After cooling at room temperature, the mixtures were neutralised to pH 7 with 10 N KOH, centrifuged for 20 min at 3,800 × g, and the pellet was discarded to remove insoluble particulates. The centrifuged samples were stored at -20°C until analysis. In order to obtain an hydrolysate that could be effectively used in agriculture, the method of Aaslyng et al. [46], which uses hydrochloric acid (HCl) and sodium hydroxide (NaOH) for neutralisation, thus obtaining sodium chloride (NaCl) as the final salt, was partially modified, because NaCl has a phytotoxic effect on plant leaves. Specifically, we used H₂SO₄ and neutralised it with KOH, leading to the production of potassium sulphate (K₂SO₄), which is a common fertiliser [47].

Determination of the degree of hydrolysis

The degree of hydrolysis (DH) is defined as the percentage of cleaved peptide bonds according to the following equation:

$$DH = \frac{h}{h_{\text{tot}}} \times 100\%$$

where h is the number of hydrolysed bonds, and h_{tot} is the total number of peptide bonds per protein equivalent [48]. The degree of hydrolysis was calculated using the *o*-phthalaldehyde (OPA) method, as first described by Church et al. [49], which is based on the reaction between amino groups released during hydrolysis and *o*-phthalaldehyde, in the presence of dithiothreitol (DTT, ThermoFisher Scientific, Waltham, MA, USA), forming a compound detectable at 340 nm in a spectrophotometer (Ultrospec 3100, Amersham Bioscience, Little Chalfont, UK). In particular, the OPA solution was prepared according to Nielsen et al. [48], as follows. Firstly, 7.62 g di-Na-tetraborate decahydrate (Sigma-Aldrich) and 200 mg Na-dodecyl-sulphate were completely dissolved in 150 ml of deionised water. At the same time, 160 mg OPA (Sigma-

Aldrich) was dissolved in 4 ml of ethanol (Sigma-Aldrich) and this was then added to the aforementioned solution. Finally, 176 mg of DTT were added, and the final OPA solution was made up to 200 ml with deionised water. Serine was chosen as the standard, since in reactions it shows a response very close to the average response of other amino acids [48], and the standard solution was prepared as follows: 50 mg L-serine (Sigma-Aldrich, St. Louis, MO, USA) was diluted in 500 ml of deionised water (0.9516 meqv/l). After preparing all the reagents, 3 ml of OPA solution were added to all the test tubes. For standard, blank and sample optical density (OD) measurements with a spectrophotometer (Ultrospec 3100), 400 µl of serine standard, deionised water or each protein hydrolysate were added to the test tubes respectively. To estimate DH, determination of h in the OPA method was calculated according to Nielsen et al. [48], with the following equation:

$$\text{Serine-NH}_2 = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}) \times 0.9516 \text{ meqv/l} \times V \times 100 / X \times P$$

where serine-NH₂ is meqv serine NH₂/g protein, OD is the optical density of the sample, V is the sample volume (in litres), X is the sample dry weight (in grams) and P is the protein content (as a percentage) in the sample.

The expression for h , defined by Adler-Nissen [50] as the concentration of protein in amino groups formed during hydrolysis, in milliequivalents (meqv)/g, was then calculated as:

$$h = (\text{serine-NH}_2 - \beta) / \alpha \text{ meqv/g protein}$$

where the values reported by Adler-Nissen [50] were used for β (=0.4) and α (=1).

The value of h_{tot} , defined as hydrolysis equivalent at complete hydrolysis to amino acids, was calculated by summing the content of the individual amino acids in 1 g of protein [50], and fixed to 8 g equivalent/kg protein, because for most proteins the average molecular weight of amino acids is 125 g/mol [48].

Protein concentration and peptide and amino acid composition analysis

The protein concentration of hydrolysates was determined by measuring the OD of the samples at 205 nm (A_{205}) in the spectrophotometer, as described by Simonian [51]. In particular, a calibration curve prepared with standard solutions of bovine serum albumin (BSA, Sigma-Aldrich) was used to calculate the protein concentration of each sample. The identification of peptides and amino acids was performed on hydrolysed and non-hydrolysed samples by an external service company (ISB Srl, Ion Source and Biotechnologies, Milan, IT). Briefly, chromatographic runs were obtained using an Ultimate 3000 HPLC (ThermoFisher, San Jose, USA) combined with HCT ultra mass spectrometer (Bruker Daltonics, Breme, Germany). The samples were diluted 1:10 in HPLC grade water, and injected for LC-MS/MS analysis. Mobile phases A and B were composed of 0.2% formic acid and acetonitrile (CH₃CN) respectively. The column was a Phenomenex Luna C18 (2.0 × 50 mm, particle size 3 µm), and the volume of injection was 15 µl. Peptide annotation was obtained using the plant GPM database (http://plant.thegpm.org/tandem/thegpm_tandem.html), with specific searches in *G. max*, *B. napus* and Viridiplantae proteomes for soybean, rapeseed and guar samples respectively. Based on peptide peak areas, the peptides identified in hydrolysed samples were compared with those found in non-hydrolysed ones, and quantitation ratios were calculated for similar peptides (sequence identity higher than 70%). The free amino acid content was quantified (µg/mL) by ISB Srl (Ion Source

and Biotechnologies, Milan, IT) using liquid chromatography coupled with mass spectrometry for quantification.

Evaluation of the efficacy of protein hydrolysates against courgette powdery mildew under greenhouse conditions

Enzymatic and acid protein hydrolysates were tested against powdery mildew on courgette plants (cv Nero Milano) at a dosage of 1 g/l, in two and three independent experiments respectively. Briefly, courgette plants were grown in individual 2.5 l-pots containing a mixture of peat and pumice (3:1), as described by Nesler et al. [26]. In all the experiments, plants were grown in a greenhouse at 25±1°C (day and night), with 65±5% relative humidity (RH) and a 14 h photoperiod. On plants with two fully developed leaves, both surfaces of each leaf were treated with a hand sprayer, allowed to dry, and inoculated with a water suspension of *P. xanthii* conidia (1×10⁵ conidia/ml). The inoculum was obtained from infected leaves of untreated courgette plants, and was maintained by subsequent inoculations under greenhouse conditions at 25 ± 1° C with 80 ± 10% RH. As a control, plant leaves were sprayed with water and with non-hydrolysed protein sources, in order to detect a possible effect of the original protein sources against the pathogen. In particular, for the enzymatic process the non-hydrolysed sample followed the hydrolysis steps without the addition of any enzyme, while for the acid process the original plant sources were subjected to the hydrolysis procedure, but replacing sulphuric acid with deionised water. As an additional control for acid hydrolysis, courgette leaves were treated with a 0.11 M K₂SO₄ solution, corresponding to the highest quantity of the salt created in the final hydrolysates. Four replicates (plants) were analysed for each treatment, and powdery mildew severity was scored at 14 days post-inoculation (dpi) on all leaves by assessing the percentage of infected leaf area covered by white powdery mildew sporulation, according to the standard guidelines of the European and Mediterranean Plant Protection Organization [52]. The efficacy of each treatment was calculated according to the following formula:

$$\text{Efficacy} = (\text{SC} - \text{ST}) / \text{SC} \times 100$$

where SC is the disease severity of water-treated plants (control) and ST is the disease severity of plants treated with a tested molecule.

Evaluation of direct effect on the germination of *Podosphaera xanthii* conidia

The effect of enzymatic and acid protein hydrolysates on *P. xanthii* conidial germination was analysed following the method previously described by Romero et al. [53], with some modifications. Healthy courgette plants were grown for four weeks under greenhouse conditions. Leaves were surface sterilised by incubation in 0.5% hypochlorite for 5 min, and rinsed three times in sterile water for 5 min under orbital shaking at 80 rpm. Leaf disks (19 mm diameter) were cut out and placed (adaxial surface uppermost) on wet sterilised filter paper (three foils) in Petri dishes, and then homogeneously sprayed with enzymatic and acid hydrolysates at a dosage of 1 g/l using a small hand sprayer. As a control, leaf discs were sprayed with water and non-hydrolysed protein sources. For acid hydrolysates, a 0.11 M K₂SO₄ solution was also tested as an additional control. Leaf disks were then dried under a laminar hood for 1 h, and conidia from young leaves carrying fresh sporulation of *P. xanthii* at 14 dpi were brushed gently with a paint brush. Plates were incubated for 48 h at 23 ± 1°C with a RH of 99% and a 16 h photoperiod to allow conidia germination. Conidia were removed from the leaf disc surface using a piece of transparent adhesive tape (2 × 3 cm), and stained with a drop of Cotton Blue staining solution, according to Peries [54]. The percentage of germinated conidia was assessed by counting under a light microscope (Eclipse 80i, Nikon, Amsterdam, the Netherlands),

and conidia were scored as germinated when their germ tube length was greater than their lateral radius [55]. Two replicates of three disks were assessed for each treatment, by counting 30 conidia for each leaf disk.

Statistical analysis

Data were analysed with Statistica 13.1 software (Dell, Round Rock, TX, USA). An *F*-test was used to demonstrate the non-significant effect of experiments ($p > 0.05$), before pooling the data. After validation of normal distribution (K-S test, $p > 0.05$) and variance homogeneity (Cochran's test, $p > 0.05$) of the data, analysis of variance (ANOVA) was carried out, and Fisher's LSD test ($p \leq 0.05$) was applied to detect significant differences between treatments. Amino acid and peptide composition data for hydrolysed and non-hydrolysed samples were plotted using Principal Component Analysis (PCA) with Statistica 13.1 software. Pearson's analysis ($p \leq 0.05$), performed on all replicates (potted plants), was used to reveal potential correlations between efficacy levels and the compositional data of hydrolysates, such as DH values, concentrations of detected free amino acids and the quantitation ratios of detected peptides, in order to reveal the properties of different hydrolysis methods. The percentage of identity between different peptide sequences was obtained using BLAST pairwise alignment of protein sequences.

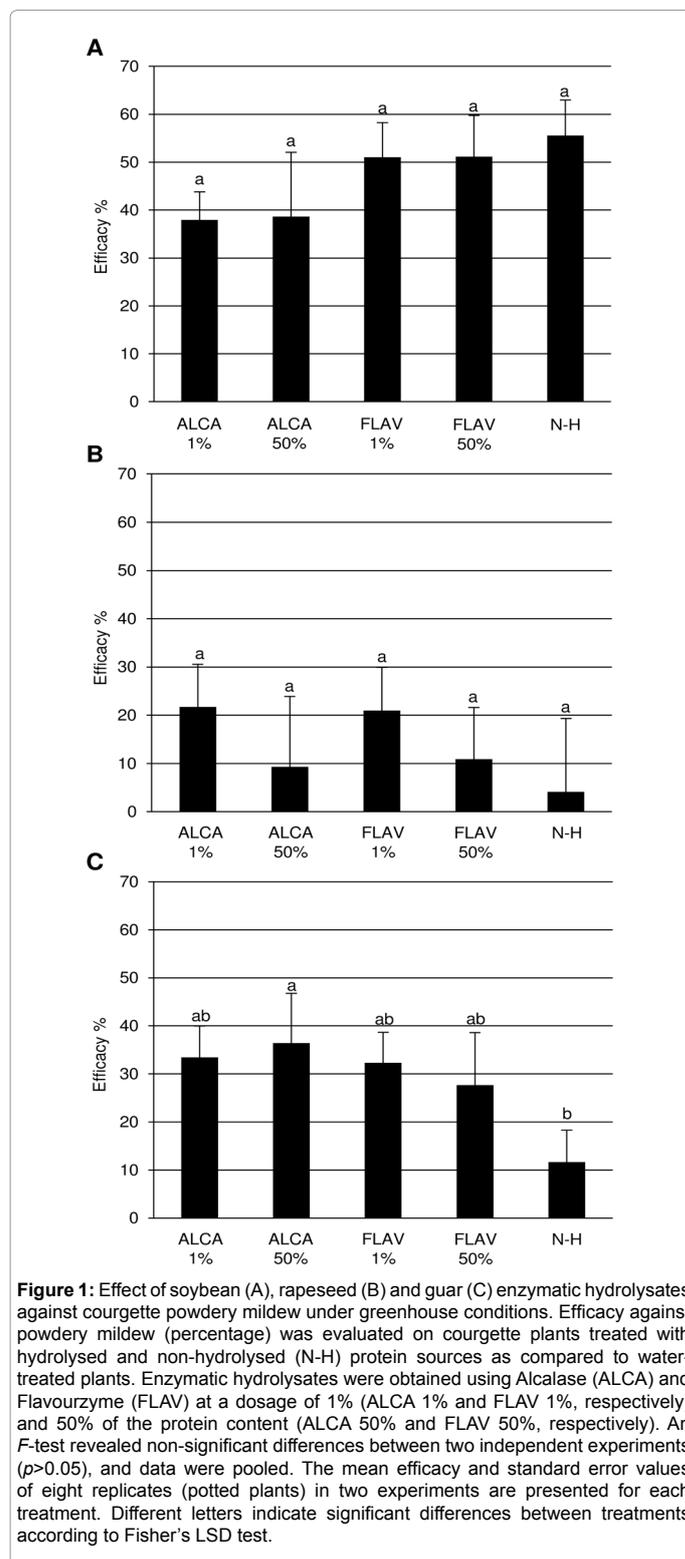
Results

Evaluation of the efficacy of enzymatic and acid hydrolysates against courgette powdery mildew

Soybean, rapeseed and guar enzymatic hydrolysates produced using Alcalase and Flavourzyme at two different E/S ratios (1% and 50%) were tested against powdery mildew on courgette plants (Figure 1). Foliar treatments with soybean and rapeseed enzymatic hydrolysates demonstrated an efficacy in disease reduction comparable to treatments with the non-hydrolysed protein source (Figures 1A and 1B). Conversely, the efficacy against powdery mildew was higher after the application of the guar hydrolysate produced with Alcalase at 50%, as compared to the non-hydrolysed protein source (Figure 1C). No direct effect on *P. xanthii* conidia germination was observed after application of guar enzymatic hydrolysates produced with Alcalase ($40.5 \pm 12.4\%$ and $35 \pm 8.8\%$ respectively) and Flavourzyme ($36.3 \pm 5.4\%$ and $31.1 \pm 7.4\%$, respectively) at 1% and 50% as compared with H_2O -treated leaf disks ($62.8 \pm 4.2\%$; ANOVA, $p > 0.05$; Fisher's LSD test). Similarly, conidia germination was not affected by treatments with soybean enzymatic hydrolysates produced with Alcalase ($28.3 \pm 5.3\%$ and $48.9 \pm 1.4\%$, respectively) and Flavourzyme ($30.6 \pm 4.7\%$ and $36.1 \pm 5.7\%$, respectively), and with rapeseed enzymatic hydrolysates produced with Alcalase ($30.6 \pm 4.5\%$ and $37.8 \pm 4.4\%$, respectively) and Flavourzyme ($32.8 \pm 6.3\%$ and $34.4 \pm 4.1\%$, respectively) at 1% and 50% E/S ratio, as compared with H_2O -treated leaf disks ($52.2 \pm 3.2\%$; ANOVA, $p > 0.05$; Fisher's LSD test).

Soybean, rapeseed and guar acid hydrolysates obtained using 6 N H_2SO_4 with two different time and temperature conditions were tested against courgette powdery mildew (Figure 2). Foliar treatments with soybean and rapeseed acid hydrolysates showed an efficacy in disease reduction comparable to that obtained with K_2SO_4 and non-hydrolysed protein sources (Figures 2A and 2B). In contrast, the efficacy against powdery mildew was significantly higher after application of the guar hydrolysate produced with 6 N H_2SO_4 at 100 °C for 8 h (6N B), as compared to K_2SO_4 and the non-hydrolysed protein source (Figure 2C). The estimated contribution of K_2SO_4 and hydrolysed proteins to the control of powdery mildew symptoms is presented separately, with

green and red bars respectively (Figure 2). In detail, K_2SO_4 contributed to disease reduction with a mean efficacy of $39.5 \pm 4.6\%$, whereas the additional effect of hydrolysed peptides and free amino acids ranged from $11.5 \pm 2.2\%$ to $17.5 \pm 3.9\%$, from $0.8 \pm 4.4\%$ to $18.5 \pm 2.5\%$ and from $10.5 \pm 2.8\%$ to $28.9 \pm 2.1\%$ for soybean, rapeseed and guar 6N A and 6N B hydrolysates, respectively. More specifically, the levels of



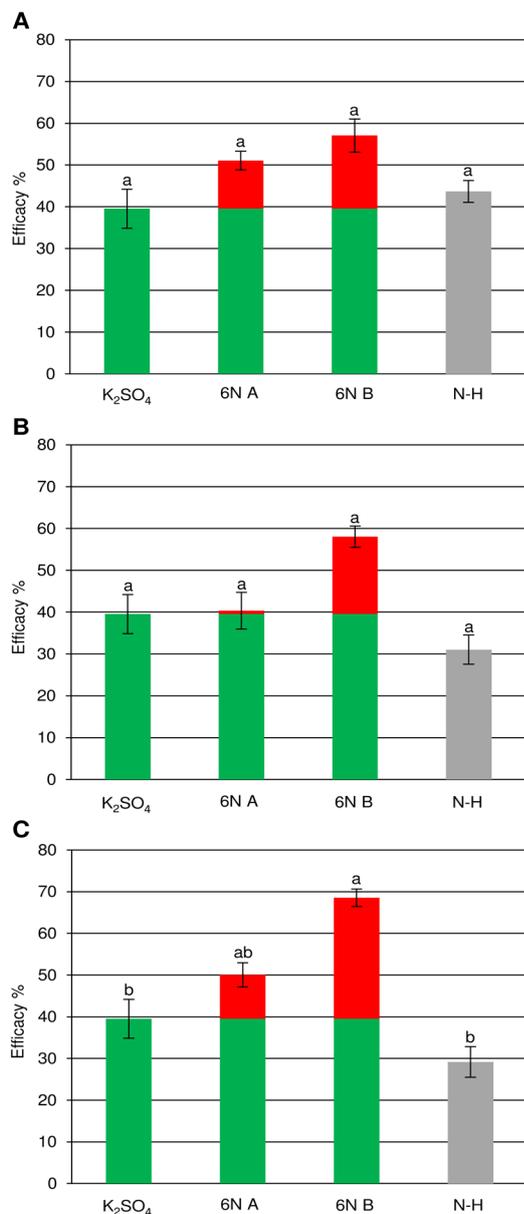


Figure 2: Effect of soybean (A), rapeseed (B) and guar (C) acid hydrolysates against courgette powdery mildew under greenhouse conditions. Efficacy against powdery mildew (percentage) was evaluated on courgette plants treated with hydrolysed and non-hydrolysed (N-H) protein sources or with 0.11 M K₂SO₄ as compared to water-treated plants. Acid hydrolysates were obtained by incubation of the protein source with 6 N H₂SO₄ at 121°C for 15 min (6N A) and at 100°C for 8 h (6N B). An *F*-test revealed non-significant differences between three independent experiments ($p > 0.05$), and data were pooled. Mean efficacy and standard error values of 12 replicates (potted plants) in three experiments are presented for each treatment. Different letters indicate significant differences between treatments according to Fisher's LSD test. For each hydrolysate, the contribution of K₂SO₄ and hydrolysed proteins to the efficacy in disease reduction was visually presented using green and red bars respectively.

efficacy considered without the contribution of K₂SO₄ are shown in Figure S1 of the Supplementary File, and reveal the potential impact of hydrolysed proteins alone, confirming the higher efficacy of the guar acid hydrolysate H₂SO₄, 6N B. The percentage of conidia germination

on guar 6N A- and 6N B-treated leaf disks ($12.8 \pm 2.9\%$ and $18.3 \pm 2.8\%$, respectively) was comparable (ANOVA, $p > 0.05$; Fisher's LSD test) to that on K₂SO₄-treated leaves ($20 \pm 5.4\%$), and significantly lower as compared with H₂O-treated leaf disks ($52.2 \pm 3.2\%$, ANOVA, $p \leq 0.05$; Fisher's LSD test). Similar results were obtained for soybean and rapeseed acid hydrolysates (data not shown).

Composition of enzymatic and acid hydrolysates

No significant Pearson's correlations ($p > 0.05$) were detected between the efficacy and DH values of enzymatic hydrolysates for all protein sources (Table 1). In contrast, a positive correlation ($R^2 = 0.41$; $p = 0.048$) was found for guar acid hydrolysates (Table 1), while no significant correlation emerged for soybean and rapeseed acid hydrolysates. In order to better understand the modes of action of protein hydrolysates, the content of peptides (Tables S1, S2 and S3) and free amino acids (Tables S4, S5 and S6) present in soybean, rapeseed and guar samples was analysed. The peptide composition varied according to the hydrolysis protocol for each protein source, and PCA analysis mostly discriminated samples of enzymatic hydrolysis (Figure 3). Specifically, differences in peptide composition were mainly observed between Alcalase 1%, Flavourzyme 1% and 50% hydrolysates of soybean and rapeseed protein sources (Figures 3A and 3B), and between Flavourzyme 50%, Alcalase 1% and 50% hydrolysates of the guar protein source (Figure 3C). On the other hand, the non-hydrolysed sample clustered with 6N A and 6N B acid hydrolysates for each protein source. Pearson's correlation analysis between the efficacy against powdery mildew and peptide quantitation ratios in guar hydrolysates revealed a moderate negative correlation for one peptide of enzymatic hydrolysates (Peptide 1; Table 2). Moreover, positive and negative correlations respectively were observed for two (Peptide 2 and Peptide 5) and one (Peptide 3) peptides for acid hydrolysates (Table 2). Correlation analysis of peptide composition data was not performed for soybean and rapeseed samples, since these hydrolysates were not effective against courgette powdery mildew.

PCA analysis of amino acid composition discriminated 6N A and 6N B acid hydrolysates on the first axis and Alcalase 1% and 50% hydrolysates on the second axis for each protein source (Figure 4). Moreover, non-hydrolysed samples clustered with Flavourzyme 1% and 50% hydrolysates. As regards guar samples, hydrolysates with significant efficacy against courgette powdery mildew (namely Alcalase 50% and 6N B samples) clustered with PCA analysis (Figure 4C). Pearson's correlation analysis between the efficacy against powdery mildew and amino acid concentrations detected a positive correlation for DL-Homophenylalanine and L-Glutamine of guar enzymatic hydrolysates, and for all amino acids of guar acid hydrolysates, except for L-Leucine/Isoleucine, L-Aspartic acid and L-Methionine (Table 3). Pearson's

Treatment	Protein sources	R ² value	p-value
Enzymatic hydrolysates	Soybean	-0.289	NS
	Rapeseed	-0.069	NS
	Guar	0.009	NS
Acid hydrolysates	Soybean	-0.226	NS
	Rapeseed	0.156	NS
	Guar	0.408	0.048

Pearson's correlation analysis between efficacy values (%) against powdery mildew and degree of hydrolysis (DH) values (%) of enzymatic and acid hydrolysates of soybean, rapeseed and guar was performed on all replicates (plotted plants). Correlation (R² value) and significant (p -value ≤ 0.05) or non-significant (NS) values were calculated.

Table 1: Pearson's correlation between the efficacy against powdery mildew and the degree of hydrolysis of enzymatic and acid hydrolysates.

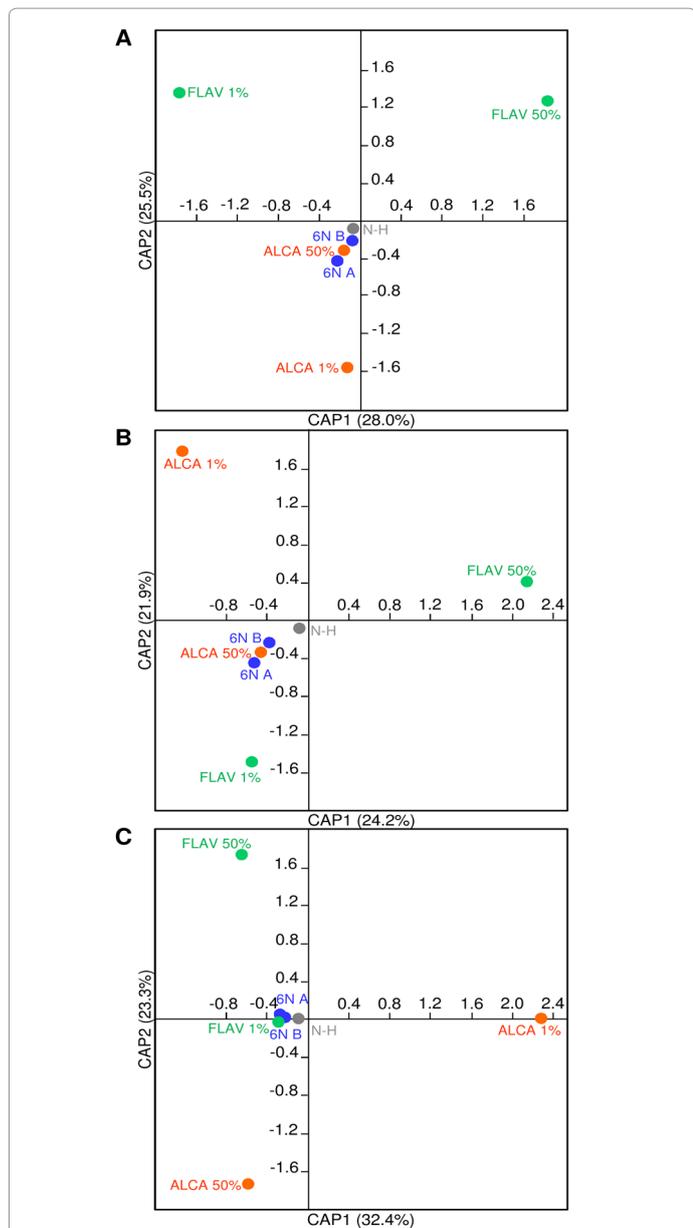


Figure 3: Principal component analysis (PCA) of hydrolysed samples according to their amino acid composition. PCA was obtained for soybean (A), rapeseed (B) and guar (C) samples using data for non-hydrolysed samples (N-H; grey), acid hydrolysates (blue) obtained by incubation with 6 N H₂SO₄ at 121°C for 15 min (6N A) or at 100°C for 8 h (6N B), and enzymatic hydrolysates obtained using Alcalase (ALCA, orange) and Flavourzyme (FLAV, green) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) or 50% of the protein content (ALCA 50% and FLAV 50% respectively). The two first principal components are plotted with the proportion of variance explained by each component in brackets.

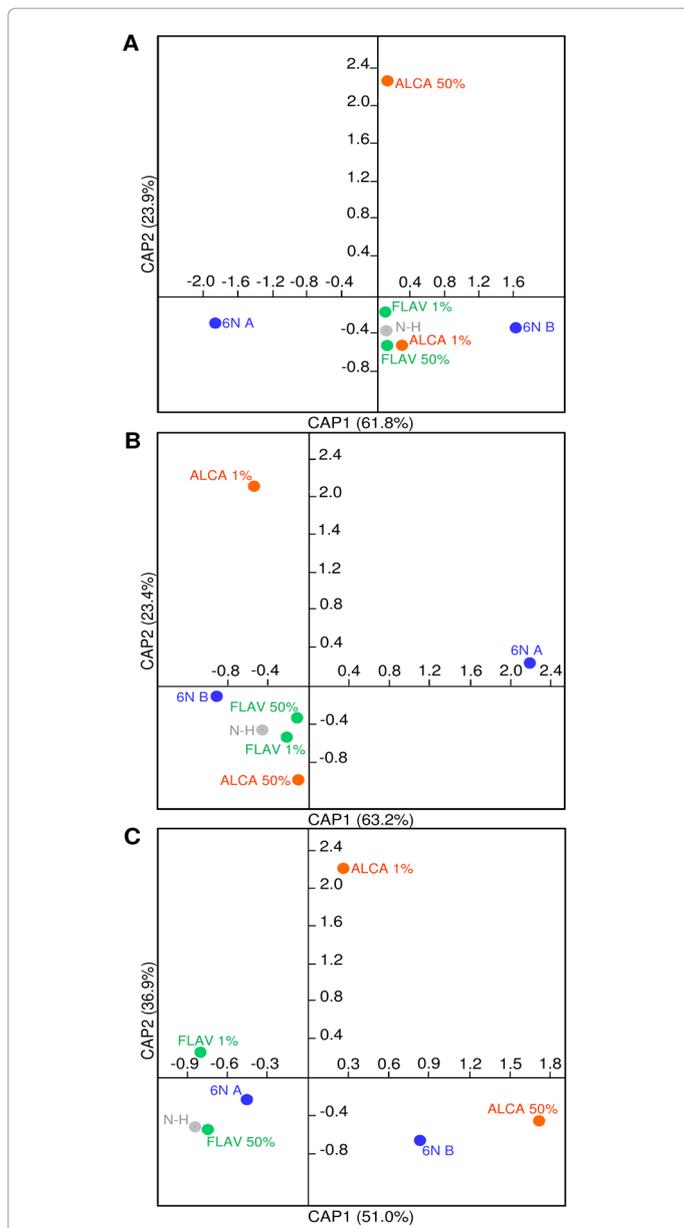


Figure 4: Principal component analysis (PCA) of hydrolysed samples according to their peptide composition. PCA was obtained for soybean (A), rapeseed (B) and guar (C) samples using data for non-hydrolysed samples (N-H; grey), acid hydrolysates (blue) obtained by incubation with 6 N H₂SO₄ at 121°C for 15 min (6N A) or at 100°C for 8 h (6N B), and enzymatic hydrolysates obtained using Alcalase (ALCA, orange) and Flavourzyme (FLAV, green) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) or 50% of the protein content (ALCA 50% and FLAV 50%, respectively). The two first principal components are plotted with the proportion of variance explained by each component in brackets.

analysis performed on soybean enzymatic and acid hydrolysates revealed no significant correlation between efficacy levels and amino acid concentrations (Table S7), as in the case of rapeseed enzymatic hydrolysates (Table S8). On the other hand, 14 positive correlations were found between efficacy levels and the amino acid concentrations of rapeseed acid hydrolysates (Table S8).

Discussion

Protein-derived DAMPs resulting from pathogen infection were

proved to be involved in triggering and amplifying plant immunity [11]. Likewise, artificial protein hydrolysates were demonstrated to act as resistance inducers [25-27] and their biocontrol activity was affected by the original protein source, hydrolysis method and degree of hydrolysis [20,28], as well as by their biochemical properties [18,56]. On the basis of these findings, we investigated and compared the impact of different plant protein sources and different hydrolysis methods on the efficacy of protein hydrolysates in controlling courgette powdery mildew, in order to clarify the possible role of specific peptide fragments and amino acids

Peptide number	Peptide sequences	Enzymatic hydrolysates		Acid hydrolysates	
		R ² value	p-value	R ² value	p-value
1	EWVDSAGAGAGGGGAPGTDVSCVGK	-0.348	0.028	-0.082	NS
2	GGTGGAQLPGRDGMVLVAYAPALVAAAASVPGAVEGLR	-0.042	NS	0.729	0.000
3	HEGSPGAAEQGADQQGGGLAVAAAGEPDGDGGGVRR	-0.068	NS	-0.707	0.000
4	ISASGVVAPPVPPPPPPPPPPHKLK	-	-	0.071	NS
5	TNHRFSEIEIDMISLNVIEVFPAIQQLI	-	-	0.561	0.001
6	QKIGGGGGGGGGGGGGGGGPPK	0.108	NS	-	-
7	IGGGGGGGGGGGGGGGGPPK	0.108	NS	-	-
8	TTMALAGAAAGMGKGNLSSSSMHSVAR	0.108	NS	-	-
9	FYGSGEGLMPGGMPGAGGPGGFPAGGPGAGHGDDGPTVEEVD	0.170	NS	-	-
10	EGAGAGAAAATTGGAAAAR	0.170	NS	-	-
11	MGGGHDMGGMAMAPPPAAAAAAAHGGNK	0.084	NS	-	-
12	SVDDLIMNVGSGGGGAPMAVATTAGGGDAGGTPPHFSFTR	-0.013	NS	-	-
13	ELIGGGGGGGGCC	-0.013	NS	-	-

Pearson's correlation analysis between efficacy values (%) against powdery mildew and quantitation ratios of peptides detected in enzymatic and acid hydrolysates of the guar meal was performed on all replicates (plotted plants). Correlation (R² value) and significant (p-value ≤ 0.05) or non-significant (NS) values were calculated.

Table 2: Pearson's correlation between the efficacy against powdery mildew and the quantitation ratios of peptides detected in guar enzymatic and acid hydrolysates.

Amino acid	Enzymatic hydrolysate		Acid hydrolysate	
	R ² value	p-value	R ² value	p-value
L-Glicine	0.214	NS	0.468	0.007
L-Alanine	0.210	NS	0.481	0.005
L-Proline	0.169	NS	0.598	0.000
L-Threonine	0.222	NS	0.381	0.031
L-Leucine/Isoleucine	0.107	NS	0.317	NS
L-Histidine	0.190	NS	0.733	0.000
L-Aspartic acid	-0.0181	NS	0.169	NS
L-Arginine	0.235	NS	0.559	0.001
DL-Homophenylalanine	0.318	0.046	0.750	0.000
L-Aspartic acid	0.195	NS	0.668	0.000
L-Cysteine	0.287	NS	0.422	0.016
L-Glutamic acid	0.217	NS	0.755	0.000
L-Glutamine	0.351	0.026	0.686	0.000
L-Lysine	0.211	NS	0.658	0.000
L-Methionine	0.204	NS	0.078	NS
L-Phenylalanine	0.309	NS	0.742	0.000
L-Serine	0.186	NS	0.756	0.000
L-Valine	0.285	NS	0.593	0.000
Glycated L-Lysine	0.204	NS	0.603	0.000
Glycated L-Arginine	-	-	0.561	0.001

Pearson's correlation analysis between efficacy values (%) against powdery mildew and free amino acid concentrations (µg/mL) was performed on all replicates (plotted plants) for enzymatic and acid hydrolysates of the guar meal. Correlation (R² value) and significant (p-value ≤ 0.05) or non-significant (NS) values were calculated

Table 3: Pearson's correlation between the efficacy against powdery mildew and the free amino acid concentrations of guar enzymatic and acid hydrolysates.

in the induction of resistance.

Our results showed that both enzymatic and acid methods significantly enhanced the efficacy of guar against courgette powdery mildew, in particular when hydrolysis was carried out with Alcalase 50% and with H₂SO₄ 6N condition B, respectively. Conversely, the biocontrol activity of soybean and rapeseed protein sources against courgette powdery mildew was not improved by the tested hydrolysis processes. In agreement with previous findings [57], our results confirmed that the

extent to which the functional properties of a protein may be altered by hydrolysis is very much dependent on the degree to which the protein has been hydrolysed. Indeed, a positive correlation was found between efficacy and the DH values of guar acid hydrolysates, suggesting that high hydrolysis time and temperature conditions could increase the biocontrol activity of this protein source. DH affects the physico-chemical characteristics of protein hydrolysates and could in turn affect their functionality [17]. Among other things, amino acid and peptide compositions were proved to modulate the biological activity of protein

hydrolysates [56], depending on molecular size, structure and specific sequence [18]. In particular, peptide concentrations were usually higher in enzymatically-derived protein hydrolysates than in chemically-derived ones [14], because proteolytic enzymes, which do not need high temperature to exert their function, usually target specific peptide bonds, producing low-salted mixtures of different length peptides [14]. As a result, PCA analysis performed on the peptide composition data of each protein source highlighted major differences between enzymatic hydrolysates, while acid hydrolysates clustered with the non-hydrolysed sample. For guar enzymatic and acid hydrolysates, significant correlations were detected between efficacy values and the quantitation ratios of specific peptide sequences, suggesting their crucial role against powdery mildew. Indeed, specific peptides of plant origin have been demonstrated to act as non-toxic signalling molecules for innate plant defence [32]. More specifically, the concentrations of Peptide 2 (GGTGGAQLPGRDGMILVAYAPALVAAAASVVPGAVEGLR) and Peptide 5 (TNHRFSEIEIDMISLNVIEVFPAIQSLI) of guar increased after acid hydrolysis and correlated with increased efficacy against powdery mildew. Thus, similar peptides found in the 6N A acid sample (EMGGKGGGGGGGGGGGGGGGPG) and the 6N B acid sample (DGGGGGSGAGAVVG and TNHRFSEIEIDMISLNVIEVFPAIQSLI) may be responsible for plant defence activation against powdery mildew, possibly by mimicking the biological activity of endogenous natural DAMPs. In particular, peptides deriving from cytosolic proteins, such as the active form of systemin [58] and the AtPep1 peptide [59], were shown to be internal signals for plant defence mechanisms in the soybean and *Arabidopsis thaliana* respectively. A similar function was demonstrated for peptides originating from secreted precursors, such as hydroxyproline-rich systemins in the potato [60] and phytosulphokines in *A. thaliana* [61]. Furthermore, several peptides released from the degradation of proteins with primary functions were shown to elicit plant defence responses, such as the inceptin family in the cowpea [62] and other peptide fragments in the soybean [58,63] for example. All of them seem to be active as elicitors and can activate the expression of typical defence marker genes [11]. However, sequences of guar Peptide 2 and Peptide 5 do not have similarities with the above-mentioned peptides, previously referred to as DAMPs [11]. Conversely, guar Peptide 1 and Peptide 3 were negatively correlated to efficacy levels in enzymatic and acid hydrolysates respectively, indicating that they may interfere with the activation of plant defence. In addition, guar enzymatic hydrolysates did not affect conidia germination on leaf disks, suggesting a mode of action mainly based on the stimulation of plant resistance mechanisms, as observed for other protein extracts. Indeed, peptide fragments of different origin have been shown to elicit grapevine defence mechanisms by the up-regulation of defence-related genes [24-27]. However, further analysis will be required to demonstrate how these peptide sequences could be involved in plant immunity regulation.

Although chemical hydrolysis has several drawbacks as compared to enzymatic hydrolysis (e.g., an increase in salinity) [14,29], both methods were investigated, in order to understand whether the potential efficacy in terms of disease control was caused by the hydrolysis process itself or eventually by the use of specific enzymes. Acid hydrolysis needs high temperatures to be carried out and randomly attacks all peptide bonds, leading to a high DH and a high free amino acid content [14]. Hence, PCA of amino acid composition data highlighted the highest variability between 6N A and 6N B acid hydrolysates for each protein source. Interestingly, the guar hydrolysates with significant efficacy against courgette powdery mildew (Alcalase 50% and 6N B) clustered together, suggesting a similar amino acid content. A bigger difference in free

amino acid composition was observed between Alcalase 1% and 50% hydrolysates as compared to Flavourzyme 1% and 50% samples, and the latter enzyme contains both endo- and exoprotease activities [45]. For guar acid hydrolysates, positive correlations were found between efficacy values and amino acid concentrations, suggesting that they may make a contribution to efficacy against powdery mildew. Indeed, the twenty proteinogenic amino acids play essential roles in the regulation of development, growth and stress responses in plants, and previous studies have revealed the involvement of amino acid metabolism in plant disease responses [64-67]. For example, treatment of rice roots with amino acids such as glutamate induced systemic resistance against rice blast in leaves [68], and lime plants treated with methionine significantly increased plant-induced resistance against citrus cancer disease [69]. Interestingly, positive correlations were also found for rapeseed acid hydrolysates, indicating that amino acids could partially improve the biocontrol characteristics of hydrolysates against courgette powdery mildew under greenhouse conditions. In addition to amino acids and peptides, plant protein hydrolysates contain other organic compounds, such as phenols, lipids and carbohydrates [14], which have been shown to act as active signals of defence responses. Specifically, phenolic compounds are quickly synthesised at the infection site, resulting in the effective isolation of the pathogen [70,71], and likewise lipids [72,73] and carbohydrates [74] have been demonstrated to be involved in plant immunity. Conversely, animal-derived protein hydrolysates lack carbohydrates, phenols and phytohormones [14], and repeated foliar applications caused phytotoxic effects on plant growth [30] that could be attributed to an unbalanced amino acid composition [31] and a high salinity [23]. Moreover, European Regulation 354/2014 recently prohibited the application of these products to the edible parts of organic crops, because their use generates serious concerns in terms of food safety.

Our results demonstrate that the biocontrol activity of protein hydrolysates against the powdery mildew of courgettes is affected by the original protein source, the method and the degree of hydrolysis. Moreover, free amino acid and peptide composition could contribute to efficacy levels and regulate plant responses to pathogen infection. However, the use of strong acids such as H_2SO_4 during hydrolysis caused an increase in the salinity of protein hydrolysates [14], and the formation of K_2SO_4 in guar acid hydrolysates contributed to disease control. Indeed, guar acid hydrolysates significantly reduced the percentage of conidia germination on leaf disks, in contrast to enzymatic ones. Other critical aspects in acid hydrolysis are the destruction of several amino acids and other thermolabile compounds and the phenomenon called racemisation, namely the conversion of free amino acids from L-form to D-form, which cannot be used by plants in their metabolism, making the hydrolysate less effective or even potentially phytotoxic [29,14].

The possibility of controlling crop diseases with the foliar application of low-cost protein hydrolysates represents an innovative approach, especially with a view to reducing pesticides in integrated pest management programs. Our results indicate the efficacy of guar protein hydrolysates against courgette powdery mildew, and two specific hydrolysis methods led to the formation of bioactive products. Preventive foliar application of plant-derived industrial by-products may offer considerable environmental and economic benefits. However, if expensive commercial enzymes need to be used in the hydrolysis process, the economic advantages of using agricultural by-products (such as protein meal deriving from oil extraction) may be nullified. Furthermore, knowledge of the application of protein hydrolysates to crops is far from being complete and further studies are required, in order to fully clarify their mechanisms of action and the effects on phyllosphere microbial communities.

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Author Contributions

Conceived and designed the experiments: MC, MP, IP. Carried out the experiments and acquired the data: MC AN OG. Analysed the data: MC. Wrote the manuscript: MC. Revised the manuscript: MP AN OG IP. All authors have read the manuscript and agree with its content.

Conflict of Interest

The authors declare that they have no conflict of interest.

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