UNIVERSITA' DEGLI STUDI DI UDINE

In agreement with FONDAZIONE EDMUND MACH – Trento

PhD School 'Agricultural Science and Biotechnology'
XXX Cycle
Coordinator: Prof. Giuseppe Firrao

Doctoral Thesis

UPDATE ON THE BIOPESTICIDE REGULATION AND DEVELOPMENT OF PROTEIN-BASED PRODUCTS FOR THE BIOCONTROL OF CROP DISEASES

PhD Candidate

Martina Cappelletti
ID 126603

Supervisor
Prof. Dr. Ilaria Pertot

Tutor
Dr. Michele Perazzolli

DEFENCE YEAR 2018

In memory of
Giulio Regeni

'The fishermen know that the sea is dangerous and the storm terrible,
but they have never found these dangers sufficient reason for remaining ashore'

Vincent Van Gogh

CONTENTS

ABSTRACT – English

In the last decades, concerns on the negative drawbacks of chemical pesticides on human health and environment have raised interest in safer alternatives; biopesticides, such as biogenic elicitors, represent an encouraging solution. The regulatory approach in the European Union does not distinguish biopesticides as a specific category of plant protection, and for this reason they are subjected to the same regulations as synthetic chemicals, requiring several authorization steps for the final approval and marketing. Among others, protein-based products and peptide fragments can stimulate plant growth, and represent a wide category of elicitors able to reduce the symptoms of common crop diseases, by acting as stimulators of plant defence and influencing systemic resistance processes, which can be regarded as effective alternative to synthetic chemical pesticides. The final objective of the current doctoral project was to provide new insights on the use of bioactive protein-based products against crop diseases, to further develop new sustainable strategies for organic agricultural practices. More in details, as well as providing an updated overview of the regulatory procedures for the authorization of biopesticides, the specific goals of the present research were i) to characterize the mode of action of protein-based products against crop diseases and ii) to optimize a method for the low-cost production of bioactive protein-based products. For this purpose, we analysed the mechanisms of action of a protein derivative called nutrient broth (NB) against grapevine downy mildew (caused by *Plasmopara viticola*), focusing on its roles as resistance inducer and nutritional source for phyllosphere microbial populations (Chapter 2). We showed that NB reduced downy mildew symptoms and induced the expression of defence-related genes in greenhouse- and *in vitro*-grown plants, indicating the activation of grapevine defence processes. Furthermore, NB increased the number of culturable phyllosphere bacteria, and altered the composition of bacterial and fungal populations on grapevine leaves. Thus, modifications in the structure of leaf populations caused by NB application could partially contribute to downy mildew control by competition for space/nutrients with the pathogen or other biocontrol strategies. Particularly, changes in the abundance of phyllosphere microorganisms may provide a contribution to the resistance induction, partially affecting the hormone-mediated signalling pathways involved. Later, we optimized an experimental procedure to develop low-cost protein hydrolysates starting from plant agro-industrial by-products, since animal-derived protein derivatives may create concerns about food safety (Chapter 3). Particularly, we

compared the effect of enzymatic and acid hydrolysis on different plant protein sources (soybean, rapeseed and guar protein meals), in terms of efficacy against the powdery mildew of Cucurbitaceae (caused on courgette plants by *Podosphaera xanthii*), investigating the potential contribution of amino acids and peptide fragments generated during the hydrolysis to the activation of plant resistance. Our results showed that the original protein source affected the biocontrol properties of protein hydrolysates, and two hydrolysis processes improved the functional properties of guar protein meal against powdery mildew. A positive correlation was found between the efficacy and degree of hydrolysis of guar acid hydrolysates, suggesting that the hydrolysis method may enhance the functional properties of the original protein source. In addition, significant correlations were revealed between the efficacy of guar hydrolysates and concentrations of specific peptide fragments and amino acids, which may be involved in the regulation of the plant defence response. Specifically, guar enzymatic hydrolysates did not present a direct toxic effect against the germination of pathogenic conidia, suggesting a mode of action mainly based on the stimulation of plant resistance mechanisms, as observed for NB.

The possibility of controlling crop diseases with the preventive foliar application of protein-based products represents an innovative approach, especially in a view of reducing harmful chemical pesticides in integrated pest management programs. However, further studies are required to fully clarify their modes of action and the impact on phyllosphere microorganisms under field conditions.

RIASSUNTO – Italiano

Negli ultimi anni, la preoccupazione della popolazione per le conseguenze negative dell'uso intensivo dei pesticidi chimici tradizionali sulla salute umana e sull'ambiente sta portando le comunità scientifica ed i rappresentanti politici verso un crescente interesse per i prodotti fitosanitari a base biologica, considerati un'alternativa sicura da inserire nei programmi di lotta integrata. Ad oggi, l'approccio legislativo dell'Unione Europea non riconosce i cosiddetti 'biopesticidi' come categoria di prodotti fitosanitari e per questo essi sono soggetti alle stesse regolamentazioni dei pesticidi tradizionali, con molti livelli di valutazione per arrivare all'approvazione finale e alla commercializzazione. Oltre alla loro ben nota azione biostimolante per il metabolismo e la crescita della pianta, i prodotti a base proteica rappresentano una vasta categoria di induttori di resistenza, composti non tossici capaci di ridurre i sintomi delle malattie più comuni grazie alla presenza di aminoacidi e peptidi, che vengono riconosciuti come molecole segnale dalla pianta e permettono l'attivazione dei meccanismi naturali di difesa, costituendo di conseguenza una valida alternativa ai prodotti fitosanitari di sintesi chimica. Lo scopo finale di questo progetto di dottorato è stato quello di sviluppare nuove conoscenze riguardo l'utilizzo di prodotti a base proteica nella lotta contro i fitopatogeni, per ipotizzare l'introduzione di nuove strategie sostenibili per la protezione delle colture. Più in dettaglio, oltre a presentare la versione aggiornata delle procedure regolative necessarie alla registrazione dei 'biopesticidi' in Europa e Stati Uniti d'America, gli obiettivi specifici sono stati quelli di i) caratterizzare il modo di azione dei prodotti a base proteica biologicamente attivi contro le malattie delle colture e ii) ottimizzare un metodo low-cost per la loro produzione su larga scala. Per questo scopo, abbiamo analizzati il meccanismo di azione del derivato proteico nutrient broth (NB) contro la peronospora della vite (causata da *Plasmopara viticola*), approfondendo anche il suo ruolo di fonte nutrizionale per le comunità microbiche fogliari (Capitolo 2). I trattamenti fogliari preventivi con NB permettono di ridurre i sintomi della peronospora della vite tramite l'attivazione di geni di difesa in piante cresciute in serra ed *in vitro*. Inoltre, NB aumenta il numero dei batteri coltivabili, e altera la composizione delle popolazioni di batteri e funghi residenti sulle foglie delle piante in serra. Le modifiche delle comunità microbiche dovute al trattamento con NB potrebbero parzialmente contribuire al controllo della peronospora, attraverso la competizione per spazio o nutrienti con il patogeno. Inoltre, i cambiamenti nelle dinamiche del microbioma fogliare potrebbero fornire un contributo all'induzione di resistenza, influenzando parzialmente i

segnali ormono-dipendenti coinvolti. Inoltre, è stata ottimizzata una procedura sperimentale per l'ottenimento di idrolizzati proteici da matrici proteiche a basso costo e di origine vegetale, considerando le preoccupazioni riguardo la sicurezza alimentare create dai prodotti a base proteica di origine animale (Capitolo 3). In particolare, è stato confrontato l'effetto dell'idrolisi chimica e enzimatica su differenti fonti proteiche vegetali (farine di soia, colza e guar), in termini di efficacia di biocontrollo contro l'oidio dello zucchino (causato da *Podosphaera xanthii*), analizzando il potenziale contributo dato da aminoacidi e peptidi all'attivazione della resistenza nella pianta. I risultati hanno dimostrato come l'attività di biocontrollo degli idrolizzati proteici vegetali dipenda dalla fonte proteica originale e dal metodo di idrolisi. In particolare, due specifici processi di idrolisi hanno permesso di intensificare le proprietà funzionali delle proteine di guar contro i sintomi dell'oidio. Le analisi hanno rivelato una correlazione positiva tra i livelli di efficacia degli idrolizzati chimici e il loro grado di idrolisi, suggerendo come questo metodo possa rafforzare le proprietà funzionali della fonte proteica originale. Inoltre, sono state trovate correlazioni significative tra i valori di efficacia e le concentrazioni di specifici aminoacidi e peptidi, che potrebbero essere coinvolti nella regolazione della risposta innata di difesa della pianta.

In conclusione, l'applicazione fogliare degli estratti proteici in agricoltura può rappresentare un approccio innovativo da inserire nei programmi di lotta integrata di molte colture, soprattutto al fine di ridurre l'uso dei prodotti fitosanitari di sintesi chimica. Tuttavia, saranno necessari ulteriori studi per approfondire il loro meccanismo di azione e gli effetti sui microorganismi fogliari delle piante in condizioni di campo.

CHAPTER 1.
Introduction

1.1 Crop diseases and the use of pesticides

Humans and animals directly or indirectly depend on plant for food. Unfortunately, several external agents can cause disease in plants, such as pathogenic microorganisms (e.g. viruses, bacteria, fungi, protozoa and nematodes) and unfavorable environmental conditions (e.g. lack or excess of nutrients, moisture and light, and the presence of toxic chemicals in air or soil). In addition, plants suffer from competition with other plants (weeds), and they are often damaged by attacks of insects. In the last 100 years, the control of plant diseases and other plant pests was increasingly relying on the use of toxic pesticides (Agrios, 2005). Using chemical pesticides presents several positive outcomes, such as the improving of crop quality and production and the resulting increased farm and agribusiness profits. However, their extensive application has produced a concomitant impact on the environment (Skevas et al., 2013). Indeed, only a tiny part of the sprayed amount of pesticides reaches the target organism (W. J. Zhang et al., 2011), while the rest ends up in the soil or on the crops, from where the pesticides can diffuse to other environmental sectors or enter the food chain (Storck et al., 2017), posing serious and irreversible environmental risks and costs. Hence, chemical pesticides have toxic effects on humans, livestock and wildlife (Schulz et al., 2002), while among the risks they pose are related to the toxic residues in food, water and soil, which harm the agro-ecosystems, have adverse effects on untarget biota, and develop pest resistance (Levitan, 2000; Looser et al., 2000; Smalling et al., 2013). More in details, these substances can be dangerous for human health when the level of exposure exceeds the safety levels. Additionally, the decline in the number of beneficial pest predators has led to the proliferation of various pests with adverse impacts on fauna and flora (Pimentel & Greiner, 1997), and some chemicals applied to crops could end up in ground and surface water pollution (Sharpley et al., 2001). Finally, pesticide-resistant weeds and pests can trigger increased pesticide applications to reduce the damage, resulting in higher economic costs for farmers. Therefore, numerous formerly authorized and widely used pesticides are now banned because unexpected and/or unacceptable risks emerged after their initial introduction to the market. Moreover, much of modern research in plant pathology aims at finding environmentally friendly means of control of plant diseases, and the most promising approaches include conventional breeding and genetic engineering of disease-resistant plants, the application of disease-suppressing cultural practices, RNA and gene-silencing techniques, of plant defense promoting, non-toxic substances, and, to some extent, the

use of biological agents antagonistic to the microorganisms that cause plant disease (Agrios, 2005). The member states of the European Union (EU) have a long history of controlling pesticide use through many country-specific programmes. However, the increase in pesticide use and the increasing presence of pesticides in aquatic environments, together with the fact that the current pesticide regulatory framework does not sufficiently address the reduction of use of pesticides, has led the and the European Commission (EC) to consider an overhaul of the pesticide regulations (FAO, 2004).

1.2 Registration of plant protection products in Europe and its difference to the US procedure

Over the past 50 years, pest management in the industrialized countries has been based on the intensive application of synthetic chemical pesticides, in order to keep up with the increasing need of food availability (Chandler et al., 2011). Because of negative side effects on human health and the environment (Tilman, 1999), and the development of resistance in pest populations (van Emden & Service, 2004), the introduction of new regulations has resulted in the withdrawal from the market of many synthetic active substances (Czaja et al., 2015). Hence, biological control has started to play a central role in the cultivation of many crops, and scientific community and policy makers have turned the attention to alternative and more sustainable pest management tactics. Integrated pest management (IPM) is a systems approach that combines different crop protection practices with careful monitoring of pests and their natural enemies, in order to balance agricultural production and health and environmental demands (Barzman et al., 2014). Generally, biopesticides are a set of crop protection tools used in IPM, and they constitute a special group of active substances for plant protection that occur naturally, or are nature-identical synthetic substances. They also include a number of living organisms, having different modes of action (Czaja et al., 2015). They may be used as fungicides, insecticides and herbicides, leading to many positive outcomes, such as decreased pesticide residues in food, low-risk to non-target organisms and fast degradation (Czaja et al., 2015). For this reason, there is strong interest for their use in IPM practices (Villaverde et al., 2014). The related legislation varies greatly worldwide, because countries have different requirements, guidelines and legal limits for plant protection products (Handford et al., 2015). The number of biopesticides authorisations in the EU and

United States is increasing: in the EU almost 100 biopesticide active ingredients have been registered (around 40 microorganisms, 30 pheromones and semiochemicals, 25 botanicals and other alternatives), whereas in the US more than 430 biopesticide active ingredients and 1320 active product registrations have been recorded until 2013. The pesticide registration process in the US is under the responsibility of the Environmental Protection Agency (EPA), and it is primarly based on the Regulation No. 40 CFR, Part 158 (EPA, 1991). In order to assess whether the use of proposed pesticides may pose unreasonable risks of harm to human health and the environment, including non-target organisms, EPA requires that registrants submit a variety of data about the composition, toxicity, degradation and other physical/chemical characteristics of the pesticide, and is committed to extensively review this information (Leahy et al., 2014). Aiming to encourage a more sustainable approach to crop protection, EPA promotes the development and application of low risk biological pesticides. In addition to biochemical and microbial pesticides, the definition of biopesticide in the US includes plant-incorporated protectants, namely substances produced by plants containing added genetic material. Contrary to EU law, the registration of biopesticides in the US follows a separate regulation compared to synthetic chemicals, with different requirements. In particular, the Biopesticides and Pollution Prevention Division was established in 1994 in EPA's Office Pesticide Programs, and it is responsible for all regulatory activities associated with biologically-based pesticides. Since biopesticides are usually less toxic than conventional pesticides, their registration may require a significantly reduced data set, and there are limited associated fees to help registration processes. This reduces the registration time from 3-5 years for synthetic pesticides to 1.5-2.5 years for biopesticides. Accordingly, biochemical and microbial pesticides are subject to different data requirements for registration than conventional chemicals, which are listed in 40 CFR Part 158 (Subpart U Biochemical Pesticides 158.2000 and Subpart V: Microbial Pesticides 158.2100) (EPA, 1991). Furthermore, EPA has published a guidance for developing these data in the Biochemical Pesticides Test Guidelines OSCPP Series 880, and in the Microbial Pesticides Test Guidelines OSCPP Series 885, respectively. Briefly, the active components of the product must be chemically identified, and the active component must be shown to be responsible for the pesticide activity. Even if characterized, mixtures of several active components or several organisms can be difficult and costly to register. Most successful products involve a single active component. The main types of data include information on the manufacturing and characterization of the product, toxicology/safety data,

environmental safety data, and product performance. The two most important factors in biochemical classification are that the product must be of natural origin and that it must have a non-toxic mode of action against the target pest (Braverman et al., 2003).

In Europe, the term ‘plant protection product’ (PPP) is often used interchangeably with ‘pesticide’; however, pesticide is a broader term that also covers non plant/crop uses, for example biocides (EU, 2017). PPPs protect crops or desirable or useful plants, and contain at least one active substance (or active ingredient, namely the active component against pests/plant diseases), and may also include other components such as safeners and synergists. The EC evaluates each active substance for safety before it reaches the market as a commercial product, and substances must be proven to be safe for human health, including their residues in food, animal health and the environment. In details, marketing and use of PPPs is covered by Regulation EC 1107/2009 (EC, 2009), and specific data requirements for applications are contained in Regulation EU 283/2013 (EC, 2013a), and Regulation EU 284/2013 (EC, 2013b). Only active substances registered on the EU's list of approved active substances and subsequently authorized as PPPs by each EU Member State can be released into the environment, and the authorization is granted only if proposed uses are not expected (or known) to have harmful effects on environmental, animal, or human health. After the submission of a dossier by a pesticide company, the first step of the authorization process is the production of an initial draft assessment report (DAR) by an EU designated rapporteur member state (RMS) (Figure 1). The RMS can modify and amend, and concludes on the risk assessment provided by the pesticide company including (i) the identity of the active substance and its biological efficacy, (ii) its toxicology and metabolism in mammals, (iii) its metabolic pathway and its residues in plants, crops and livestock and the risk for consumers, (iv) its environmental fate and behavior in soil, water and air, (v) its ecotoxicological impact on several non-target soil and aquatic organisms, including soil microorganisms and (vi) any relevant information found in the literature, if available. The second step is the peer review of the RMS's dossier, including the risk assessment, which is coordinated by the Pesticides Unit of EFSA. In the third step, the Pesticides Unit of EFSA produces a conclusion report. Risk managers of the Standing Committee on Plants, Animals, Food and Feed analyzes the EFSA's conclusions, and decide whether or not to register the compound on the EU's list of approved active substances (step 4). The first approval of an active substance must not exceed 10 years (15 years for low-risk substances), but review of the

approval can be requested by the EC at any time if risk concerns emerge (according to article 21 of 1107/2009).

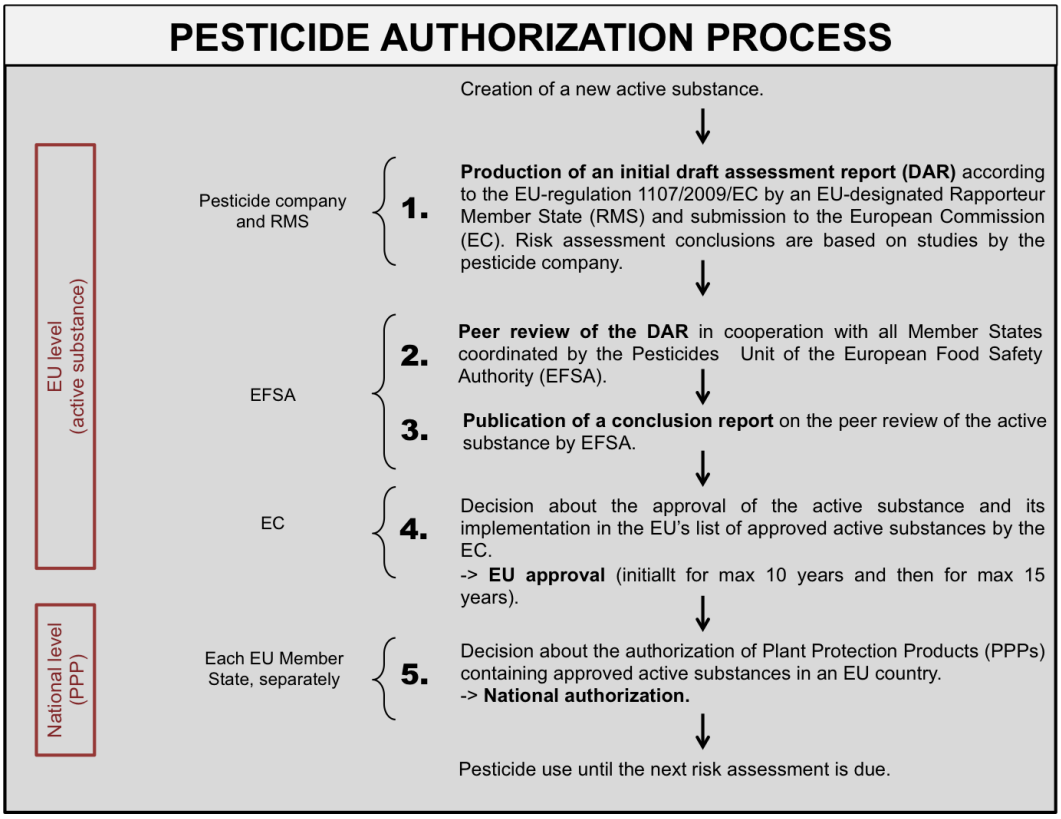

Figure 1. Schematic presentation of the stepwise process of pesticide authorization in the EU, modified from Storck et al. (2017).

The review of an approval should not exceed 15 years. In the same manner as for the initial approval, the review of the approval starts with the designation of an RMS by the EC in charge of providing a renewed assessment report (RAR) taking into consideration data resulting from post-authorization monitoring and literature search. The RAR is then subjected to the same procedure as the DAR. Finally, risk managers of each EU Member State separately take the final decision at the national level of granting or not granting authorization to use an EU-authorized active substance in a PPP without EFSA being involved (step 5) (Storck et al., 2017). EU-Regulation 1107/2009/EC introduced for the first time the division of Europe into three geographical zones according to pedo-climatic criteria (i.e. Scandinavian, temperate, and Mediterranean zones proposed in Annex I) to harmonize and speed up the regulatory process in different Member States. Within the same zone, the peer review procedure on PPPs is similar to that of active substances. First, the RMS evaluates the

registration report of the PPP, then the other Member States can comment on the first evaluation. The final decision is then taken by national governments based on the proposal of risk managers. National authorities may also impose risk mitigation measures and monitoring after authorization of a PPP. Contrary to US, the regulatory approach in Europe does not recognise biopesticides as a regulatory category of plant protection active ingredients, and for this reason they are subjected to the same regulations as synthetic chemicals. Subsequently, registration of new biopesticides usually requires the same or even more time as compared to conventional pesticides. Generally, biopesticides may be divided into several groups: products with pheromones or other semiochemicals as the active ingredient; microbial pesticides including fungi, bacteria and viruses; products containing living organisms such as invertebrates; plant-extract and vegetable-oil-based product (Czaja et al., 2015). Subsequently, biologically active agents classified as biopesticides present the most varied sources, and therefore the application of the same safety criteria to all of them is very difficult, and their approval and registration as active PPPs present several difficulties (Czaja et al., 2015). To avoid any confusion, EU recently introduced categories of low-risk active substances (LRAS) and basic substances (Huber, 2016). LRAS are substances, which have been evaluated as having a low-risk, fulfilling certain criteria such as being not carcinogenic, mutagenic or toxic to reproduction. A standard authorisation is still required for these products, and the general approval process is similar to conventional chemicals. A major benefit for applicants is that the active substance are approved for 15 years instead of the standard 10 years for the first approval, and products are authorised in a few months, differently from conventional PPPs. For this reason, biopesticides are candidates for being approved as LRAS. On the other hand, basic substances are active substances that are not predominantly used as PPPs, but which may be of value for plant protection; however, they are not placed on the market as a PPP. A basic substance is an active substance useful in plant protection either directly or in a product consisting of the substance and a simple diluent, which presents neither an immediate or delayed harmful effect on human and animal health nor an unacceptable effect on the environment. For basic substances, the application is submitted by the applicant (including Member States) directly to the EC, who then forwards the application to EFSA for its scientific evaluation. A basic substance is already evaluated in accordance with other Community legislation (food, cosmetic, commodity chemical). An application can be made by any interested part, including member states themselves, according to the guidance document on Basic substances

(EU, 2017), template listed in Annex I. The authorisation as basic substances is granted by the Commission after a scientific evaluation by EFSA and after consulting all Member States. The entire procedure takes approximately one year. This authorisation has no expiration data (unlimited time), and has no data protection (EFSA, 2017). In conclusion, the future EU policy schemes aims at the sustainable use of pesticides in European agriculture, involving the reduction of risks and impacts of pesticide use on human health and the environment. Accordingly, the design of optimal pesticide policies requires insight into the relationships between production decisions on crop yields and their quality, the environmental and health spillover impacts of pesticide use, and how policies and regulations influence production decision making. Furthermore, a key policy consideration should be balancing the incentives for economic growth against the adverse impact on the environment, which includes the management of land, water and air, as well as the overall stability and biodiversity of the ecological system (Skevas et al., 2013).

1.3 The two study pathosystems

1.3.1 Grapevine (*Vitis vinifera* L.) and the pathogen *Plasmopara viticola*

The grapevine has been a part of the human culture since the establishment of agricultural societies, and nowadays has become economically the most important fruit species in the world, because of the numerous uses of its fruit for the production of wine, juice, table grapes, dried fruits and organic compounds (Ferreira et al., 2004). The grapevine belongs to the kingdom of Viridiplantae, order Vitales and family Vitaceae, which comprises about 60 inter-fertile wild *Vitis* species distributed in Asia, North America and Europe under subtropical, Mediterranean and continental–temperate climatic conditions. Some species as *V. rupestris*, *V. riparia* or *V. berlandieri* are principally used as rootstocks or genetic sources for breeding due to their resistance against pathogens (Terral et al., 2010). However, *V. vinifera* is currently the main cultivated species because of the high quality for the wine production. In 2014 grapevine crop covers an area of 7.1 million hectares, with a global production of 74,5 million tons, and Europe was the biggest grapes producer, especially in the Mediterranean countries owing to the favourable climatic conditions (FAO, 2014). *Vitis vinifera* is greatly affected by a large number of pathogens that cause diseases in pre- and post-harvest periods, affecting fruit yield and quality, as well as production,

processing and export (Armijo et al., 2016). The causal agents of grapevine diseases are diverse and include viruses, mycoplasma, bacteria, insects, worms, arthropods, oomycetes and fungi. Among others, some of the most destructive diseases are the grey mould, powdery mildew and downy mildew, caused by the necrotrophic deuteromycete *Botrytis cinerea*, the biotrophic ascomycete *Erysiphe necator* and the oomycete *Plasmopara viticola*, respectively.

Downy mildew is one of the most dangerous and destructive disease of grapevine, particularly under warm and wet climates (Gessler et al., 2011). It originates from North East of the USA, imported in 1834 and spread to Europe by the end of the Century, where it caused widespread damages to the grape industry. *Plasmopara viticola* is an obligate biotrophic organism belonging to the Peronosporaceae family, and attacks all green parts of the plant. Sexual reproduction of *P. viticola* involves specialized reproductive structures where meiosis can occur and gametes are formed. In particular, oospores are often produced at the end of the growing season from the fertilisation of oogonia by antheridia, and are resistant to survive until conditions are suitable for new growth (Burruano, 2000). The primary infection starts early in the season, when oospores in fallen leaves or mycelium in dormant twigs are activated by adequate climate conditions to produce sporangia. In the presence of water, the mature sporangium releases self-motile biflagellate zoospores that infect plants tissues. Zoospores are able to place on the abaxial surface of leaves close to stomata, then germinate and penetrate through the stomatal cavities, where they form a substomatal vesicle. This vesicle gives rise to primary hyphae and mycelium, which grows through intercellular spaces, enclosed by the veins of the leaf and enters to the cell of the mesophyll by its cell-wall-penetrating and feeding haustoria, which invaginates the plasma membrane of the parenchyma cells. Sporangiophores arise and emerge through stomata, and sporangia may be carried by wind and rain to nearby healthy plants, germinate quickly and produce many zoospores that causes secondary infections and thus spread the disease (Gessler et al., 2011). All young grapevine tissues of *V. vinifera* are susceptible to downy mildew infection. More specifically, foliar symptoms appear as yellow circular spots with an oily appearance, and the affected area becomes soon brown or necrotic, until after warm and humid nights a white downy fungal growth appears on the abaxial leaf side (a phenomenon called sporulation). Severely infected leaves may curl and fall down, and this defoliation can reduce sugar level in the developing fruit and final yield. Moreover, in older leaves the lesions are restricted by veins to form small, angular, yellow to reddish-brown spots, which

combine to form a mosaic-like symptom. Young shoots, tendrils and petioles can also be infected by downy mildew. Indeed, infected shoot tips generally curl and become white due to downy mildew sporulation. Eventually, shoots turn and become brown or die at the late stages of infection. Flowers and young berries are also susceptible to this pathogen. At the flowering stage, infected bunches appear greyish in colour, and acquire a typical curl with an “S” shape. Infected bunches and berries are covered by downy mildew sporulation after raining periods, and they become brown at the late stages of infection. Young fruits can become resistant to infection from three to four weeks after bloom, because skin tissues evolve and stomata close. However, *P. viticola* can penetrate form the stomata of rachis or peduncles, and this infection causes brown rot symptoms, characterized by dry berries with a dark green and brown colour (Gessler et al., 2011). Downy mildew control is generally achieved by widespread applications of chemical fungicides in vineyards. Farmers agree to zonal warning, which take into account current meteorological conditions and grapevine phenological stages. Indeed, risks of infection are higher with warm and rainy weather, and during the period of intensive growth (i.e. from the end of May to the middle of July). However, in order to maintain their efficiency and to avoid the resistance of pest populations, it is necessary to alternate phytosanitary treatments, either with different mechanisms of action or with new active substances (Delaunois et al., 2014). In organic farming, this pathogen is mainly monitored by regular sprays with products based on copper (Gessler et al., 2011). However, these products do not eradicate existing infections, and concerns about their environmental effects are rising.

1.3.2 Cucurbitaceae family and the pathogen *Podosphaera xanthii*

The Cucurbitaceae or cucurbit family is one of the most important groups known worldwide of about 130 genera and 800 species, mainly distributed in tropical and substropical regions (Dhiman et al., 2012). Cultivars developed by breeders, especially of pumpkin (*Cucurbita pepo*), melon (*Cucumis melo*), cucumber (*Cucumis sativus*), and water melon (*Citrullus lanatus*) present economically important uses in food, agriculture and cosmetic industries (Ielciu et al., 2016), and the commercial role of derivatives from medicinal species is increasing rapidly (Kocyan et al., 2007). The courgette (*Cucurbita pepo* L.) is extremely diverse in fruit characteristics. Native to semi-arid and temperate regions of North America, it was domesticated at least twice, in Mexico over 10,000 years ago and in the United States over 4000 years ago (Paris, 2016). The world

growing area of cucurbits both in the field and under cover of 8.6 million hectares is almost two times higher than the growing area of tomatoes (4.6 million ha) (FAO, 2014). There are over 200 known cucurbit diseases of diverse aetiologies, and viruses borne by aphids, whiteflies, and other insects are some of the most destructive of *Cucurbita pepo*, tending to vary from region to region and year to year (Paris, 2016). In addition, other pathogens limit the production of pumpkins and squash, and the two powdery mildew fungi, namely the obligate biotrophic ectoparasites *Podosphaera xanthii* and *Golovinomyces orontii*, can be devastating and difficult to combat (Lebeda et al., 2016), because they weaken plants over the course of the season, causing reduction in plant growth, premature desiccation of the leaves and consequent reduction of the quality and marketability of the fruits. In Italy, powdery mildew is particularly serious on crops such as melon and zucchini (Gilardi et al., 2012).

Podosphaera xanthii, previously named *Sphaerotheca* (*Podosphaera*) *fusca emend.* (*s. lat*), *Spaerotheca fulginea* f. *cucurbitae* (Erysiphaceae family) usually grows on the plant surface, obtaining nutrients from the host epidermal cells by means of haustoria. With respect to the asexual life cycle, after landing on a susceptible host, conidia produce a short germ tube, ending in a primary differentiated appressorium with a primary haustorium. Then, a primary hypha arises, forming first secondary appressoria and haustoria, and later the branches of secondary hyphae. Conidiophores emerge from some of the secondary hyphae, and conidia are produced in chains. The mat of secondary hyphae and conidia forms the white mycelium on the surface of the plant (Perez-Garcia et al., 2009). In addition, the sexual reproduction occurs with the production of a chasmothecium from the union of two hyphae of opposite mating types. Chasmothecia are, in general, considered to be overwintering sources of inoculum. Like other powdery mildew diseases, the symptoms of *P. fusca* are characterized by the whitish, talcum-like, powdery fungal growth that develops on both leaf surfaces, petioles and stems (Perez-Garcia et al., 2009). The fungus feeds the plant nutrients, reduces photosynthesis and causes yellowing, and sometimes the death of leaves. A severe infection may kill the plant. Crop yields can be reduced because of reduced size or number of fruits, and fruit from affected plants can have low quality (Perez-Garcia et al., 2009). The most common strategy to control the powdery mildew of *C. pepo* includes the use of resistant cultivars, but actually traditional fungicides are the most effective means of control. Unfortunately, fungicides generally have a high risk of developing resistance in pest populations, because they have specific modes of

action, and *P. xanthii* has a high potential for resistance development (McGrath, 2001). Most of the fungicides to control powdery mildew are primarily preventive. Other non-systemic fungicides, such as sulfur and copper, have some efficacy to control powdery mildew outbreaks. Sulfur is one of the oldest natural fungicides to control powdery mildews. However, sulfur only provides a moderate level of control, and phytotoxicity as scorch occurs when sulfur is applied to the leaves (Nuñez-Palenius et al., 2006).

1.4 The plant self-protection system

Plants are sessile organisms, constantly exposed to a wide range of harmful pests. Despite the lack of specialized immune cells or organs, plants are surprisingly resilient to pathogen attacks, relying entirely on innate immune responses (Jones & Dangl, 2006). Although plants evolved an array of constitutive defences, such as structural barriers and preformed antimicrobial metabolites to prevent or at least attenuate the invasion, many microbes succeed in breaking through this pre-invasive layer of defence. However, a broad spectrum of inducible plant defences can be recruited to limit further pathogen entrance (Pieterse et al., 2009). For this purpose, plants employ a two-tier innate immune system that involves plasma membrane-localized and intracellular immune receptors (Zipfel, 2014). So far, these levels have been called basal or horizontal disease resistance and gene-based (R) or vertical disease resistance, respectively; however, the two forms are currently defined by a new terminology that found immediate acceptance within the scientific community. The primary immune response recognises common features of pathogens, such as flagellin, chitin, glycoproteins and lipopolysaccharides, referred to as Pathogen- or Microbial-Associated Molecular Patterns (PAMPs or MAMPs), and activate Pattern-Recognition Receptors (PRRs), which in turn initiate a multitude of downstream signalling events that result in the activation of a basal resistance named PAMP-Triggered Immunity (PTI; Figure 2a). In addition, plant molecules that are breakdown products of wounding or infection, known as Damage-Associated Molecular Patterns (DAMPs), can also induce PTI following interaction with host PRRs (Boller & Felix, 2009; Miller et al., 2017). During the co-evolutionary arms race between pathogens and their hosts, pathogens acquired effector molecules that are transported into the host cell to suppress PTI and promote virulence of the pathogen, resulting in Effector-Triggered Susceptibility (ETS; Fig 2b). In turn, plants acquired resistance (R) proteins

that recognize these attacker-specific effectors, resulting in a secondary immune response called Effector-Triggered Immunity (ETI; Fig. 2c) (Pieterse et al., 2009).

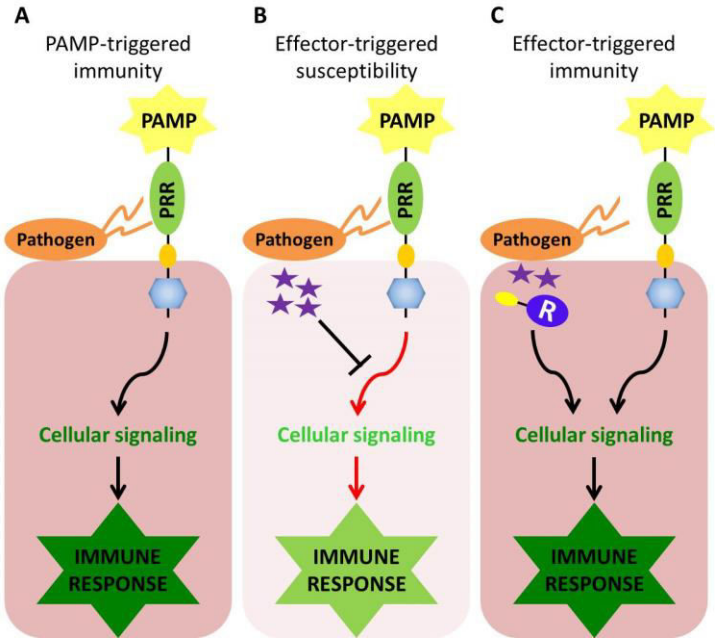

Figure 2. Simplified schematic representation of the plant immune system, modified from Pieterse et al. (2009).

The outcome of the battle depends on the balance between the ability of the pathogen to suppress the plant's immune system and the capacity of the plant to recognize the invader and to activate effective defences (Chisholm et al., 2006; Jones & Dangl, 2006; Gohre & Robatzek, 2008; Pieterse et al., 2009). Typically, PAMPs are widely conserved across genera, making the PTI responsible for non-host-specific resistance, whereas effectors and ETI are often strain-specific (van Wees et al., 2000; Spoel & Dong, 2012). Nevertheless, the nature of the defence responses activated during PTI and ETI shows substantial overlap (Tsuda et al., 2008), and immediately leads to transcriptional and metabolic modulations, such as cell wall fortification through the synthesis of callose and lignin (Kim et al., 2005) and production of antimicrobial secondary metabolites, among which phytoalexins (Harborne, 1999). Early cellular events comprise also the increase of $[Ca^{2+}]$ cytosolic (Lecourieux et al., 2002), the production of nitric oxide (NO) (Delledonne et al., 1998), and subsequent rapid activation of mitogen-activated protein kinases (MAPKs) (Jonak et al., 2002). The recognition of pathogen-specific effectors through the ETI system is followed by a burst

of Reactive Oxygen Species (ROS) that culminates in a programmed hypersensitive cell death and necrosis at the site of invasion, leading to local resistance, in order to keep the pathogen isolated from the rest of the plant and prevent further damage (DeWit, 1997; Spoel & Dong, 2012). This local hypersensitive response can also immunise plants (Spoel & Dong, 2012). Indeed, once plant defence responses are activated at the site of infection, a systemic defence response is induced in distal plant parts to protect undamaged tissues against subsequent attacks. This long-lasting and broad-spectrum resistance is referred to as Systemic Acquired Resistance (SAR) (Durrant & Dong, 2004), and is characterized by the coordinate activation of a specific set of Pathogenesis-Related (PR) genes, encoding PR proteins with antimicrobial activity, that currently comprise 17 families including defensin proteins and lytic enzymes such as chitinase, glucanase and protease (van Loon et al., 2006). The onset of SAR requires the accumulation of Salicylic Acid (SA) (Yalpani et al., 1991), locally at the primary site of infection and often also systemically in non-infected separated tissues (Mishina & Zeier, 2007; Tsuda et al., 2008), although SA does not appear to be the primary long-distance signal (Ryals et al., 1996). Beneficial soil-borne microorganisms, such as mycorrhizal fungi and plant growth–promoting rhizobacteria and their metabolites, can induce a phenotypically similar form of systemic immunity, known as Induced Systemic Resistance (ISR) (van Wees et al., 2008). Like those of pathogens, different beneficial MAMPs are recognized by the plant, resulting in a mild but effective activation of the immune response in systemic tissues (Pieterse et al., 1996; Pieterse et al., 1998; van Wees et al., 2000; Pieterse et al., 2009). In contrast to SA-dependent SAR, ISR triggered by beneficial microorganisms is commonly regulated by jasmonic acid (JA)- and ethylene (ET)-dependent signalling pathways (Pieterse et al., 2009), and provides advantages in terms of energy costs for the plant, because is associated with the priming, defined as the physiological state resulting in a stand-by of defences after an initial stimulus. In particular, primed plants show faster and/or stronger activation of defence responses when they are subsequently challenged by biotic or abiotic stress, which frequently results in a better tolerance compared to non-primed plants (Conrath et al., 2006; van der Ent et al., 2009). Subsequently, three endogenous plant signalling molecules, namely SA, JA and ET are involved in plant defence (Kunkel & Brooks, 2002). Evidences are emerging that SA-dependent and the SA-independent pathways do not function independently, but rather influence each other through a complex network of regulatory interactions (Kunkel & Brooks, 2002). In other words, the cross-talk between them provides great regulatory potential for

activating multiple resistance mechanisms in varying combinations (Pieterse & van Loon, 1999).

1.5 The induction of resistance in grapevine and Cucurbitaceae

Concerns about the negative impact of chemicals on human health and the environment have sparked increasing interest in developing safer alternative strategies. An attractive sustainable approaches to manage crop diseases is the boost of plant self-protection against pathogens, and therefore studies have increasingly targeted living microorganisms and exogenous molecules that are able to stimulate and/or potentiate the plant defence responses, called elicitors (Boller & Felix, 2009). Elicitors can be derived from biological origins or synthetic analogues of plant signalling molecules (Wiesel et al., 2014), and include compounds belonging to different chemical families, such as proteins and glycoproteins, glycans and lipids. They can be constituents of the pathogen or secreted by it, or they are released from the plant or pathogen cell walls by hydrolytic enzymes from the pathogen or the plant (Garcia-Brugger et al., 2006). The perception by the host cell of these warning signals activate a battery of defence reactions, including accumulation of host-synthesized phytoalexins, deposition of phenolics, lignin or callose-like materials, increased activity of PR proteins with hydrolytic activity, and often hypersensitive response. Altogether, these reactions are able to reduce the pathogen growth (Garcia-Brugger et al., 2006).

In last decades, several elicitor compounds have been tested on grapevine. In details, resistance mechanisms have been shown to be activated by the application of fosetyl-aluminum (Dercks & Creasy, 1989), ß-aminobutyric acid (BABA) (Hamiduzzaman et al., 2005) and benzothiadiazole-7-carbothioic acid S-methyl ester (BTH) (Perazzolli et al., 2008; Banani et al., 2014), as well as vitamins like thiamine and riboflavin (Boubakri et al., 2012; Boubakri et al., 2013). Likewise, bacterial elicitors such as flagellin and harpin proteins stimulated innate immunity in grapevine (Qiao et al., 2010; Chang & Nick, 2012; Trdà et al., 2014), and ß-1-3-glucan laminarins and sulfated laminarins derived from the brown alga *Laminaria digitata* were also proved to act as efficient elicitor agents (Aziz et al., 2003; Trouvelot et al., 2008; Trouvelot et al., 2014). Similarly, oligogalacturonides were shown to induce grapevine resistance against *B. cinerea* (Aziz et al., 2004; Allegre et al., 2009), as well as native or sulfated oligoglucuronans (Caillot et al., 2012), elicitins from *Pythium oligandrum* (Mohamed et al., 2007), rhamnolipids (Varnier et al., 2009) and ergosterol (Laquitaine et al., 2006).

Likewise, chitin-derivative (chitosan) elicitors were effective against *B. cinerea* and *P. viticola* (Repka, 2001; Aziz et al., 2006; Trotel-Aziz et al., 2006). The resistance against grapevine downy mildew was induced by beneficial microorganisms such, as *Trichoderma harzianum* T39 (Perazzolli et al., 2008; Perazzolli et al., 2011; Perazzolli et al., 2012) and *Aureobasidium pullulans* (Harm et al., 2011), as well as by plant extracts, for example *Rheum palmatum* (Godard et al., 2009) and *Solidago canadensis* extracts (Harm et al., 2011), and other organic amendments (Thuerig et al., 2011). An optimized chitosan formulation (Iriti et al., 2011) and a complex of chitosan fragments (COS-OGA) reduced grapevine powdery mildew severity (van Aubel et al., 2014). Likewise, an extract from the green macroalga *Ulva armoricana* controlled powdery mildew by inducing plant defence through the JA pathway (Jaulneau et al., 2010; Jaulneau et al., 2011). Moreover, knotweed extracts (giant Milsana) were reported as resistance inducers with moderate efficacy against powdery mildew (Delaunois et al., 2014). BTH activated grapevine resistance also against powdery mildew (Dufour et al., 2013).

Likewise, several elicitor compounds have been experimented on Cucurbitaceae. In particular, the systemic resistance has been induced by spraying cucumber leaves with solutions of oxalate or phosphate salts (Mucharromah & Kuc, 1991). More in details, foliar spray of phosphate salts (Reuveni et al., 1993) and application of phosphate through a hydroponic system (Reuveni et al., 2000) induced systemic resistance against powdery mildew on cucumber plants. Likewise, BABA was demonstrated to activate defence mechanisms in Cucurbitaceae against several pathogens (Cohen, 2002; Zeighaminejad et al., 2016), and a laminarin-based product was proved to manage powdery mildew in squash (Zhang et al., 2016). Application of osthol, a cumarinic compound extracted from dried fruits of *Cnidii monnieri*, induces a resistance response against powdery mildew in pumpkin leaves (Shi et al., 2007), and the product Milsana, the commercial name given to extracts from leaves of the giant knotweed *Reynouthria sachalinensis*, induced resistance in powdery mildew-infected cucumber plants, correlating with the induction of chalcone synthase and chalcone isomerase (Fofana et al., 2002). Other chemical inducers for resistance induction against powdery mildew of cucumber under greenhouse conditions are flusilazole, SA, potassium dihydrogen phosphate, magnesium sulfate, ferrous sulfate, oxalic acid and potassium monohydrogen phosphate (Hamza et al., 2017). Several microbiological strategies have been proposed to control powdery mildew in cucurbits, using mycoparasites or microbial antagonists, such as *Bacillus spp*. (Romero et al., 2004;

Romero et al., 2007), *Streptomyces lydicus* (S. A. Zhang et al., 2011) and *Ampelomyces quisqualis* (Angeli et al., 2016), but little is known about the use of microorganisms as resistance inducers. Prior inoculation with avirulent or hypovirulent pathogens or with non-pathogenic isolates, such as *Alternaria cucumarina* or *Cladosporium fulvum*, activated resistance mechanisms in the non-inoculated leaves, and provided long-lasting protection (Reuveni & Reuveni, 2000). Likewise, the induction of systemic defence responses against *P. xanthii* was observed after foliar application of a water extract of a species of the cyanobacterium *Anabaena* (Roberti et al., 2015) on *C. Pepo.* Furthermore, specific strains of *Bacillus spp*. can elicit resistance that results in reduction in disease severity in watermelon and cucumber by a broad range of pathogens (Kloepper et al., 2004).

In agriculture, treatments of plants with elicitors in the absence of virulent pathogens can lead to a defence response, and provide protection against subsequent pathogen challenges (Wiesel et al., 2014). However, elicitors confers highly variable and incomplete protection (40–80%), while conventional pesticides have a protective efficacy of around 90–100% (Dagostin et al., 2011). This could be explained by their composition, which includes molecules with broader modes of action compared to conventional molecules targeting metabolic cycles in specific pathogens. Moreover, the variability of the results could be related to external conditions, and environment/plant/pathogen systems (Atkinson & Urwin, 2012).

1.6 Protein-based products and their physicochemical properties

In the past two decades, increasing attention has been paid to the bioactive role of protein-based products manufactured from various sources (Clemente, 2000). Among others, protein hydrolysates are mixtures of active peptide fragments and free amino acids obtained by hydrolysis of protein contained in agro-industrial by-products of animals (i.e. leather, viscera, feathers, blood, collagen 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 (Maini, 2006; Schaafsma, 2009; Calvo et al., 2014; Colla et al., 2015). Proteolysis enhances the functional properties of the original protein (Chabanon et al., 2007), allowing the activation of the latent biological activities of peptides encrypted in the protein structure (Sinha et al., 2007). The biological activity of protein hydrolysates is modulated by changes in amino acid and peptide compositions (Jamdar et al., 2010). Indeed, the efficiency of a protein hydrolysate is

linked to the type and composition of peptides generated during hydrolysis (Mahmoud, 1994; Panyam & Kilara, 1996), and peptide functionalities depend on molecular size, structure and amino acid sequences (Chabanon et al., 2007). 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 (Adler-Nissen, 1982; Cheison et al., 2009). Indeed, 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 (Spellman et al., 2003). Characteristics and functional properties of hydrolysates are influenced by the method used for hydrolysis, and by the choice of the original protein source (Pecha et al., 2012). The chemical hydrolysis, performed under acid or alkaline conditions, is favourable from an economical point of view, but it presents several disadvantages compared to the enzymatic process (Lisiecka et al., 2011; Colla et al., 2015). Because the high temperatures used, the process is very aggressive, and attacks randomly all peptide bonds of proteins, leading to the destruction of some essential amino acids (e.g. tryptophan) and other thermolabile compounds (e.g. vitamins) (Colla et al., 2015), and produces a high content of free amino acids in total (high DH), which can encumber the final osmotic balance (Jeewanthi et al., 2015). Another critical aspect of the chemical hydrolysis is the phenomenon called racemisation, namely the conversion of free amino acids from L-form to D-form, which cannot be used from plants in their metabolism, making the hydrolysate less effective or even potentially toxic for plants (Chen et al., 2016). Finally, the use of strong acids or alkalis during hydrolysis causes an increase of salinity of protein hydrolysates (Colla et al., 2015). On the other hand, the enzymatic hydrolysis is performed by proteolytic enzymes, which hydrolyse proteins more gently. They do not need high temperature to exert their function, and usually target specific peptide bonds, producing low-salted well-defined mixtures of amino acids and peptides of different length. Finally, enzymatic hydrolysis requires low energy, resulting more environmental friendly (Colla et al., 2015). Moreover, the biological activity of hydrolysates can be affected by the choice of the original protein source (Pecha et al., 2012). Cerdan et al. (2009) demonstrated that repeated foliar applications of animal-derived protein hydrolysates caused phytotoxic effect on plant growth, and this negative effect could be attributed to an unbalanced amino acid composition (Oaks et al., 1977), a higher concentration of free amino acids (Moe, 2013) and a high salinity (Colla et al., 2014). Because the use of animal-derived protein hydrolysates generates heavy concerns in terms of food safety, the European

Regulation 354/2014 recently prohibited the application of these products on the edible parts of organic crop. Conversely, specific peptides from plant origin have been demonstrated to act as non-toxic signalling molecules for plant defence, growth and development (Ryan et al., 2002). Compared to animal-based product, plant-derived protein hydrolysates present a higher amount of minerals and other compounds, which can be involved in the biocontrol action, such as fats, carbohydrates and phenols (Ertani et al., 2013).

1.6.1 The biostimulant action of protein-based products

The products manufactured from animal- (Kumar et al., 2013) and plant-derived proteins (Gibbs et al., 2004) are widely investigated as natural antioxidant compounds, with interesting applications in food science and nutrition and pharmaceutical preparations, and influence numerous biological processes, evoking hormonal and immunological responses (Phelan et al., 2009). Moreover, protein hydrolysates produce biostimulant effects on crops, especially under stressed environmental conditions (du Jardin, 2015; EBIC, 2017), decreasing the need of chemical fertilizers by up to 50% (Subbarao et al., 2015). When applied to plants, they are mostly used as foliar applications, but soil applications and seed coating also exist with some of them (du Jardin, 2015). In particular, foliar applied biostimulants were shown to reach mesophyll cells by absorption through cuticle and epidermal cells (Fernández & Brown, 2013), and when supplied through soil, the absorption occurs through root epidermal cells and gets redistributed through xylem (Chen, 1964). Although they are commonly used in small quantities, bioactive peptide fragments and amino acids can be readily absorbed through diffusion processes and easily reach active sites (Stiegler et al., 2013). The protein hydrolysates can act as growth regulators, and improve the performance of several horticultural crops, by enhancing the activity of the antioxidant system and boost plant metabolism, including increased shoot and root biomass, thus promoting the productivity and fruit quality of several crops (Lisiecka et al., 2011; Colla et al., 2014; Ertani et al., 2014; Colla et al., 2015). For example, the treatment of plant leaves and roots with protein hydrolysates has been demonstrated to increase nitrogen and iron metabolism, nutrient uptake, and water and nutrient use efficiencies for both macro and microelements (Ertani et al., 2009; Cerdan et al., 2013). Their effects on carbon and nitrogen metabolism, and plant primary and secondary metabolism have been also reported by Maini (2006), Parrado et al. (2008) and Schiavon et al. (2008).

Owing to the presence of specific peptides and precursors of phytohormone biosynthesis, such as tryptophan, protein hydrolysates could also interfere with the phytohormone balance of the plant, thus affecting plant development (Colla et al., 2014). In addition, several bioactive peptides have been identified to have hormone-like activities (Ito et al., 2006; Kondo et al., 2006), and the application of plant-derived protein hydrolysates triggered auxin- and gibberellin-like activities, promoting crop performances (Schiavon et al., 2008). Moreover, protein hydrolysates have been shown to improve the quality of fruits and vegetables in terms of phytochemicals (i.e. carotenoids, flavonoids, polyphenols) (Ertani et al., 2014), and to reduce undesired compounds, such as nitrates (Liu et al., 2007). Finally, protein hydrolysates applications have been displayed to avoid or reduce losses in production caused by unfavourable soil conditions and environmental stresses, including thermal stress, salinity, drought, alkalinity, and nutrient deficiency (Colla et al., 2014). More in details, the root application of plant-derived protein hydrolysates has been proved to facilitate the uptake and subsequent assimilation of iron in plants grown under an iron-deficiency situation (Cerdan et al., 2013), and treatments with a biostimulant composed of a complex of vitamins, amino acids, proteins and betaines produced positive effects on drought-stressed tomato plants in terms of the biomass production and chlorophyll fluorescence (Petrozza et al., 2014). Moreover, plant-protein hyrolysates increased plant yield and biomass even when plants are grown under salinity conditions, stimulating plant nitrogen metabolism and antioxidant systems (Ertani et al., 2013; Lucini et al., 2015).

1.6.2 Protein-based products as inducers of plant resistance

Beyond their well-known biostimulant activity, protein hydrolysates and peptides from various sources can act as initial triggers, mediators or amplifiers of plant immunity, and increasing attention has recently been devoted to investigation of their bioactive role in plant defence (Albert, 2013). Several peptides originating from microbial pathogens can act as MAMPs and activate the plant innate immune responses (Huffaker et al., 2006), including fungal elicitors like Pep13 (Brunner et al., 2002), AVR9 (Vandenackerveken et al., 1993) and elicitins (Kamoun, 2001), and bacterial elicitors like elf18 (Kunze et al., 2004), flg22 (Navarro et al., 2004), NPP1 (Fellbrich et al., 2002) and hrpZ (He et al., 1993). Moreover, endogenous plant peptides generated as degradation products from precursor proteins during pathogen

infection were demonstrated to act as DAMPs, showing a similar mode of action (Yamaguchi & Huffaker, 2011; Bartels & Boller, 2015; Choi & Klessig, 2016). A recent review classifies peptides relevant in plant defence response according to their origin (Albert, 2013). In details, the active form of systemin in Solanaceae family (Pearce et al., 1991; Schaller & Ryan, 1996), and the peptide *At*Pep1 in *Arabidopsis thaliana* (Huffaker et al., 2006; Pearce et al., 2008) and its homologue *Zm*Pep1 in maize (Huffaker et al., 2011) were shown to be internal signals for plant defence mechanisms derived from cytosolic proteins. A similar function was demonstrated for peptides originating from secreted precursors, such as hydroxyproline-rich systemins in potato (Bhattacharya et al., 2013) and phytosulphokines in *A. thaliana* (Igarashi et al., 2012). Finally, several peptides released from the degradation of proteins with separate primary functions were shown to elicit plant defence responses, such as the inceptin family in the cowpea (Schmelz et al., 2007), and other peptide fragments like a subtilisin-like protein in the soybean (Pearce et al., 2010; Yamaguchi & Huffaker, 2011). All of them are active as elicitors, and evoke typical molecular steps involved in the immune signalling pathway (Ma et al., 2013), such as the increase of cytosolic [Ca^{2+}], the production of NO and ROS, the activation of MAPKs and the expression of typical defence marker genes (Schaller, 2001; Albert, 2013). Recently, casein and soybean hydrolysates have been shown to control green mould of citrus (Lachhab et al., 2015), and elicit grapevine defence mechanisms against downy mildew (Lachhab et al., 2014) and grey mould caused by *B. cinerea* (Lachhab et al., 2016) by the up-regulation of *PR* genes. Likewise, a protein derivative was effective in controlling the powdery mildew of courgette and grapevine under file conditions, and activated the expression of defence-related genes in grapevine, suggesting the stimulation of plant resistance mechanisms (Nesler et al., 2015). 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 (Pecha et al., 2012; Baglieri et al., 2014).

1.7 The phyllosphere microbiota

Besides the biostimulant effect and plant resistance activation, proteins and peptides contained in hydrolysates can also serve as nutritional substrate for microbial

phyllosphere populations (Colla et al., 2015). Indeed, plant leaves are naturally inhabited by epiphytic communities of bacteria and fungi, which metabolize resources such as carbohydrates, small peptide fragments, amino acids and organic acids passively leaked by plants (Leveau & Lindow, 2001; Trouvelot et al., 2014). Their composition and functional properties can be affected by environmental (e.g. UV radiation, pollution, nitrogen fertilization, water availability) and biotic (e.g. leaf age, invading microorganisms, host genotype) factors (Lindow & Brandl, 2003; Vorholt, 2012; Copeland et al., 2015), as well as by farming practices and applications of pesticides and fertilizers (Berlec, 2012). Most phyllosphere-colonizing microorganisms live as beneficial commensals on their host plants (Mueller & Ruppel, 2014), and frequently show positive influences on plant health and growth (Penuelas & Terradas, 2014). Specifically, epiphytes of aerial plant surfaces are involved in processes as carbon and nitrogen cycle cycles (Lindow & Brandl, 2003), and live closely related to the air microbiome, especially to air-borne pathogens (Lindow & Brandl, 2003; Rastogi et al., 2013). In addition, some phyllosphere microorganisms are recognised to act as natural biological control agents, thanks to their ability to reinforce natural plant defences and to their antagonism to pathogens (Vorholt, 2012; Ritpitakphong et al., 2016) through the induction of plant resistance, production of antimicrobial compounds, competition for space and nutrients, parasitism, or by combinations of these mechanisms (Pal & McSpadden Gardener, 2006). In this respect, the phyllosphere represents a niche with great agricultural and environmental significance (Whipps et al., 2008), and many studies have recently shown that plant-microbe interactions are not only crucial for better investigating plant growth and health, but also to develop new sustainable strategies in crop protection (Berg et al., 2014). Understanding the population dynamic balance between the organisms of the phyllosphere as an ecological system should lead to new approaches in agronomy, crop protection and breeding that enhance sustainability (Newton et al., 2010). In details, grapevine is naturally colonised by a wide variety of both prokaryotic and eukaryotic microorganisms that interact with the plant, having either beneficial or phytopathogenic effects, and play a great role in fruit yield, grape quality and evolution of grape fermentation and wine production, affecting the final quality (Pinto et al., 2014). In addition, the microbial community can activate the plant defence pathways, inducing the accumulation of PR proteins of grapevine as a protection against fungal pathogen attacks or other biological stresses (Ferreira et al., 2004).

1.8 Aim of the study

Increasing concerns about the negative impacts of chemical pesticides on human health and the environment require the development of safer alternatives, and scientific community have turned the attention to more sustainable pest management strategies, including the use of low-risk substances and biopesticides. Besides the biostimulant action, protein-based products contain a large variety of amino acids and bioactive peptide fragments which could potentially be recognised by plants as elicitors and act as stimulators of plant immunity.

The final objective of the current doctoral project was to provide new insights on the application of bioactive protein-based products against crop diseases, to further develop new sustainable alternatives to be included in organic integrated pest management programs. For this purpose, my doctoral thesis critically presents the current regulations for the biopesticides registration, describing the procedures required in Europe and overseas for their authorization process. In order to analyse the multiple mechanism of action of the protein derivative nutrient broth (NB), recently proved to control the grapevine powdery mildew in vineyards by the activation of innate defence responses, we tested its efficacy against grapevine downy mildew symptoms and its ability to up-regulate some defence-related genes on *in vitro*- and greenhouse grown-grown plants, using a standard resistance inducer based on laminarins (LAM) as control. Moreover, a metabarcoding approach on NB- and LAM-treated plants was performed to investigate the possible contribution of the phyllosphere microbiota (Chapter 3). Since the use of animal-derived products generates heavy concerns in terms of food safety, the current doctoral project focused on the analysis of the biocontrol activity of plant-derived protein hydrolysates (Chapter 4). Initially, we optimised an experimental procedure to create hydrolysates by testing different hydrolysis methods and protein sources, namely low-cost agro-industrial by-products such as soybean, rapeseed and guar meals, commonly used in animal feeding fot their high protein content. Then, we investigated the potential contribution of amino acids and peptide fragments generated during hydrolysis to the activation of plant resistance, in order to compare the effect of acid and enzymatic hydrolysis in terms of biocontrol efficacy against the powdery mildew of Cucurbitaceae under greenhouse conditions.

CHAPTER 2.

Leaf treatments with a protein-based resistance inducer partially modify phyllosphere microbial communities of grapevine

Published on Frontiers in Plant Science
Vol. 7, Art. 1053
July 19, 2016
https://doi.org/10.3389/fpls.2016.01053

frontiers in Plant Science

ORIGINAL RESEARCH
published: 19 July 2016
doi: 10.3389/fpls.2016.01053

Leaf Treatments with a Protein-Based Resistance Inducer Partially Modify Phyllosphere Microbial Communities of Grapevine

Martina Cappelletti[1,2†], *Michele Perazzolli*[1*†], *Livio Antonielli*[3], *Andrea Nesler*[1], *Esmeralda Torboli*[1], *Pier L. Bianchedi*[4], *Massimo Pindo*[1], *Gerardo Puopolo*[1] *and Ilaria Pertot*[1]

[1] Department of Sustainable Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy, [2] Department of Agricultural and Environmental Sciences, University of Udine, Udine, Italy, [3] Bioresources Unit, Department of Health and Environment, Austrian Institute of Technology, Tulln and der Donau, Austria, [4] Technology Transfer Center, Fondazione Edmund Mach, San Michele all'Adige, Italy

OPEN ACCESS

Edited by:
Victor Flors,
Universitat Jaume I, Spain

Reviewed by:
Biswapriya Biswavas Misra,
University of Florida, USA
Jaime Cubero,
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain

***Correspondence:**
Michele Perazzolli
michele.perazzolli@fmach.it

[†] These authors have contributed equally to this work.

Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: 16 May 2016
Accepted: 05 July 2016
Published: 19 July 2016

Citation:
Cappelletti M, Perazzolli M, Antonielli L, Nesler A, Torboli E, Bianchedi PL, Pindo M, Puopolo G and Pertot I (2016) Leaf Treatments with a Protein-Based Resistance Inducer Partially Modify Phyllosphere Microbial Communities of Grapevine. Front. Plant Sci. 7:1053. doi: 10.3389/fpls.2016.01053

Protein derivatives and carbohydrates can stimulate plant growth, increase stress tolerance, and activate plant defense mechanisms. However, these molecules can also act as a nutritional substrate for microbial communities living on the plant phyllosphere and possibly affect their biocontrol activity against pathogens. We investigated the mechanisms of action of a protein derivative (nutrient broth, NB) against grapevine downy mildew, specifically focusing on the effects of foliar treatments on plant defense stimulation and on the composition and biocontrol features of the phyllosphere microbial populations. NB reduced downy mildew symptoms and induced the expression of defense-related genes in greenhouse- and *in vitro*-grown plants, indicating the activation of grapevine resistance mechanisms. Furthermore, NB increased the number of culturable phyllosphere bacteria and altered the composition of bacterial and fungal populations on leaves of greenhouse-grown plants. Although, NB-induced changes on microbial populations were affected by the structure of indigenous communities originally residing on grapevine leaves, degrees of disease reduction and defense gene modulation were consistent among the experiments. Thus, modifications in the structure of phyllosphere populations caused by NB application could partially contribute to downy mildew control by competition for space or other biocontrol strategies. Particularly, changes in the abundance of phyllosphere microorganisms may provide a contribution to resistance induction, partially affecting the hormone-mediated signaling pathways involved. Modifying phyllosphere populations by increasing natural biocontrol agents with the application of selected nutritional factors can open new opportunities in terms of sustainable plant protection strategies.

Keywords: resistance induction, gene expression, *Vitis vinifera*, *Plasmopara viticola*, phyllosphere microbiota, biological control

INTRODUCTION

Increasing concerns about the negative impacts of chemical pesticides on human health and the environment require the development of safe alternatives to conventional disease control methods (Fantke et al., 2012). Elicitors, including beneficial microorganisms and exogenous molecules of both biological and synthetic origin, can stimulate plant defenses against pathogens (Boller and Felix, 2009; Walters et al., 2013), and they represent some of the most promising complementary/alternative strategies to reduce the massive use of fungicides (Delaunois et al., 2014). The mechanism of action of these compounds relies on the rapid activation of sophisticated defense systems after perception by plant cells, leading to specific transcriptional and metabolic modulations, such as the up-regulation of genes encoding pathogenesis related (PR) proteins (Wu et al., 2014). Two main pathways are known to be activated by elicitors: the systemic acquired resistance that is mediated by salicylic acid (SA)-dependent processes, and the induced systemic resistance that is mediated by jasmonic acid (JA)- and ethylene-regulated pathways (Pieterse et al., 2009; Walters et al., 2013).

Because grapevine (*Vitis vinifera* L.) is a major fruit crop worldwide, it represents an ideal model for studying interactions between the plant, elicitor and pathogen. Commercial grapevine cultivars are highly susceptible to a destructive disease (namely downy mildew) caused by the obligate parasite *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni, and require frequent fungicide applications to avoid yield and quality losses (Gessler et al., 2011). Several elicitors are known to activate defense responses against grapevine downy mildew, including *Solidago canadensis* extracts (Harm et al., 2011), organic amendments (Thuerig et al., 2011), and fungi such as *Trichoderma harzianum* T39 (Perazzolli et al., 2008), or *Aureobasidium pullulans* (Harm et al., 2011). Likewise, the application of fosetyl-aluminum (Dercks and Creasy, 1989), ß-aminobutyric acid (Hamiduzzaman et al., 2005), and benzothiadiazole-7-carbothioic acid S-methyl ester (Perazzolli et al., 2008) has been shown to induce grapevine resistance mechanisms. Bacterial proteins, such as harpin and flagellin (Qiao et al., 2010; Chang and Nick, 2012; Trdà et al., 2014), oligosaccharides, as oligogalacturonide (Allegre et al., 2009), and vitamins, such as thiamine and riboflavin (Boubakri et al., 2012, 2013), have been demonstrated to elicit grapevine defense responses. Resistance induction to downy mildew in grapevine includes primarily the up-regulation of defense-related genes, such as genes encoding PR-1, PR-2, PR-4, chitinase 3 (CHIT-3), and osmotin (OSM-1 and OSM-2) proteins (Perazzolli et al., 2011, 2012).

Abbreviations: CAP, canonical analysis of principal coordinates; CFU, colony forming units; CHIT-3, chitinase 3; Exp1, experiment 1; Exp2, Experiment 2; H_2O, water; ITS, internal transcribed spacer; JA, jasmonic acid; LAM, commercial laminarin-based product; NB, nutrient broth; OSM-1, osmotin 1; OTU, operational taxonomic units; PERMANOVA, permutational multivariate analysis of variance; PR, pathogenesis related; PR-1, pathogenesis related protein 1; PR-2, pathogenesis related protein 2; PR-4, pathogenesis related protein 4; qPCR, quantitative real-time PCR; SA, salicylic acid; T0, just before *Plasmopara viticola* inoculation; T1, 1 day after *Plasmopara viticola* inoculation; UNT, untreate

Generally speaking, carbohydrates and proteins or peptides represent a wide category of plant resistance inducers (Albert, 2013; Trouvelot et al., 2014). Protein hydrolysates consist of a mixture of small peptide fragments and free amino acids, originating from animal, plant and microbial proteins by chemical or enzymatic hydrolysis, which could act as biostimulants by influencing the growth process or by directly stimulating plant defense responses (Colla et al., 2015). For example, some plant-derived protein hydrolysates have been shown to increase the activity of the plant antioxidant system and others have demonstrated beneficial effects on plant metabolism, including shoot and root growth (Colla et al., 2015). Recently, casein and soybean hydrolysates have been shown to act as elicitors of grapevine defense mechanisms against downy mildew and gray mold by the up-regulation of *PR* genes (Lachhab et al., 2014, 2016). Likewise, a protein derivative (nutrient broth, NB) showed a high efficacy in controlling powdery mildew in vineyards by inducing the expression of defense-related genes which demonstrate stimulation of plant defense mechanisms (Nesler et al., 2015). Similarly, increasing interest has been devoted to the use of carbohydrates to stimulate plant resistance against diseases, either as elicitors of plant defenses or signaling molecules that mimic phytohormones (Trouvelot et al., 2014). For instance, chitin, chitosan, oligogalacturonides, and storage polysaccharides, such as ß-1-3 glucans extracted from the brown alga *Laminaria digitata* (namely laminarin), have been reported to stimulate plant defense reactions against several phytopathogens (Trouvelot et al., 2014). In grapevine, chitosan (Aziz et al., 2006), laminarin (Aziz et al., 2003), sulfated laminarin (Trouvelot et al., 2008), β-glucans and oligogalacturonides (Allegre et al., 2009) act as resistance inducers against *P. viticola*. In addition to plant resistance activation, protein hydrolysates (Colla et al., 2015) and carbohydrates (Trouvelot et al., 2014) can also serve as nutritional sources for microbial phyllosphere communities. Indeed, plant leaves are colonized by complex microbial communities, whose structure can be affected by environmental (such as UV radiation, pollution, and nitrogen fertilization) and biotic (such as leaf age and invading microorganisms) factors (Vorholt, 2012). When protein- or carbohydrate-based treatments are applied on leaves, they may change the nutrient availability, exerting a selective pressure on structure, dynamics, and functional properties of phyllosphere communities. Phyllosphere microbial communities frequently show positive influences on plant health and growth (Peñuelas and Terradas, 2014). For example, some phyllosphere microorganisms are regarded as natural biological control agents, thanks to their ability to reinforce natural plant defenses and to their antagonism to pathogens (Vorholt, 2012; Ritpitakphong et al., 2016) through the production of antimicrobial compounds, competition for space and nutrients, parasitism, or by combinations of these mechanisms (Pal and McSpadden Gardener, 2006).

The aim of this research was to understand whether the efficacy of NB against pathogens is related only to induction of resistance on grapevine or also to an indirect effect from modifications of leaf microbial communities. Grapevine downy mildew was selected as the study pathosystem. The effect of NB

foliar application was assessed on plants in the absence (axenic conditions) and in the presence of phyllosphere microorganisms (greenhouse conditions), and its impact on natural grapevine phyllosphere microbiome was evaluated with both culture dependent and independent approaches in comparison to the application of a laminarin-based product (LAM) as a reference for resistance induction.

MATERIALS AND METHODS

Grapevine Treatments and Pathogen Inoculation *In vitro*

Grapevine rooted cuttings (Pinot noir ENTAV115) were grown *in vitro* on Murashige-Skoog medium half dose with 3% sucrose and 0.6% agarose in De Wit cultures tubes (Duchefa Biochemie, Haarlem, The Netherlands) for 1 month in a growth chamber at 23 ± 1°C with a photoperiod of 16 h of light. Plants were treated with sterilized water (H_2O) or with a sterilized solution of 3.0 g/l NB. NB was obtained by mixing three commercial extracts commonly used as nutritional substrates in microbiological media: 0.4 g/l meat extract (product code 70164, Fluka, Sigma-Aldrich, St. Louis, MO, USA), 0.7 g/l yeast extract (product code 70161, Fluka, Sigma-Aldrich) and 1.9 g/l peptone (product code 70175, Fluka, Sigma-Aldrich), and this application dosage was previously optimized against grapevine powdery mildew (Nesler et al., 2015).

Each leaf of *in vitro*-grown plants was treated with six to eight drops (20 µl each) of H_2O or NB on the abaxial and adaxial surface and plants were incubated for 3 days in the growth chamber to maximize the phenotypic response of grapevine induced resistance (Perazzolli et al., 2008; Nesler et al., 2015). Each leaf was then dried with a sterile filter paper under sterile conditions and immediately inoculated with a sterile suspension of *P. viticola* (4×10^4 sporangia/ml) as described by Algarra Alarcon et al. (2015) and the disease severity was assessed visually as percentage of leaf area covered by sporulation after 7 days (EPPO, 2001). For gene expression analyses, samples were collected in triplicates just before (T0) and 1 day after (T1) *P. viticola* inoculation. This time point was chosen because it is associated with leaf colonization by primary hyphae (Lenzi et al., 2015) and with modulation of defense-related genes for the establishment of resistance responses (Hamiduzzaman et al., 2005; Trouvelot et al., 2008; Polesani et al., 2010; Perazzolli et al., 2012). Each sample comprised two leaves from the second-fourth node of one plant. Six plants were analyzed for each treatment in a randomized complete block design and the experiment was carried out twice.

Grapevine Treatments and Pathogen Inoculation under Greenhouse Conditions

Two-year-old plants of the susceptible grapevine cultivar Pinot noir ENTAV115 grafted onto Kober 5BB were grown for 2 months under greenhouse conditions as described by Perazzolli et al. (2012). Plants were kept untreated (UNT) or treated with H_2O, 3.0 g/l of NB, or 0.75 ml/l laminarin-based commercial product (LAM, dosage according to the manufacturer's instruction of Vacciplant, Belchim Crop Protection, Londerzeel, Belgium) used as a reference of resistance inducers from natural origin in grapevine (Aziz et al., 2003). Treatments were applied for three consecutive days (1, 2, and 3 days before *P. viticola* inoculation), in order to maximize the phenotypic response of grapevine induced resistance (Perazzolli et al., 2008; Nesler et al., 2015). One day after the last treatment, plants were inoculated as described by Perazzolli et al. (2012), and the disease severity was assessed visually after 7 days (EPPO, 2001). The disease reduction (efficacy) was calculated according to the following formula: (disease severity of H_2O-treated plants—disease severity in plants treated with a tested molecule)/(disease severity of H_2O-treated plants) × 100. Three replicates (pool of two plants each) were collected just before (T0) and 1 day after (T1) *P. viticola* inoculation for each treatment. Each sample comprised four half-leaves (collected from the fourth-sixth node of two plants) and 50 leaves (randomly collected from two plants) for the gene expression and microbial community analysis, respectively. Twelve plants were analyzed for each treatment in a randomized complete block design, and the experiment was carried out twice (namely Exp 1 and Exp 2). Under greenhouse conditions, UNT samples were used to compare indigenous microbial populations originally residing on grapevine leaves in the two different experiments, while effects of treatments tested were evaluated considering H_2O-treated plants as reference control at each time point.

RNA Extraction and Gene Expression Analyses

Total RNA extraction, DNase treatment, cDNA synthesis, and quantitative real-time PCR (qPCR) reactions were carried out as previously described (Lenzi et al., 2015) using specific primers (Table S1). Cycle threshold values and reaction efficiencies were calculated with the LightCycler 480 SV1.5.0 software (Roche, Branford, CT, USA) and the LinRegPCR 11.1 software (Ruijter et al., 2009), respectively. Relative expression levels of each gene were calculated with the Pfaffl equation (Pfaffl, 2001) on three replicates and two independent experiments, using the grapevine *Actin* gene for normalization (Polesani et al., 2010; Perazzolli et al., 2012).

Isolation of Grapevine Phyllosphere Microorganisms

Phyllosphere microorganisms were collected by leaf washing as described by Perazzolli et al. (2014). Each sample was plated on Nutrient Agar supplemented with 100 mg/l cycloheximide and on Potato Dextrose Agar supplemented with 0.25% lactic acid to isolate culturable bacteria and fungi, respectively. Plates were incubated at 25°C for 48 h, the colony forming units (CFU) per unit of leaf area (CFU/cm2) were calculated, and representative isolates were selected visually for each treatment and experiment based on morphological analysis of bacterial colonies.

Functional Characterization of Culturable Phyllosphere Bacteria

Proteolytic activity, siderophore production and antagonist activity against *Phytophthora infestans* were evaluated for culturable bacteria as described by Puopolo et al. (2010). Three replicates were analyzed for each bacterial isolate and each assay was carried out twice.

For the assay against *P. viticola*, each bacterial isolate was grown in 1 ml of Luria Bertani medium under orbital shaking at 80 rpm at 27°C for 24 h. The bacterial suspension was centrifuged (5000 g for 15 min), washed three times in 1 ml of isotonic solution (NaCl 0.85%), and adjusted to an optical density of 0.2 at 600 nm. Each bacterial suspension was mixed with an equal volume of a sterile suspension of *P. viticola* sporangia (2×10^4 sporangia/ml). Surface-sterilized leaf disks were prepared according to Perazzolli et al. (2014), inoculated with three 10 µl-drops of inoculum suspension for each disk and incubated overnight in the dark at 25 ± 1°C. Disks were dried under laminar flow and incubated under greenhouse conditions for 7 days before visual assessment of disease severity (EPPO, 2001). Five replicates (five dishes with five leaf disks each) were analyzed for each bacterial isolate and the experiment was carried out twice. The two bacterial isolates with biocontrol activity against *P. viticola* were identified by amplification of the V6-V8 region of the 16S rRNA by colony PCR, followed by sequencing with an ABI PRISM 3730xl DNA analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and alignment against the database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Sequences were deposited at the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra) under the accession numbers KU596386 (*Pseudomonas* spp. isolate T1_NB_7 of Exp 1) and KU596387 (*Enterobacter* spp. isolate T1_NB_13 of Exp 2).

DNA Extraction, Amplification, and Pyrosequencing

Microbial pellets were obtained from leaf-washing suspensions as described by Perazzolli et al. (2014), and DNA was extracted from microbial pellets using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA). Bacterial sequences were amplified with primer pairs (Pinto et al., 2014) that amplify the V6-V8 region of the 16S rRNA (Baker et al., 2003), and fungal sequences were amplified with primer pairs that align to the ITS3 and ITS4 regions of the internal transcribed spacer (ITS) fragment (White et al., 1990). Fusion primers with the Lib-L Primer sequences for unidirectional pyrosequencing (Roche) were used (Table S2), and amplicons were obtained from 100 ng of extracted DNA, using the FastStart High-Fidelity PCR system (Roche) with 0.25 mM deoxynucleoside triphosphates, 1% (w/v) bovine serum albumin, 4% (v/v) dimethyl sulfoxide, 0.3 µM of each primer, and 2.5 U of FastStart High-Fidelity DNA polymerase (Roche) in 50 µl of reaction. Amplification reactions were carried out in triplicate with the following protocol: denaturation at 95°C for 5 min, 32 cycles of amplification at 95°C for 30 s, annealing at 60 and 58°C for 1 min for bacteria and fungi, respectively, extension at 72°C for 45 s, and final extension at 72°C for 10 min. No amplification of 16S and ITS fragments was obtained from leaf-washing suspensions of *in vitro*-propagated plants, confirming that these plants were grown under axenic conditions.

Library construction and pyrosequencing were carried out as described by Perazzolli et al. (2014). Briefly, PCR products were purified using an AMPure XP bead kit (Beckman Coulter, Brea, CA, USA), quantified using a Roche 454 Titanium library quantification kit (KAPA Biosystems, Boston, MA, USA) and pyrosequenced using a GS FLX+ system (Roche) with the XL+ chemistry (Roche). Sequences have been deposited at the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra) under the accession number SRP065898 and BioProject number PRJNA301108.

Bioinformatics Analysis and 16S rRNA Gene and Its Sequence Processing

Bacterial and fungal sequences were processes as reported by Touceda-Gonzalez et al. (2015) with some modifications. Sequence quality check and filtering were carried out with PRINSEQ (Schmieder and Edwards, 2011) (http://prinseq.sourceforge.net/) and FlowClus (https://github.com/jsh58/FlowClus), respectively. For quality filtering, reads shorter than 150 bases or longer than 1000 bases were discarded, and homopolymer runs longer than six bases were excluded, as well as ambiguous sequences longer than six bases. A Phred quality score greater than 25 in a sliding window of 50 bases was considered as the minimum average allowed, and one barcode correction and two primer mismatches were accepted. Quality filtered reads were processed using V-Xtractor (Hartmann et al., 2010) (http://www.microbiome.ch/Tools.html) and ITSx (Bengtsson-Palme et al., 2013) (http://microbiology.se/software/itsx), in order to obtain highly reliable 16S V6-V8 rRNA and ITS2 sequences, respectively. USEARCH v7 (Edgar, 2013) (http://www.drive5.com) was used to de-replicate and sort the extracted regions. Chimeras were removed with UCHIME (Edgar et al., 2011) (http://drive5.com/usearch/manual/uchime_algo.html) using the ChimeraSlayer's database (http://microbiomeutil.sourceforge.net/#A_CS) (Haas et al., 2011) and the UNITE reference sequences (Koljalg et al., 2013) for bacterial and fungal sequences, respectively.

Clustering of operational taxonomic units (OTU) was carried out using the USEARCH v7 tool with 97% of pairwise sequence identity (Edgar, 2013). QIIME (Caporaso et al., 2010) (http://qiime.org) was used for taxonomy assignments of bacterial and fungal OTU with a naïve Bayesian RDP classifier and a minimum confidence of 0.8 (Wang et al., 2007) against the Greengenes database (August, 2013) (http://greengenes.secondgenome.com and UNITE database) (March, 2015) (http://www2.dpes.gu.se/project/unite/UNITE_intro.htm), respectively. After taxonomic classification in the Greengenes database, OTU corresponding to chloroplasts and mitochondrial sequences were discarded. The percentage of the total OTU that were sequenced in each sample was estimated using the Good's coverage estimator (Good, 1953).

Statistical Analysis

Statistical analysis of bacterial and fungal data were carried out as reported by Touceda-Gonzalez et al. (2015) with some modifications. The BIOM table generated by the 16S rRNA gene and ITS analysis was subsampled via multiple rarefaction in QIIME (Caporaso et al., 2010). For alpha-diversity metrics, the Chao1 index (Chao, 1984) and the Simpson's diversity index (Simpson, 1949) were calculated to estimate OTU richness and microbial diversity, respectively. For beta-diversity metrics, the BIOM table was processed with the metagenomeSeq Bioconductor package (Paulson et al., 2013; McMurdie and Holmes, 2014) (https://bioconductor.org/packages/release/bioc/html/metagenomeSeq.html) and a multivariate analysis was performed with an unsupervised Principal Component Analysis (data not shown) followed by its constrained ordination counterpart, i.e., a Canonical Analysis of Principal coordinates (CAP) (Anderson and Willis, 2003), a permutation test (Legendre and Legendre, 1998), and a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) implemented in the vegan R package (https://cran.r-project.org/web/packages/vegan/index.html). Significant differences among communities were assessed using the Bray-Curtis dissimilarity distance (Bray and Curtis, 1957) and ordination analyses were carried out with the phyloseq R package (McMurdie and Holmes, 2013) (https://joey711.github.io/phyloseq). When significant ($P < 0.05$) differences of treatment and/or experiment were detected, pairwise comparisons between treatments were carried out by the RVAideMemoire package with false discovery rate corrections for multiple testing (https://cran.r-project.org/web/packages/RVAideMemoire/index.html).

Data on disease severity, observed species, Chao1 and Simpson indexes, microbial relative abundances, and gene expression levels were processed using Statistica 9 Software (StatSoft, Tulsa, OK, USA). Three replicates (namely A, B, C) were analyzed for each treatment and each time point in two independent experiments (Exp 1 and Exp 2). Relative abundances of bacteria and fungi were normalized by arcsine transformation. Disease severity scores, CFU counts, and fold change values were transformed by square root, Log_{10}, and the equation $y = Log_{10}(1 + x)$ (Casagrande et al., 2011), respectively. When the *F*-test demonstrated non-significant treatment-experiment interactions ($\alpha > 0.05$), data from the two experiments were pooled. After validating data for normal distribution (K-S test, $P > 0.05$) and variance homogeny (Cochran's test, $P > 0.05$), analysis of variance (one-way ANOVA) was carried out using Fisher's test ($\alpha = 0.05$) to reveal significant differences among treatments and time points.

RESULTS

Effects of Nutrient Broth against Downy Mildew under Axenic Conditions

Under axenic conditions, foliar applications of NB reduced downy mildew symptoms on *in vitro*-grown grapevines in two independent experiments. An *F*-test demonstrated non-significant effect of the experiment ($P = 0.79$), and dat... pooled. Disease severity was significantly lower (Fisher test; $\alpha = 0.05$) in NB-treated (disease severity: 1.2 ± 0.9%; average ± standard error) with respect to H_2O-treated plants (disease severity: 19.1 ± 5.6%). In order to investigate the molecular mechanisms induced by NB in grapevine, expression levels of six defense-related genes (Table S1) were analyzed by qPCR in leaves collected at T0 and T1 of *P. viticola* inoculation (**Figure 1**). Under these axenic conditions, the expression of *PR-2*, *PR-4*, *CHIT-3*, *OSM-1*, and *OSM-2* was induced by NB at T0 and it remained at a high level at T1. In particular, expression of *PR-4* and *OSM-1* was further enhanced by *P. viticola* inoculation in NB-treated plants and expression of *PR-2*, *PR-4*, *CHIT-3*, *OSM-1*, and *OSM-2* was higher in NB-treated plants in comparison to H_2O-treated plants at T1. Conversely, expression of *PR-1* was not affected by NB treatment or by *P. viticola* inoculation under axenic conditions.

Assessment of Grapevine Resistance against Downy Mildew under Greenhouse Conditions

Under greenhouse conditions, foliar applications of NB reduced downy mildew severity as compared with H_2O-treated and UNT plants in the two different greenhouse experiments (**Figure 2**). Although a slight effect of the experiment was present (*F*-test, $P = 0.046$), the reduction of disease severity was greater in NB-treated plants (60.0 ± 1.3%) than in LAM-treated plants (34.6 ± 3.5%). Expression levels of the six previously mentioned defense-related genes (Table S1) were analyzed by qPCR in leaves collected at T0 and T1 of *P. viticola* inoculation (**Figure 3**). As expected, the expression levels of all tested genes were comparable in UNT and H_2O-treated plants at T0, excluding the contribution of H_2O treatment on defense gene modulation. The expression of all tested genes was induced by NB at T0 and it remained at a high level at T1; only the expression of *CHIT-3* was further enhanced at T1 in Exp 1. The expression levels of the defense genes *PR-1*, *PR-2*, *OSM-1* were higher in NB-treated plants with respect to H_2O-treated plants at T0 and T1 (more than three- and two-fold, respectively), as well as those of *PR-4* at T0, *CHIT-3*, and *OSM*-2 at T0 and T1 of Exp 1. Conversely, *PR-4* in Exp 1 and Exp 2, *CHIT-3*, and *OSM-2* in Exp 2 showed comparable expression levels in NB and H_2O-treated plants at T1. LAM treatment induced the expression of all tested genes at T0 and they remained at high expression levels at T1, with a further reinforcement of *CHIT-3* expression at T1 in both experiments. Expression levels of some genes were higher in LAM-treated plants in comparison to NB-treated plants, such as those of *CHIT-3*, *OSM-1* at T0, *OSM-2* in Exp 2, and *PR-4* at T1 in Exp 2. In agreement with previous findings (Perazzolli et al., 2011, 2012), *P. viticola* inoculation induced the expression of *PR-1*, *PR-2*, *PR-4*, *CHIT-3*, and *OSM-1* in H_2O-treated plants at T1 in both experiments.

Effects of Grapevine Treatments on the Structure and Composition of Leaf Microbial Communities

Treatment with NB significantly increased the number of bacterial CFU per leaf unit as compared to controls (H_2O-treated

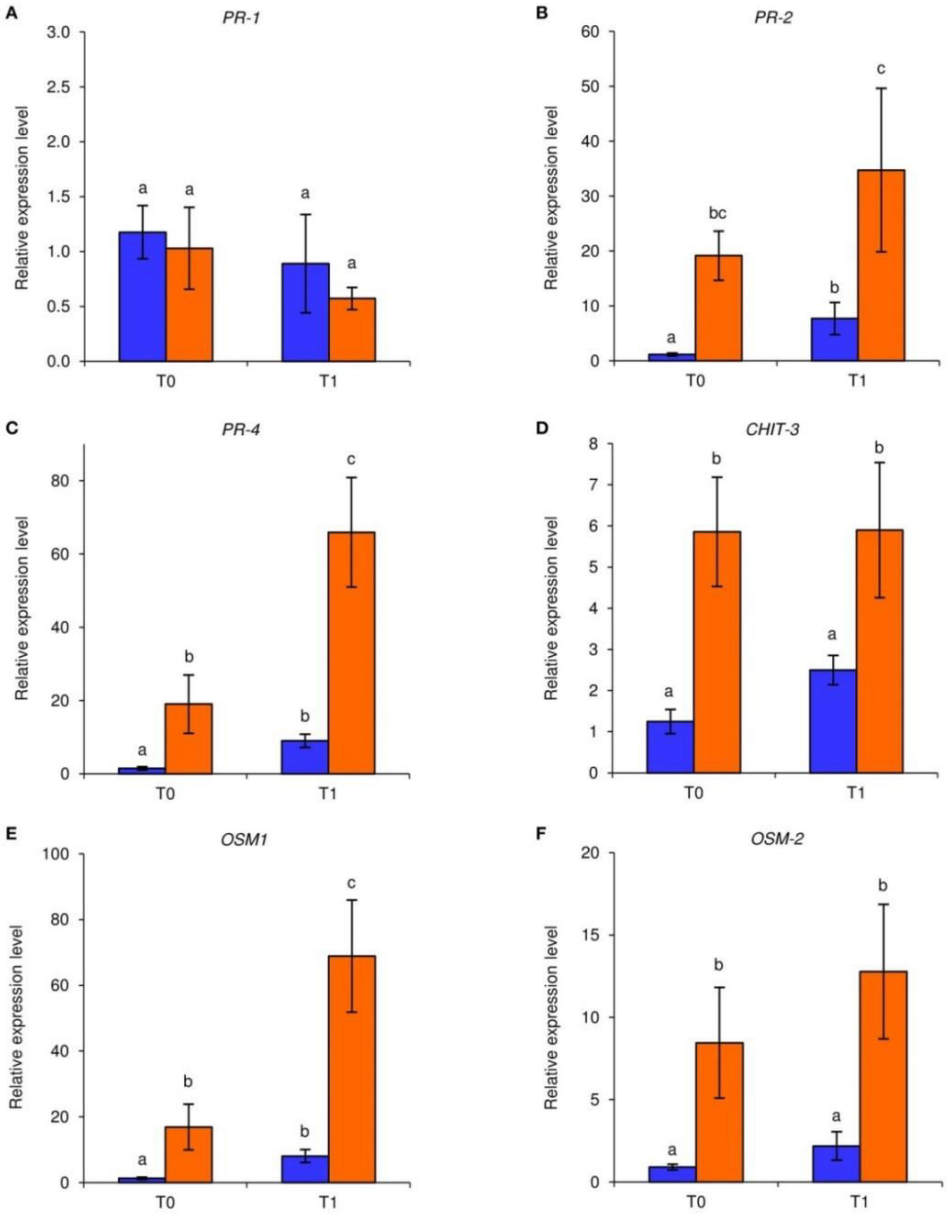

FIGURE 1 | Effect of leaf treatments on gene expression of grapevine plants under axenic conditions. *In vitro*-grown grapevines were treated with water (blue) or nutrient broth (orange) for three consecutive days before pathogen inoculation. Leaf samples were collected just before (T0) and 1 day after (T1) inoculation with *Plasmopara viticola*. Relative expression levels of genes encoding the pathogenesis-related (PR) protein 1 (*PR-1*; **A**), *PR-2* **(B),** *PR-4* **(C)**, chitinase 3 (*CHIT-3*; **D)**, osmotin 1 (*OSM-1*; **E**), and *OSM-2* **(F)** were assessed by quantitative real-time PCR. Relative expression levels were calculated using *Actin* as constitutive gene for normalization, and data were calibrated on water-treated plants at T0. An *F*-test revealed non-significant differences between experiments (*P*-values ranged from 0.06 to 0.91 for the genes tested), and data from the two experiments were pooled. Mean levels of relative expression and standard errors of six replicates (plants) pooled from two experiments are presented for each treatment and time point. For each gene, different letters indicate significant differences according to Fisher's test ($\alpha = 0.05$).

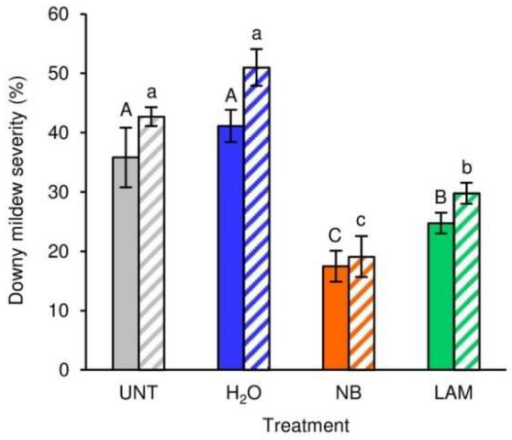

FIGURE 2 | Effect of leaf treatments on grapevine downy mildew severity under greenhouse conditions. Plants were left untreated (UNT), or treated daily with water (H_2O), nutrient broth (NB) or laminarin (LAM) three times before *Plasmopara viticola* inoculation. Disease severity was assessed as percentage of abaxial leaf area covered by *P. viticola* sporulation 7 days after inoculation. The *F*-test revealed differences between the experiments ($P = 0.046$) and each experiment was analyzed separately. Mean severity and standard error values of 12 replicates (plants) are presented for each treatment and experiment. Different uppercase and lowercase letters indicate significant differences among treatments according to Fisher's test ($\alpha = 0.05$) in experiment 1 (solid bars) and experiment 2 (striped bars), respectively.

and UNT plants) at T0 and T1 in both experiments (File S1, Figures S1A,B). Conversely, H_2O and LAM treatments did not affect bacterial CFU as compared to UNT plants. Considering the representative bacterial isolates originated from treated leaves, NB did not increase the percentage of bacteria with protease activity, siderophore production, or antagonistic activity against the oomycete *P. infestans* compared to H_2O treatment (File S1, Table S3). Although the percentage of bacterial isolates with biocontrol activity against *P. viticola* did not increase after NB application, two isolates (*Pseudomonas* spp. and *Enterobacter* spp.) from NB-treated plants significantly reduced downy mildew severity on grapevine leaf disks (File S1, Figure S2). Culturable fungi were not affected by the treatments tested, except for the slight increase of fungal CFU in NB-treated plants of Exp 1 (Figures S1C,D).

The composition of bacterial and fungal communities was analyzed on leaves collected from UNT, H_2O-, NB- and LAM-treated plants at T0 and T1 for the two independent greenhouse experiments. For bacterial (16S rRNA gene) and fungal (ITS) regions, 678,811 and 153,401 quality filtered reads were obtained, respectively (Tables S3–S5). Rarefaction curves (Figures S3, S4), Good's coverage and Chao1 indexes (Tables S4, S5) confirmed that the estimated microbial richness was sufficiently covered by the sequencing effort (File S1). OTU numbers, richness and microbial diversity estimated by the Simpson index highlighted differences of bacterial and populations among experiments and grapevine treatments (File S1, Figures S5, S6).

Almost the totality of bacterial reads (99.95 and 98.4%, respectively) were assigned to taxa at phylum (File S1, Figure S7, and Table S6) and family level (1404 OTU); 84 different bacterial families were identified in total, and the 15 dominant families were selected (more than 0.5% of relative abundance in at least one sample; **Figure 4**). Although all plants originated from the same nursery stock and were grown under the same controlled conditions, indigenous communities on UNT leaves differed between Exp 1 and Exp 2. In particular, the Enterobacteriaceae family comprised almost the totality of identified bacteria of UNT plants in Exp 2, while greater bacterial diversity was present on leaves of Exp 1. H_2O treatment did not affect the proportions of bacterial families as compared with UNT plants in both experiments, except for Sinobacteraceae and Nocardioidaceae in Exp 1 (**Figure 4A**) and Streptococcaceae in Exp 2 (**Figure 4B**), and H_2O-treated plants were used as reference control for treatment comparisons. In Exp 1, the abundance of Exiguobacteraceae significantly increased on NB-treated plants in comparison to H_2O-treated plants on leaves collected at T0, while the proportions of Pseudonocardiaceae, Xanthomonadaceae, Halomonadvaceae, Sinobacteraceae, Legionellaceae, Peptococcaceae, Streptomycetaceae, Streptococcaceae, Hyphomicrobiaceae, and Nocardioidaceae decreased (**Figure 4A**). Furthermore, lower abundance of Halomonadaceae, Sinobacteraceae, and Nocardioidaceae was observed on LAM-treated plants in comparison to H_2O-treated plants. At T1, relative abundances of bacterial families were comparable on H_2O-, NB-, and LAM-treated plants. For T0 samples of Exp 2, the abundance of Enterobacteriaceae was lower on NB-treated plants with respect to H_2O-treated plants, while that of Pseudomonadaceae was greater (**Figure 4B**). The presence of Exiguobacteraceae increased on NB-treated plants as compared to H_2O-treated plants at T1, while proportions of Enterobacteriaceae and Moraxellaceae decreased. The relative abundance of all the dominant families was comparable on LAM- and H_2O-treated plants at both time points.

Of bacterial reads, 87.3% were assigned to taxa at the genus level (885 OTU), 150 and 70 different bacterial genera and species were identified, respectively. Relative abundances of the dominant genera (**Figure 5**) and dominant species (Figure S8) differed by treatment, time point and experiment. H_2O treatment did not modify genera proportions as compared with UNT plants, only in Exp 1 the genus *Serratia* decreased, while the Unknown and *Enhydrobacter* genera increased (**Figure 5A**). On leaves collected at T0, levels of the *Serratia* and *Exiguobacterium* genera significantly increased on NB-treated plants in comparison to H_2O-treated plants in Exp 1, whereas those of Unknown, *Saccharopolyspora*, *Halomonas*, *Dokdonella*, *Alkanindiges*, *Rhodanobacter*, *Enterobacter,* and *Enhydrobacter* decreased (**Figure 5A**). Furthermore, lower abundances of *Halomonas* and *Alkanindiges* were observed on LAM-treated plants in comparison to H_2O-treated plants. At T1, relative abundances of bacterial genera were comparable on H_2O-, NB-, and LAM-treated plants. In Exp 2, the abundance of *Pseudomonas* was increased by NB with respect to H_2O-treated

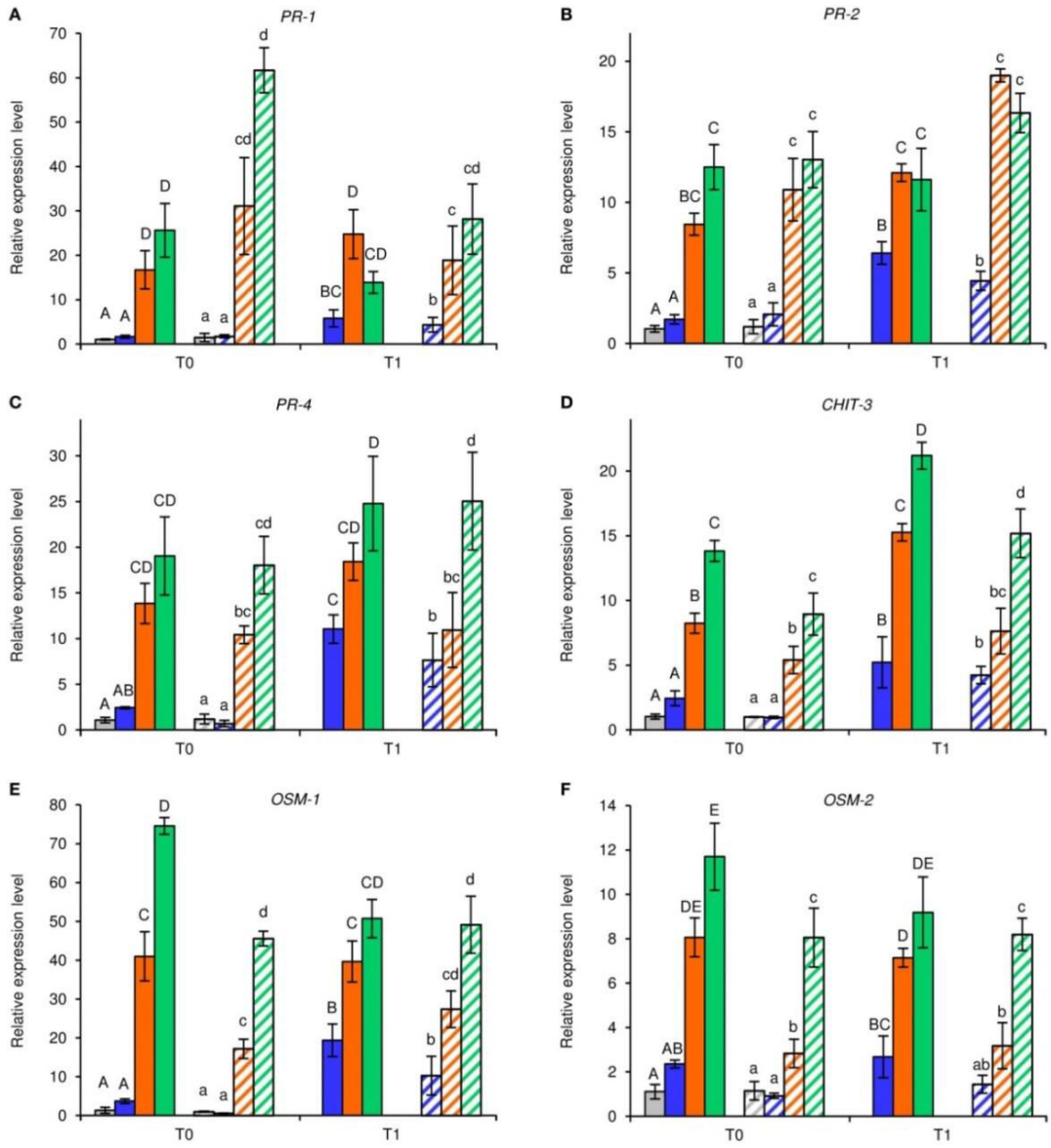

FIGURE 3 | Effect of leaf treatments on gene expression of grapevine plants under greenhouse conditions. Grapevine plants were left untreated (gray), treated daily with water (blue), nutrient broth (orange) or laminarin (green) three times before *Plasmopara viticola* inoculation. Leaf samples were collected just before (T0) and 1 day after (T1) pathogen inoculation. Relative expression levels of genes encoding the pathogenesis-related (PR) protein 1 (*PR-1*; **A**), *PR-2* **(B)**, *PR-4* **(C)**, chitinase 3 (*CHIT-3*; **D**), osmotin 1 (*OSM-1*; **E**), and *OSM-2* **(F)** were assessed by quantitative real-time PCR. Relative expression levels were calculated using *Actin* as constitutive gene for normalization, and data were calibrated on untreated plants at T0. The *F*-test revealed differences between the experiments (*P*-values ranged from 0.0003 to 0.038 for the genes tested), and each experiment was analyzed separately for each gene. For each time point, mean levels and standard errors of relative expression are calculated based on three replicates (plants) for each treatment and experiment. For each gene, different uppercase and lowercase letters indicate significant differences among treatments and time points according to Fisher's test ($\alpha = 0.05$) in experiment 1 (solid bars) and experiment 2 (striped bars), respectively.

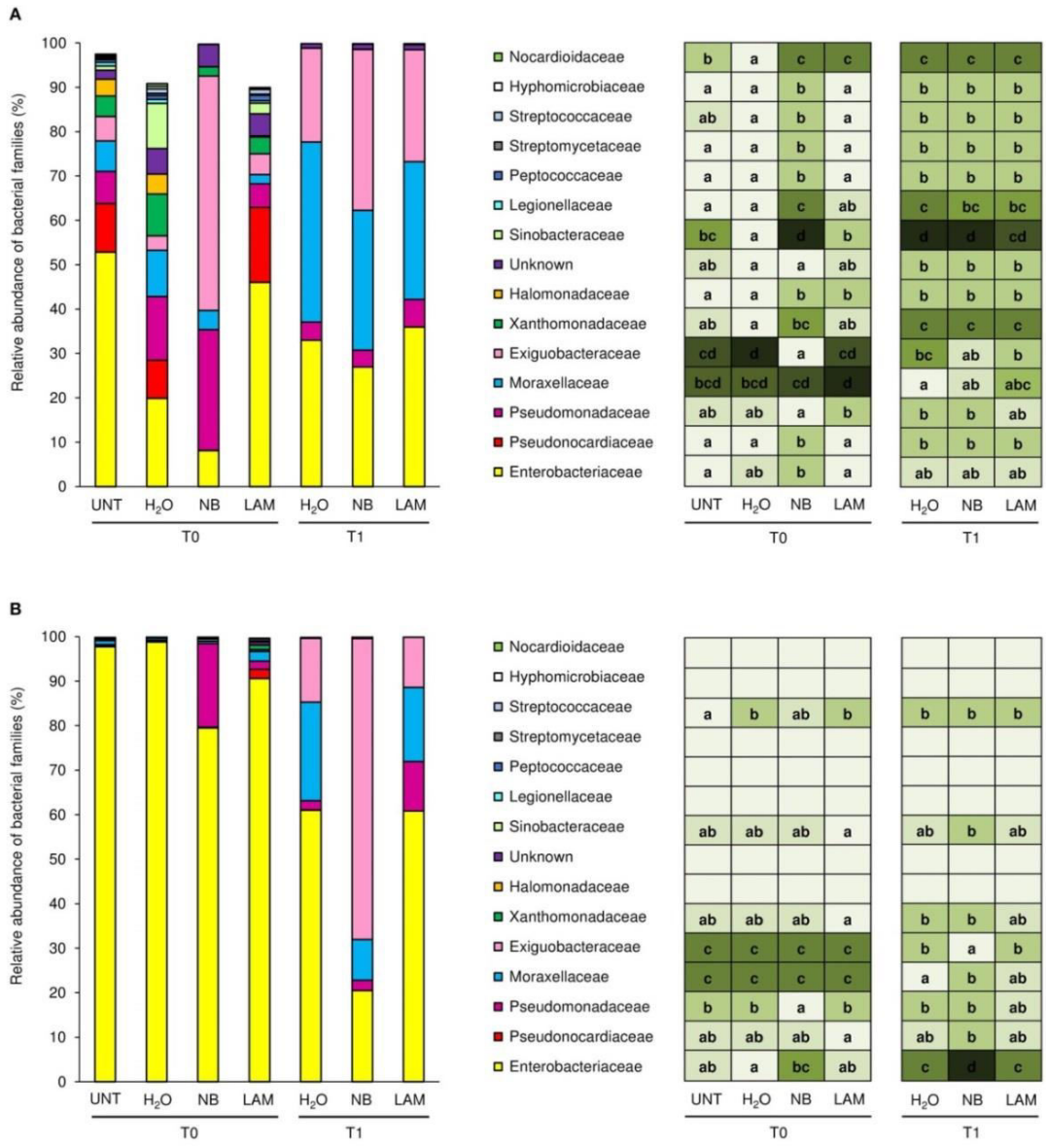

FIGURE 4 | Relative abundance of the dominant (more than 0.5% of relative abundance in at least one sample) bacterial families on grapevine leaves. Percentages of relative abundance were determined for microbial populations of leaves of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB), or laminarin (LAM) collected just before (T0) and 1 day after (T1) *Plasmopara viticola* inoculation in the Experiment 1 **(A)** and Experiment 2 **(B)**. Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. For each taxon, the intensity of the color gradient and letters reported in the table indicate significant differences among treatments and time points according to Fisher's test ($\alpha = 0.05$).

plants (**Figure 5B**). At T1, the presence of *Exiguobacterium* increased on NB-treated plants in comparison to H_2O-treated plants, while the proportions of Unknown, *Acinetobacter* and *Pantoea* were reduced. Relative abundances of all dominant genera were comparable on LAM- and H_2O-treated plants at both time points, except for an increase in *Dokdonella*

proportions at T0. Although abundances of genera related to the biocontrol of downy mildew were scarcely affected by grapevine treatments, it is remarkable that in Exp 2 the abundance of *Lysobacter* was greater on NB-treated plants in comparison to H_2O-treated plants at T0 (**Table 1**).

Concerning fungal populations, 34, 53, and 87 different families, genera and species were identified in total, respectively (Table S7). Proportions of the ten dominant families (Figure S9), seven dominant genera (Figure S10) and 15 dominant species (Figure S11) were homogeneous between the two experiments and only slightly affected by treatments and time points (File S1). As regard to fungal genera related to the biocontrol of downy mildew, in Exp 1 greater abundance of *Alternaria* spp. was detected on NB-treated plants in comparison to H_2O-treated plants at T1, while in Exp 2 the relative abundance of *Trichoderma* spp. was greater on NB-treated plants in comparison to H_2O-treated plants at T1 (**Table 1**).

Global effects of experiments, treatments and time points on bacterial and fungal diversity were examined using PERMANOVA and CAP analyses. PERMANOVA of bacterial samples collected at T0 indicated significant differences ($P = 0.0001$) among experiments and treatments (Table S8). CAP validated these results, and the first principal coordinate discriminated samples of Exp 1 from those of Exp 2 at T0, while the second axis highlighted differences among treatments (**Figure 6A**), with significant differences among experiments ($P = 0.001$) and treatments ($P = 0.002$) according to permutation tests on CAP (Table S8). Permutation pairwise comparisons showed significant differences between NB-treated and UNT plants ($P = 0.0044$), between NB- and H_2O-treated plants ($P = 0.0062$), but not between LAM- and H_2O-treated ($P = 0.0788$) and LAM-treated and UNT ($P = 0.1536$) plants (Table S8). Considering samples collected at T0 and T1, PERMANOVA identified significant differences among treatments ($P = 0.0077$), time points ($P = 0.0001$) and experiments ($P = 0.0001$; Table S8). CAP discriminated the two time points and the two experiments on the first and the second axis, respectively (**Figure 6B**), and permutation tests on CAP supported significant differences of bacterial communities among time points ($P = 0.001$), experiments ($P = 0.001$) and treatments ($P = 0.011$). Permutation pairwise comparisons obtained for samples collected at T1 revealed no significant effects of grapevine treatments (Table S8), and indicated that effects on bacterial populations occurred at T0. The CAP of fungal data discriminated the two experiments on the first axis considering samples collected at T0 (**Figure 6C**) or at T0 and T1 (**Figure 6D**), and permutation tests confirmed significant differences between experiments ($P = 0.001$; Table S9). PERMANOVA detected no significant difference among treatments and time points, in agreement with permutation test results applied to CAP (Table S9).

DISCUSSION

Several alternatives have been proposed to reduce the massive use of chemical pesticides in viticulture (Gessler et al., Delaunois et al., 2014) and the induction of plant resistance through the use of protein-based elicitors seems to be a promising additional tool (Lachhab et al., 2014, 2016; Nesler et al., 2015). In particular, the protein derivative named NB does not raise toxicological or ecotoxicological concerns, and it could represent a valid control product for integrated plant protection programs (Nesler et al., 2015). However, beyond its properties as a resistance inducer, NB could affect the composition of phyllosphere microbial populations, which in turn might contribute to resistance induction and/or display direct biocontrol properties.

Under axenic conditions, NB strongly reduced downy mildew symptoms and induced the expression of five defense-related genes (*PR-2, PR-4, OSM-1, OSM-2,* and *CHIT-3*), suggesting that it was effective against *P. viticola* through the induction of grapevine resistance. The expression of these genes remained at a high level even after pathogen inoculation (at T1), indicating that grapevine resistance induced by NB plays a major role in limiting host colonization during the early stages of *P. viticola* infection. Likewise, the relevance of a rapid up-regulation of defense genes, i.e. within a few hours after inoculation, has been demonstrated for the response against downy mildew in resistant genotypes (Polesani et al., 2010; Casagrande et al., 2011). Our data indicate that the preventive foliar treatment with NB reduced downy mildew symptoms under greenhouse conditions, through the induction of all defense-related genes tested, including *PR-1*. Moreover, the expression of three genes was further enhanced in NB-treated plants in response to *P. viticola* inoculation, such as *PR-4* and *OSM-1* under axenic conditions, and *CHIT-3* in Exp 1 under greenhouse conditions. Thus, marker genes of SA and JA pathways, such as *PR-1* and *PR-4*, respectively (Hamiduzzaman et al., 2005), were induced by NB under greenhouse conditions, suggesting the activation of both signaling pathways (Nesler et al., 2015). Expression profiles of *in vitro*-grown plants partially differed from those of greenhouse-grown plants, and the SA marker (*PR-1*) was not induced by NB under axenic conditions. Although different expression profiles between axenic and greenhouse conditions could be related to different growing conditions of the plants, they could also be associated with changes induced by NB in the phyllosphere microbiota of greenhouse-grown plants. Some components of protein-derived products can be metabolized by the phyllosphere microorganisms, thus modifying the properties and efficacy of the originally applied product (Colla et al., 2015). In particular, NB might stimulate the JA-mediated pathways under axenic conditions, and the phyllosphere microorganisms could contribute to the activation of SA pathways under greenhouse conditions. Therefore, changes in phyllosphere microbiota composition could modify the plant signals stimulated by the protein-based resistance inducer. This second scenario, coupled with the fact that plants are naturally exposed to a wide variety of microorganisms under natural conditions and that plant resistance might be already partially activated, could explain the greater efficacy of NB under axenic conditions with respect to greenhouse conditions. Although LAM induced the expression of all defense-related genes tested, with a higher expression of some of them in comparison with

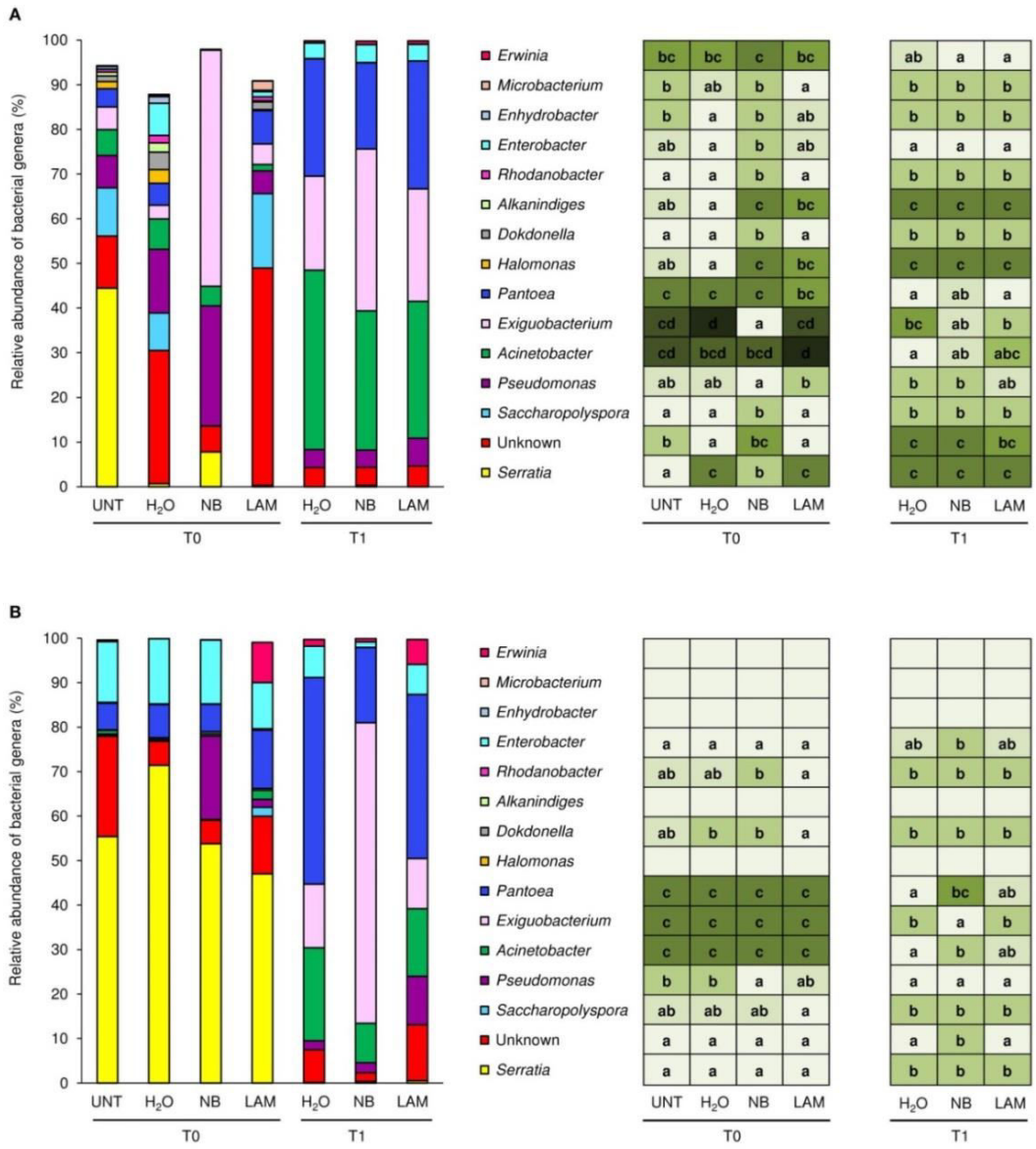

FIGURE 5 | Relative abundance of the dominant (more than 2% of relative abundance in at least one sample) bacterial genera on grapevine leaves. Percentages of relative abundance were determined for leaves of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB), or laminarin (LAM) collected just before (T0) and 1 day after (T1) *Plasmopara viticola* inoculation in the experiment 1 **(A)** and experiment 2 **(B)**. Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. For each taxon, the intensity of the color gradient and letters reported in the table indicate significant differences among treatments and time points according to Fisher's test ($\alpha = 0.05$).

NB treatment (*CHIT-3*, *OSM-1*, *OSM-2*, and *PR-4*), it showed lower efficacy than NB against downy mildew. These results suggest that multiple mechanisms of action are involved in the biocontrol activity of NB and that additional biotic factors, i.e. the phyllosphere microbiota could influence the efficacy against downy mildew.

TABLE 1 | Relative abundance of bacterial and fungal genera in the phyllosphere microbial populations of experiment 1 and experiment 2, which comprise known biocontrol agents against *Plasmopara viticola*.

Genus[1]	Relative abundance[2]						
	UNT T0	H_2O T0	NB T0	LAM T0	H_2O T1	NB T1	LAM T1
EXPERIMENT 1							
Bacillus	0.00 ± 0^{a}	0.02 ± 0.02^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}
Lysobacter	0.64 ± 0.24^{a}	0.78 ± 0.29^{a}	0.57 ± 0.46^{a}	0.19 ± 0.12^{ab}	0.00 ± 0^{b}	0.00 ± 0^{b}	0.00 ± 0^{b}
Stenotrophomonas	0.99 ± 0.85^{ab}	0.16 ± 0.02^{abc}	1.41 ± 1.04^{a}	0.16 ± 0.10^{abc}	0.00 ± 0^{c}	0.02 ± 0.02^{bc}	0.00 ± 0^{c}
Alternaria	0.00 ± 0^{c}	0.02 ± 0.02^{bc}	0.00 ± 0^{c}	0.00 ± 0^{c}	0.00 ± 0^{c}	0.10 ± 0.05^{a}	0.03 ± 0.02^{b}
Aureobasidium	0.00 ± 0^{b}	0.05 ± 0.03^{a}	0.02 ± 0.02^{ab}	0.00 ± 0^{b}	0.00 ± 0^{b}	0.00 ± 0^{b}	0.05 ± 0.03^{a}
Fusarium	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.03 ± 0.03^{a}
Trichoderma	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.02 ± 0.02^{a}	0.00 ± 0^{a}
Penicillium	4.95 ± 1.00^{a}	1.48 ± 0.18^{b}	2.21 ± 0.79^{ab}	2.27 ± 0.99^{ab}	2.68 ± 0.90^{ab}	2.34 ± 0.70^{ab}	2.37 ± 0.94^{ab}
Acremonium	2.47 ± 0.93^{a}	0.98 ± 0.16^{a}	0.98 ± 0.36^{a}	4.38 ± 2.96^{a}	1.77 ± 1.16^{a}	1.35 ± 070^{a}	2.95 ± 0.49^{a}
EXPERIMENT 2							
Bacillus	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}
Lysobacter	0.02 ± 0.02^{ab}	0.00 ± 0^{b}	0.16 ± 0.13^{a}	0.07 ± 0.07^{ab}	0.00 ± 0^{b}	0.00 ± 0^{b}	0.00 ± 0^{b}
Stenotrophomonas	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}
Alternaria	0.00 ± 0^{b}	0.00 ± 0^{b}	0.00 ± 0^{b}	0.07 ± 0.03^{a}	0.00 ± 0^{b}	0.07 ± 0.07^{ab}	0.00 ± 0^{b}
Aureobasidium	0.02 ± 0.02^{b}	0.00 ± 0^{b}	0.02 ± 0.02^{b}	0.00 ± 0^{b}	0.10 ± 0.03^{ab}	0.56 ± 0.31^{a}	0.34 ± 0.22^{a}
Fusarium	0.03 ± 0.03^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.12 ± 0.12^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}	0.00 ± 0^{a}
Trichoderma	0.02 ± 0.02^{b}	0.00 ± 0^{b}	0.05 ± 0.05^{ab}	0.02 ± 0.02^{b}	0.00 ± 0^{b}	0.08 ± 0.02^{a}	0.00 ± 0^{b}
Penicillium	17.15 ± 4.62^{a}	5.44 ± 0.50^{a}	12.35 ± 8.27^{a}	19.44 ± 10.79^{a}	6.79 ± 1.08^{a}	5.22 ± 1.35^{a}	4.21 ± 1.17^{a}
Acremonium	11.49 ± 8.34^{a}	1.55 ± 0.59^{a}	10.21 ± 9.88^{a}	4.29 ± 2.02^{a}	9.43 ± 3.80^{a}	2.71 ± 1.73^{a}	14.40 ± 13.47^{a}

[1]*Bacterial genera comprising known biocontrol agents against downy mildew: Bacillus spp., Stenotrophomonas maltophilia (Gessler et al., 2011), Lysobacter capsici (Puopolo et al., 2014), Alternaria alternata (Musetti et al., 2006), Aureobasidium pullulans (Harm et al., 2011), Fusarium proliferatum (Falk et al., 1996), Trichoderma harzianum (Perazzolli et al., 2011), Penicillium chrysogenum (Thuerig et al., 2006), and Acremonium byssoides (Burruano et al., 2008) identified on grapevine leaves in the two experiments under greenhouse conditions (Experiment 1 and Experiment 2).*

[2]*Percentages of relative abundance of bacterial and fungal genera with possible biocontrol activities were determined for leaves of untreated plants (UNT), plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM), collected just before (T0) and 1 day after (T1) Plasmopara viticola inoculation and normalized to the lowest number of quality filtered reads in the Experiment 1 and Experiment 2. The mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and each time point. For each genus, different superscript letters indicate the significant differences according to Fisher's test ($\alpha = 0.05$).*

Pyrosequencing analysis allowed the dissection of compositions and modifications of the microbial populations residing on the grapevine phyllosphere after the treatments tested. Even though plants in the study originated from the same nursery stock, and were grown under the same controlled conditions, significant differences among bacterial populations were found between the two greenhouse experiments. The plant phyllosphere act as an open system, and the structure of its microbial community reflects immigration, survival and growth of microbial colonists, which in turn is influenced by numerous environmental factors, in addition to leaf physico-chemical properties (Whipps et al., 2008a). The high variability among microbial populations residing on grapevine leaves in time and space is in agreement to what already observed in field experiments (Perazzolli et al., 2014). Subsequently, changes occurred in leaf bacterial and fungal populations after the treatments tested were affected by the composition of the originally residing microbiota. The dominant phyla were Proteobacteria and Actinobacteria, as already reported for grapevine phyllosphere (Leveau and Tech, 2011; Perazzolli et al., 2014; Pinto et al., 2014) and rhizosphere microorganisms (Zarraonaindia et al., 2015). It has been shown that microbial communities associated with grapevine leaves share a great proportion of taxa with soil populations, suggesting that the soil is the main microbial reservoir of the aboveground communities (Zarraonaindia et al., 2015). *Xanthomonadales*, *Rhizobiales*, and *Actinomycetales* were shown to be the dominant bacterial orders of grapevine root and rhizosphere communities (Zarraonaindia et al., 2015), and Xanthomonadaceae, Hyphomicrobiaceae, and Pseudonocardiaceae were among the most abundant bacterial families in our samples, respectively. Particularly, *Pseudomonas*, *Acinetobacter*, *Exiguobacterium*, *Pantoea*, *Alkanindiges*, *Enterobacter*, and *Erwinia* were among the genera with highest presence, as previously reported for grapevine leaves (Bulgari et al., 2009; Leveau and Tech, 2011; Martins et al., 2013; Perazzolli et al., 2014; Pinto et al., 2014). Bacterial community structure was globally affected by time points and by NB treatment in both experiments, while no effect was seen from LAM treatment. NB application possibly act as nutritional substrate for some bacteria and increased abundances of the *Exiguobacterium* genus as compared with H_2O treatment at T0 in Exp 1 and T1 in Exp 2. Interactions between *Exiguobacterium acetylicum* and two other bacteria (namely *Microbacterium* spp. and *Pantoea agglomerans*) have been reported to contribute to

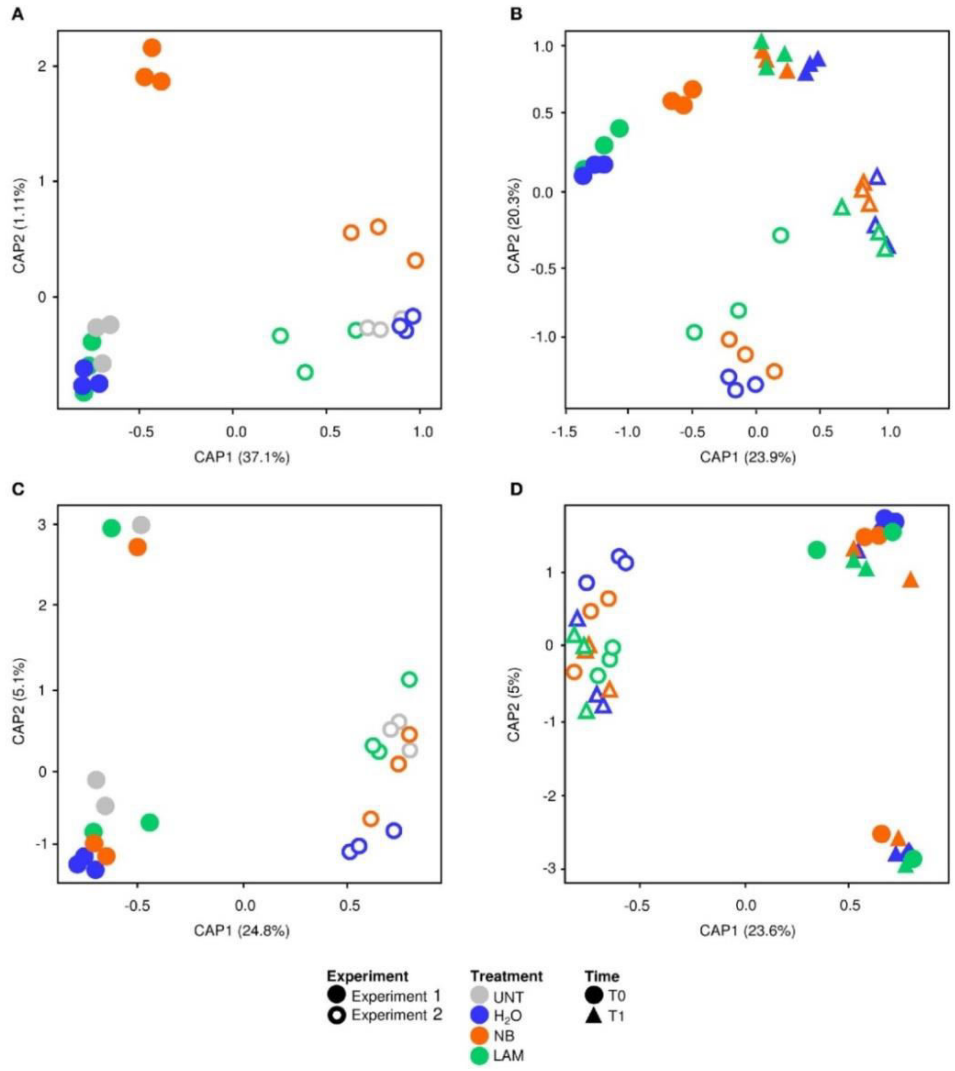

FIGURE 6 | Canonical analysis of principal coordinates (CAP) of leaf microbial communities. CAP of bacterial **(A,B)** and fungal **(C,D)** communities were obtained with the vegan package using Bray-Curtis dissimilarity matrix on data of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB), or laminarin (LAM) collected just before (T0) and 1 day after (T1) *Plasmopara viticola* inoculation. CAP was carried out for T0 samples **(A,C)**, and for T0 and T1 samples excluding UNT **(B,D)**. Results of PERMANOVA and Permutation tests obtained with ADONIS function and a Bray-Curtis dissimilarity matrix on bacterial and fungal data are reported in Tables S8, S9, respectively.

the suppression of the wheat root disease caused by *Rhizoctonia solani* (Barnett et al., 2006), indicating potential biocontrol properties of some *Exiguobacterium* strains. In Exp 2, the NB treatment increased the proportion of the Pseudomonadaceae family and the *Pseudomonas* genus as compared with UNT plants and H_2O-treated plants at T0, and *P. viridiflava* and *P. veronii* levels showed the same trend. Some species of this genera are known as active resistance inducers (Van Wees et al., 2008) and biocontrol agents, for their ability to produce proteases (Elad, 2000), siderophores (Van Wees et al., 2008) and antimicrobial metabolites (Ligon et al., 2000). Specifically for grapevine plants, members of *Pseudomonas* have been demonstrated

to effectively control *Botrytis cinerea* infections by inducing resistance mechanisms (Trotel-Aziz et al., 2008). Finally, the NB treatment increased also the proportion of *P. alcaligenes* at T0 in Exp 1, and this species has been reported as biocontrol agent against *Fusarium oxysporum* (Akhtar et al., 2010). In Exp 2, the Enterobacteriaceae family accounted for the majority of bacterial OTU at T0, and its abundance was affected by NB at both time points. One of the dominant species was *Serratia marcescens*, which significantly increased by NB as compared with respect to H_2O-treated plants at T0 in Exp 1. *S. marcescens* was reported as biocontrol agent against the soil-borne fungus *Magnaporthe poae* (Kobayashi et al., 1995) and the rice pathogen *Magnaporthe oryzae* (Jaiganesh et al., 2007). A strain of *Lysobacter capsici* reduced downy mildew symptoms in grapevine (Puopolo et al., 2014), and the abundance of the *Lysobacter* genus on grapevine leaves increased as a result of NB treatment at T0 in Exp 2. Grapevine bacterial pathogens, such as *Agrobacterium vitis*, *Xylella fastidiosa,* and *Xylophilus ampelinus* (Armijo et al., 2016) were not detected in the samples analyzed, and further studies are required to better characterize possible side effects of NB on bacterial phytopathogens. However, negligible effects on *X. fastidiosa* are highly possible, due to its transmission to new host plants exclusively by insect vectors (Armijo et al., 2016). Although more sensitive analyses are required to precisely quantify human pathogenic strains, *Salmonella* spp., *Legionella* spp., and *Escherichia* spp. were underrepresented on grapevine leaves and their abundances were not affected by the NB treatment, suggesting a minimal risk in term of increase of microorganisms potentially dangerous for human health.

The structure of fungal communities were similar in Exp 1 and Exp 2 and they were not globally affected by NB and LAM treatment. The fungal microbiota of grapevine leaves was strongly dominated by the Ascomycota phylum, as reported for other plants (Jumpponen and Jones, 2009), and by the Trichocomaceae family. A substantial part of sequenced reads was attributed to the Unknown group, which probably represented environmental sequences of unculturable fungi. The most common genera identified on grapevine leaves were *Aspergillus, Penicillium,* and *Acremonium,* as reported for other plants (Inacio et al., 2002; Whipps et al., 2008b). The NB treatment modified abundances of some specific fungal taxa, such as the *Alternaria* genus at T1 in Exp 1, and *A. alternata* was able to control *P. viticola* on leaf disks (Musetti et al., 2006). Relative abundances of *Trichoderma* spp. and *Aureobasidium* spp. were increased by the NB treatment at T1 in Exp 2. A strain of *Trichoderma harzianum* induces grapevine resistance (Perazzolli et al., 2011), and an isolate of *Aureobasidium pullulans* partially protects against downy mildew (Harm et al., 2011). Summarizing, the preventive foliar application of NB on grapevine partially alters the structures and dynamics of bacterial populations, and specific differences highlighted effects on some genera that may be related to biocontrol activity and resistance induction. On the other hand, the fungal communities on grapevine leaves were more stable than bacterial populations in the time-frame studied. This may be related to shorter generation time of the bacteria and/or the preference of bacteria for protein and amino acid nutritional source (Vorholt, 2012). Another possible reason for stability may be the longer generation time of fungi that did not afford appreciable modifications within the short time of the experiment (4 days).

Although culturable microorganisms represent a limited fraction of the community, they are the most likely to be influenced by NB, which is a laboratory microbiological medium. The increase of culturable microorganisms on NB-treated plants confirmed that the protein derivative had a nutritional role that affected mainly bacteria. However, *in vitro* assays highlighted that the NB treatment did not affect proportions of bacterial isolates with proteolytic activity, siderophore production and antagonistic activity against *P. infestans*, suggesting the absence of positive selection of potential biocontrol agents against oomycetes. Although proportions of biocontrol strains effective against *P. viticola* were not increased by the NB treatment, two isolates from NB-treated plants showed biocontrol activity against *P. viticola* on leaf disks. In short, NB leaf application on greenhouse-grown grapevines increased the number of culturable bacteria and slightly altered the structure of the residing phyllosphere microbiota. These changes may contribute to pathogen control resulting from competition for space or from other biocontrol strategies, resistance induction included. Thus, functional properties of the phyllosphere microbiota against plant diseases (Vorholt, 2012; Ritpitakphong et al., 2016) could be improved by application of nutritional substrates for leaf microorganisms.

In conclusion, NB could represent a promising alternative for the control of *P. viticola* on grapevines, considering its natural origin and the multiple mechanisms of action. The application of a protein-based resistance inducer to prevent grapevine diseases could bring appreciable advantages, such as the absence of toxicity for the environment and the activation of defense mechanisms that protects plants against different diseases, such as powdery (Nesler et al., 2015) and downy mildew. Moreover, weekly applications of NB did not produce any negative effect on grapevine growth and yield in two different seasons, indicating minimal risks for grape production and quality (Nesler et al., 2015). As demonstrated for the control of powdery mildew (Nesler et al., 2015), the reduction of downy mildew symptoms is mainly based on the induction of defense mechanisms in grapevine, involving multiple signaling pathways. Furthermore, NB increased the number of culturable phyllosphere microorganisms and changed proportions of some taxa that have previously been linked to the biological control of plant pathogens. Thus, modifications of the phyllosphere microbiota due to NB treatment may provide a partial contribution to the control of downy mildew. Although, changes in the microbial populations depend on the indigenous communities originally residing on grapevine leaves before treatment, levels of disease reduction and defense gene modulation of NB-treated plants were consistent among the experiments. The plant phyllosphere act as an open system and population dynamics are more complex than expected, suggesting that the resolution power of current meta-barcoding approaches of metagenomics is still insufficient to link

modifications in microbial composition to functional changes. Further functional analyses of NB-affected populations, for example with metatranscriptomic approaches, are required to precisely characterize their effective contribute in term of disease control.

AUTHOR CONTRIBUTIONS

MC analyzed the sequencing data and wrote the paper MPe designed the experiments, analyzed the data and wrote the paper LA carried out the bioinformatics and statistical analysis of the sequencing data AN carried out the experiments under greenhouse condition, the gene expression analysis and the characterization of culturable microorganisms ET carried out the gene expression analysis and the characterization of culturable microorganisms. PLB carried out the experiments with *in vitro*-grown plants MPi carried out the ITS and 16S sequencing GP carried out the experiments under greenhouse conditions and the characterization of culturable microorganisms IP conceived the study, designed the experiment, coordinated all research activities and wrote the paper. All authors revised and approved the final manuscript.

ACKNOWLEDGMENTS

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 289497 (project CO-FREE, theme KBBE.2011.1.2-06), no. 265865 (project PURE, theme FP7-KBBE-2010-4), and no. 324416 (project INNOVA, theme FP7-PEOPLE-2012-IAPP). We thank Oscar Giovannini and Franca Valentini for technical support with grapevine plants, and the technical staff of the FEM Sequencing Platform for technical support with pyrosequencing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01053

REFERENCES

Akhtar, M. S., Shakeel, U., and Siddiqui, Z. A. (2010). Biocontrol of *Fusarium* wilt by *Bacillus pumilus*, *Pseudomonas alcaligenes*, and *Rhizobium* sp. on lentil. *Turk. J. Biol.* 34, 1–7. doi: 10.3906/biy-0809-12

Albert, M. (2013). Peptides as triggers of plant defence. *J. Exp. Bot.* 64, 5269–5279. doi: 10.1093/jxb/ert275

Algarra Alarcon, A., Lazazzara, V., Cappellin, L., Bianchedi, P. L., Schuhmacher, R., Wohlfahrt, G., et al. (2015). Emission of volatile sesquiterpenes and monoterpenes in grapevine genotypes upon *Plasmopara viticola* inoculation *in vitro*. *J. Mass Spectrom.* 50, 1013–1022. doi: 10.1002/jms.3615

Allegre, M., Heloir, M.-C., Trouvelot, S., Daire, X., Pugin, A., Wendehenne, D., et al. (2009). Are grapevine stomata involved in the elicitor-induced protection against downy mildew? *Mol. Plant Microbe Interact.* 22, 977–986. doi: 10.1094/MPMI-22-8-0977

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral. Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

Anderson, M. J., and Willis, T. J. (2003). Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. *Ecology* 84, 511–525. doi: 10.1890/0012-9658(2003)084[0511:CAOPCA]2.0.CO;2

Armijo, G., Schlechter, R., Agurto, M., Munoz, D., Nunez, C., and Arce-Johnson, P. (2016). Grapevine pathogenic microorganisms: understanding infection strategies and host response scenarios. *Front. Plant Sci.* 7:382. doi: 10.3389/fpls.2016.00382

Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bezier, A., Lambert, B., et al. (2003). Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. *Mol. Plant Microbe Interact.* 16, 1118–1128. doi: 10.1094/MPMI.2003.16.12.1118

Aziz, A., Trotel-Aziz, P., Dhuicq, L., Jeandet, P., Couderchet, M., and Vernet, G. (2006). Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew. *Phytopathology* 96, 1188–1194. doi: 10.1094/PHYTO-96-1188

Baker, G. C., Smith, J. J., and Cowan, D. A. (2003). Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods* 55, 541–555. doi: 10.1016/j.mimet.2003.08.009

Barnett, S. J., Roget, D. K., and Ryder, M. H. (2006). Suppression of *Rhizoctonia solani* AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and *Microbacteria*. *Soil Res.* 44, 331–342. doi: 10.1071/SR05113

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., et al. (2013). Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. *Methods Ecol. Evol.* 4, 914–919. doi: 10.1111/2041-210x.12073

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Boubakri, H., Chong, J., Poutaraud, A., Schmitt, C., Bertsch, C., Mliki, A., et al. (2013). Riboflavin (vitamin B2) induces defence responses and resistance to *Plasmopara viticola* in grapevine. *Eur. J. Plant Pathol.* 136, 837–855. doi: 10.1007/s10658-013-0211-x

Boubakri, H., Wahab, M. A., Chong, J., Bertsch, C., Mliki, A., and Soustre-Gacougnolle, I. (2012). Thiamine induced resistance to *Plasmopara viticola* in grapevine and elicited host-defense responses, including HR like-cell death. *Plant Physiol. Biochem.* 57, 120–133. doi: 10.1016/j.plaphy.2012.05.016

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of Southern Wisconsin. *Ecol. Monogr.* 27, 325–349. doi: 10.2307/1942268

Bulgari, D., Casati, P., Brusetti, L., Quaglino, F., Brasca, M., Daffonchio, D., et al. (2009). Endophytic bacterial diversity in grapevine (*Vitis vinifera* L.) leaves described by 16S rRNA gene sequence analysis and length heterogeneity-PCR. *J. Microbiol.* 47, 393–401. doi: 10.1007/s12275-009-0082-1

Burruano, S., Alfonzo, A., Lo Piccolo, S., Conigliaro, G., Mondello, V., Torta, L., et al. (2008). Interaction between *Acremonium byssoides* and *Plasmopara viticola* in *Vitis vinifera*. *Phytopathol. Mediterr.* 47, 122–131. doi: 10.14601/Phytopathol_Mediterr-2615

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Casagrande, K., Falginella, L., Castellarin, S. D., Testolin, R., and Di Gaspero, G. (2011). Defence responses in Rpv3-dependent resistance to grapevine downy mildew. *Planta* 234, 1097–1109. doi: 10.1007/s00425-011-1461-5

Chang, X., and Nick, P. (2012). Defence signalling triggered by Flg22 and harpin is integrated into a different stilbene output in *Vitis* cells. *PLoS ONE* 7:e40446. doi: 10.1371/journal.pone.0040446

Chao, A. (1984). Nonparametric-estimation of the number of classes in a population. *Scand. J. Stat.* 11, 265–270.

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Delaunois, B., Farace, G., Jeandet, P., Clement, C., Baillieul, F., Dorey, S., et al. (2014). Elicitors as alternative strategy to pesticides in grapevine? Current knowledge on their mode of action from controlled conditions to vineyard. *Environ. Sci. Pollut. Res. Int.* 21, 4837–4846. doi: 10.1007/s11356-013-1841-4

Dercks, W., and Creasy, L. L. (1989). Influence of fosetyl-Al on phytoalexin accumulation in the *Plasmopara viticola* grapevine interaction. *Physiol. Mol. Plant Pathol.* 34, 203–213. doi: 10.1016/0885-5765(89)90044-1

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Elad, Y. (2000). Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot.* 19, 709–714. doi: 10.1016/S0261-2194(00)00094-6

EPPO (2001). European and Mediterranean plant protection Organization. Guidelines for the efficacy evaluation of fungicides: *Plasmopara viticola*. *EPPO Bull.* 31, 313–317. doi: 10.1111/j.1365-2338.2001.tb01000.x

Falk, S. P., Pearson, R. C., Gadoury, D. M., Seem, R. C., and Sztejnberg, A. (1996). *Fusarium proliferatum* as a biocontrol agent against grape downy mildew. *Phytopathology* 86, 1010–1017. doi: 10.1094/Phyto-86-1010

Fantke, P., Friedrich, R., and Jolliet, O. (2012). Health impact and damage cost assessment of pesticides in Europe. *Environ. Int.* 49, 9–17. doi: 10.1016/j.envint.2012.08.001

Gessler, C., Pertot, I., and Perazzolli, M. (2011). *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathol. Mediterr.* 50, 3–44. doi: 10.14601/Phytopathol_Mediterr-9360

Good, I. J. (1953). The population frequencies of species and the estimation of population parameters. *Biometrika* 40, 237–264. doi: 10.1093/biomet/40.3-4.237

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., et al. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* 21, 494–504. doi: 10.1101/gr.112730.110

Hamiduzzaman, M. M., Jakab, G., Barnavon, L., Neuhaus, J. M., and Mauch-Mani, B. (2005). ß-aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. *Mol. Plant Microbe Interact.* 18, 819–829. doi: 10.1094/MPMI-18-0819

Harm, A., Kassemeyer, H. H., Seibicke, T., and Regner, F. (2011). Evaluation of chemical and natural resistance inducers against downy mildew (*Plasmopara viticola*) in grapevine. *Am. J. Enol. Vitic.* 62, 184–192. doi: 10.5344/ajev.2011.09054

Hartmann, M., Howes, C. G., Abarenkov, K., Mohn, W. W., and Nilsson, R. H. (2010). V-Xtractor: an open-source, high-throughput software tool to identify and extract hypervariable regions of small subunit (16 S/18 S) ribosomal RNA gene sequences. *J. Microbiol. Methods* 83, 250–253. doi: 10.1016/j.mimet.2010.08.008

Inacio, J., Pereira, P., De Carvalho, M., Fonseca, A., Amaral-Collaco, M. T., and Spencer-Martins, I. (2002). Estimation and diversity of phylloplane mycobiota on selected plants in a mediterranean-type ecosystem in Portugal. *Microb. Ecol.* 44, 344–353. doi: 10.1007/s00248-002-2022-z

Jaiganesh, V., Eswaran, A., Balabaskar, P., and Kannan, C. (2007). Antagonistic activity of *Serratia marcescens* against *Pyricularia oryzae*. *Not. Bot. Horti Agrobot. Cluj Napoca* 35, 48–54. Available online at: http://www.notulaebotanicae.ro/index.php/nbha/article/view/219/216

Jumpponen, A., and Jones, K. L. (2009). Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. *New Phytol.* 184, 438–448. doi: 10.1111/j.1469-8137.2009.02990.x

Kobayashi, D. Y., Guglielmoni, M., and Clarke, B. B. (1995). Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turfgrass. *Soil Biol. Biochem.* 27, 1479–1487. doi: 10.1016/0038-0717(95)00062-J

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271–5277. doi: 10.1111/mec.12481

Lachhab, N., Sanzani, S. M., Adrian, M., Chiltz, A., Balacey, S., Boselli, M., et al. (2014). Soybean and casein hydrolysates induce grapevine immune responses and resistance against *Plasmopara viticola*. *Front. Plant Sci.* 5:716. doi: 10.3389/fpls.2014.00716

Lachhab, N., Sanzani, S. M., Bahouaoui, M. A., Boselli, M., and Ippolito, A. (2016). Effect of some protein hydrolysates against gray mould of table and wine grapes. *Eur. J. Plant Pathol.* 144, 821–830. doi: 10.1007/s10658-015-0749-x

Legendre, P., and Legendre, L. (1998). "Numerical ecology" in *Developments in Environmental Modelling*, eds P. Legendre and L. F. J. Legendre (Amsterdam: Elsevier Science BV), 852.

Lenzi, L., Caruso, C., Bianchedi, P. L., Pertot, I., and Perazzolli, M. (2015). Laser microdissection of grapevine leaves reveals site-specific regulation of transcriptional response to *Plasmopara viticola*. *Plant Cell Physiol.* 57, 69–81. doi: 10.1093/pcp/pcv166

Leveau, J. H. J., and Tech, J. J. (2011). Grapevine microbiomics: bacterial diversity on grape leaves and berries revealed by high-throughput sequence analysis of 16S rRNA amplicons. *Acta Hortic.* 905, 31–42. doi: 10.17660/ActaHortic.2011.905.2

Ligon, J. M., Hill, D. S., Hammer, P. E., Torkewitz, N. R., Hofmann, D., Kempf, H. J., et al. (2000). Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Manag. Sci.* 56, 688–695. doi: 10.1002/1526-4998(200008)56:8<688::AID-PS186>3.0.CO;2-V

Martins, G., Lauga, B., Miot-Sertier, C., Mercier, A., Lonvaud, A., Soulas, M. L., et al. (2013). Characterization of epiphytic bacterial communities from grapes, leaves, bark and soil of grapevine plants grown, and their relations. *PLoS ONE* 8:e73013. doi: 10.1371/journal.pone.0073013

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217. doi: 10.1371/journal.pone.0061217

McMurdie, P. J., and Holmes, S. (2014). Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput. Biol.* 10:e1003531. doi: 10.1371/journal.pcbi.1003531

Musetti, R., Vecchione, A., Stringher, L., Borselli, S., Zulini, L., Marzani, C., et al. (2006). Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. *Phytopathology* 96, 689–698. doi: 10.1094/PHYTO-96-0689

Nesler, A., Perazzolli, M., Puopolo, G., Giovannini, O., and Pertot, I. (2015). A complex protein hydrolysate acts as biogenic elicitor of plant resistance against powdery mildew. *Front. Plant Sci.* 6:715. doi: 10.3389/fpls.2015.00715

Pal, K. K., and McSpadden Gardener, B. (2006). Biological control of plant pathogens. *Plant Health Instr.* 10, 1094–1117. doi: 10.1094/phi-a-2006-1117-02

Paulson, J. N., Stine, O. C., Bravo, H. C., and Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* 10, 1200–1202. doi: 10.1038/nmeth.2658

Peñuelas, J., and Terradas, J. (2014). The foliar microbiome. *Trends Plant Sci.* 19, 278–280. doi: 10.1016/j.tplants.2013.12.007

Perazzolli, M., Antonielli, L., Storari, M., Puopolo, G., Pancher, M., Giovannini, O., et al. (2014). Resilience of the natural phyllosphere microbiota of the grapevine to chemical and biological pesticides. *Appl. Environ. Microbiol.* 80, 3585–3596. doi: 10.1128/AEM.00415-14

Perazzolli, M., Dagostin, S., Ferrari, A., Elad, Y., and Pertot, I. (2008). Induction of systemic resistance against *Plasmopara viticola* in grapevine by *Trichoderma harzianum* T39 and benzothiadiazole. *Biol. Control* 47, 228–234. doi: 10.1016/j.biocontrol.2008.08.008

Perazzolli, M., Moretto, M., Fontana, P., Ferrarini, A., Velasco, R., Moser, C., et al. (2012). Downy mildew resistance induced by *Trichoderma harzianum* T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. *BMC Genomics* 13:660. doi: 10.1186/1471-2164-13-660

Perazzolli, M., Roatti, B., Bozza, E., and Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. *Biol. Control* 58, 74–82. doi: 10.1016/j.biocontrol.2011.04.006

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45

Pieterse, C. M. J., Leon-Reyes, A., Van Der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., and Gomes, A. C. (2014). Unravelling the diversity of grapevine microbiome. *PLoS ONE* 9:e85622. doi: 10.1371/journal.pone.0085622

Polesani, M., Bortesi, L., Ferrarini, A., Zamboni, A., Fasoli, M., Zadra, C., et al. (2010). General and species-specific transcriptional responses to downy mildew infection in a susceptible (*Vitis vinifera*) and a resistant (*V. riparia)* grapevine species. *BMC Genomics* 11:117. doi: 10.1186/1471-2164-11-117

Puopolo, G., Giovannini, O., and Pertot, I. (2014). *Lysobacter capsici* AZ78 can be combined with copper to effectively control *Plasmopara viticola* on grapevine. *Microbiol. Res.* 169, 633–642. doi: 10.1016/j.micres.2013.09.013

Puopolo, G., Raio, A., and Zoina, A. (2010). Identification and characterization of *Lysobacter capsici* strain PG4: a new plant health-promoting rhizobacterium. *Plant Pathol.* 92, 157–164. doi: 10.4454/jpp.v92i1.25

Qiao, F., Chang, X.-L., and Nick, P. (2010). The cytoskeleton enhances gene expression in the response to the harpin elicitor in grapevine. *J. Exp. Bot.* 61, 4021–4031. doi: 10.1093/jxb/erq221

Ritpitakphong, U., Falquet, L., Vimoltust, A., Berger, A., Métraux, J.-P., and L'Haridon, F. (2016). The microbiome of the leaf surface of Arabidopsis protects against a fungal pathogen. *New Phytol.* 210, 1033–1043. doi: 10.1111/nph.13808

Ruijter, J. M., Ramakers, C., Hoogaars, W. M., Karlen, Y., Bakker, O., Van Den Hoff, M. J., et al. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37:e45. doi: 10.1093/nar/gkp045

Schmieder, R., and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27, 863–864. doi: 10.1093/bioinformatics/btr026

Simpson, E. H. (1949). Measurement of diversity. *Nature* 163, 688. doi: 10.1038/163688a0

Thuerig, B., Binder, A., Boller, T., Guyer, U., Jimenez, S., Rentsch, C., et al. (2006). An aqueous extract of the dry mycelium of *Penicillium chrysogenum* induces resistance in several crops under controlled and field conditions. *Eur. J. Plant Pathol.* 114, 185–197. doi: 10.1007/s10658-005-4512-6

Thuerig, B., Slaughter, A., Marouf, E., Held, M., Mauch-Mani, B., and Tamm, L. (2011). Site-specific field resistance of grapevine to *Plasmopara viticola* correlates to altered gene expression and was not modulated by the application of organic amendments. *Eur. J. Plant Pathol.* 129, 255–265. doi: 10.1007/s10658-010-9712-z

Touceda-Gonzalez, M., Brader, G., Antonielli, L., Ravindran, V. B., Waldner, G., Friesl-Hanl, W., et al. (2015). Combined amendment of immobilizers and the plant growth-promoting strain Burkholderia phytofirmans PsJN favours plant growth and reduces heavy metal uptake. *Soil Biol. Biochem.* 91, 140–150. doi: 10.1016/j.soilbio.2015.08.038

Trdà, L., Fernandez, O., Boutrot, F., Heloir, M.-C., Kelloniemi, J., Daire, X., et al. (2014). The grapevine flagellin receptor VvFLS2 differentially recognizes flagellin-derived epitopes from the endophytic growth-promoting bacterium *Burkholderia phytofirmans* and plant pathogenic bacteria. *New Phytol.* 201, 1371–1384. doi: 10.1111/nph.12592

Trotel-Aziz, P., Couderchet, M., Biagianti, S., and Aziz, A. (2008). Characterization of new bacterial biocontrol agents *Acinetobacter*, *Bacillus*, *Pantoea* and *Pseudomonas* spp. mediating grapevine resistance against Botrytis cinerea. *Environ. Exp. Bot.* 64, 21–32. doi: 10.1016/j.envexpbot.2007.12.009

Trouvelot, S., Heloir, M. C., Poinssot, B., Gauthier, A., Paris, F., Guillier, C., et al. (2014). Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays. *Front. Plant Sci.* 5:592. doi: 10.3389/fpls.2014.00592

Trouvelot, S., Varnier, A. L., Allegre, M., Mercier, L., Baillieul, F., Arnould, C., et al. (2008). A beta-1,3 glucan sulfate induces resistance in grapevine against *Plasmopara viticola* through priming of defense responses, including HR-like cell death. *Mol. Plant Microbe Interact.* 21, 232–243. doi: 10.1094/MPMI-21-2-0232

Van Wees, S. C. M., Van Der Ent, S., and Pieterse, C. M. J. (2008). Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* 11, 443–448. doi: 10.1016/j.pbi.2008.05.005

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* 10, 828–840. doi: 10.1038/nrmicro2910

Walters, D. R., Ratsep, J., and Havis, N. D. (2013). Controlling crop diseases using induced resistance: challenges for the future. *J. Exp. Bot.* 64, 1263–1280. doi: 10.1093/jxb/ert026

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Whipps, J. M., Hand, P., Pink, D., and Bending, G. D. (2008a). Phyllosphere microbiology with special reference to diversity and plant genotype. *J. Appl. Microbiol.* 105, 1744–1755. doi: 10.1111/j.1365-2672.2008.03906.x

Whipps, J. M., Hand, P., Pink, D. C., and Bending, G. D. (2008b). Human pathogens and the phyllosphere. *Adv. Appl. Microbiol.* 64, 183–221. doi: 10.1016/S0065-2164(08)00407-3

White, T., Bruns, T., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: A Guide to Methods and Applications*, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (New York, NY: Academic Press, Inc.), 315–322.

Wu, S., Shan, L., and He, P. (2014). Microbial signature-triggered plant defense responses and early signaling mechanisms. *Plant Sci.* 228, 118–126. doi: 10.1016/j.plantsci.2014.03.001

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., et al. (2015). The soil microbiome influences grapevine-associated microbiota. *MBio* 6:e02527-14. doi: 10.1128/mBio.02527-14

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Leaf treatments with a protein-based resistance inducer partially modify phyllosphere microbial communities of grapevine

Martina Cappelletti[1,2], Michele Perazzolli[1], Livio Antonielli[3], Andrea Nesler[1], Esmeralda Torboli[1], Pier Luigi Bianchedi[4], Massimo Pindo[1], Gerardo Puopolo[1] and Ilaria Pertot[1]

[1] Department of Sustainable Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige Italy; [2] Department of Agricultural and Environmental Sciences, University of Udine, Italy; [3] Department of Health and Environment, Bioresources Unit, Austrian Institute of Technology, Tulln and der Donau, Austria; [4] Technology Transfer Center, Fondazione Edmund Mach, San Michele all'Adige, Italy

1. **Supplementary Results**
2. **Supplementary Figures**
3. **Supplementary Tables**

1. Supplementary Results

Characterization of Culturable Leaf Microorganisms

In order to evaluate the effect of NB on phyllosphere microorganisms, viability of culturable bacteria and fungi on grapevine leaves was assessed by a plating method on selective media. Numbers of colony forming units (CFU) of culturable bacteria were greater on NB-treated plants in comparison to H_2O-treated and UNT plants at T0 and T1 in both experiments (Figures S1A and S1B). CFU numbers were generally greater at T1 in comparison to T0 on H_2O-treated plants, and the absence of bacteria in the inoculum suspension (data not shown) indicated a possible stimulation by the moist chamber used for pathogen inoculation. CFU of culturable fungi were not significantly influenced by the treatments tested, except for an increment caused by NB on grapevines of Exp 1 (Figures S1C and S1D).

Because NB application increased the number of culturable bacteria on grapevine leaves, some representative bacterial isolates (60 from the Exp 1, and 37 from the Exp 2) were selected visually based on colony morphological features from samples of H_2O- and NB-treated plants. Indicators of biological control properties were analyzed for each representative isolate: percentages of bacteria with protease activity, siderophore production and antagonistic activities against the oomycete *Phytophthora infestans* were comparable among isolates collected from NB- and H_2O-treated plants at both time points (Table S2). Furthermore, seven and six bacterial isolates were randomly selected for Exp 1 and Exp 2, respectively, and tested against *Plasmopara viticola* on leaf discs. Two bacterial isolates collected from NB-treated plants significantly reduce downy mildew severity on leaf disks with a disease reduction lower than that of the biocontrol agent *Lysobacter capsici* AZ78 (Figure S2). These isolates corresponded to a *Pseudomonas* spp. (KU596386; T1_NB_7 of Exp 1) and an *Enterobacter* spp. (KU596387; T1_NB_13 of Exp 2) by sequencing of the V6-V8 hypervariable region of the 16S rRNA gene.

Identification, Richness and Diversity of Leaf Microbial Communities

Pyrosequencing analysis of bacterial (16S rRNA gene) and fungal (ITS fragment) amplicons (Table S3) was carried out to identify microorganisms collected from grapevine leaves of plants under greenhouse conditions. After filtering out low-quality reads and short sequences, 403,900 (Exp 1) and 274,911 (Exp 2) reads of bacteria, and 78,542 (Exp 1) and 74,859 (Exp 2) reads of fungi were obtained (Tables S4 and S5). The total number of filtered reads for each replicate ranged from 2,703 to 52,719 (Exp 1) and from 1,415 to 54,709 (Exp 2) for bacteria (Table S4), and from 1,979 to 8,652 (Exp 1) and from 1,982 to 10,174 (Exp 2) for fungi (Table S5). Good's coverage was used to estimate the completeness of sampling with a probability calculation based on randomly selected sequences, and it ranged from 82.1 to 97.5% (Exp 1) and from 95.8 to 99.3% (Exp 2) for bacterial data (Table S4), and from 95.8 to 98.0% (Exp 1) and from 95.6 to 98.1% (Exp 2) for fungal data (Table S5). Likewise, rarefaction curves confirmed that a sufficient saturation was reached for both bacterial and fungal populations of each replicate (Figures S3 and S4). Chao1 index revealed that more than 88% and 74% for bacteria (Table S4) and more than 79% and 67% for fungi (Table S5) of the estimated richness was covered by the sequencing effort in Exp 1 and Exp 2, respectively.

Bacterial and fungal data were normalized to the lowest number of filtered reads (1415 in H_2O at T1 replicate A in Exp 2, and 1979 in H_2O at T1 replicate C of Exp1, respectively) and OTU were recalculated for each sample. In Exp 1, richness of bacterial communities was lower on NB-treated plants in comparison to H_2O-treated

and LAM-treated plants at T0, while OTU numbers at T1 were comparable among all treatments (Figure S5A). In Exp 2, bacterial richness was significantly lower on H_2O-treated plants at T0 with respect to all other treatments at T1. Richness of fungal populations was comparable among treatments in Exp 2, and it was greater on H_2O-treated plants at T0 in comparison to T1 (Figure S5B).

Bacterial diversity estimated by the Simpson index significantly increased by the grapevine treatments at both time points compared to UNT plants in Exp 1, except for NB-treated plants at T0 and LAM-treated plants at T1. Conversely, in Exp 2 the diversity estimator had the highest value on NB-treated leaves at T1 and the lowest value on H_2O-treated leaves at T0 (Figure S6A). For fungal populations, the Simpson index significantly differed only between T0 and T1 of H_2O-treated plants in Exp 1, and between H_2O-treated plants at T0 and LAM-treated plants at T1 in Exp 2 (Figure S6B).

Distribution of Bacterial Phyla among Experiments, Treatments and Time Points

Almost the totality of bacterial reads (99.95%) were assigned to taxa at phylum level, 11 different bacterial phyla were detected in total (Table S6) and 7 dominant phyla were presented (more than 0.4 % of relative abundance in at least one sample, Figure S7). In Exp 1, the relative abundance of Cyanobacteria, Unknown phyla and Planctomycetes was greater on H_2O-treated plants in comparison to UNT plants (Figure S7A). On leaves collected at T0, the abundance of Firmicutes was greater on NB-treated plants in comparison to H_2O-treated leaves, while levels of all other phyla were lower. Furthermore, lower abundances of Cyanobacteria, Unknown phyla and Planctomycets were detected on LAM-treated plants in comparison to H_2O-treated plants. At T1, sizes of bacterial phyla were comparable on H_2O-, NB- and LAM-treated leaves. Considering the proportions of bacterial phyla at the two time points, the levels of Actinobacteria, Cyanobacteria, Acidobacteria, Unknown phyla and Planctomycetes on H_2O-treated plants were reduced from T0 to T1. The relative abundance of dominant phyla was comparable on NB- and LAM-treated leaves at the two time points, except for the reduction of Actinobacteria on LAM-treated leaves from T0 to T1.

In Exp 2, H_2O application on grapevine leaves did not influence phyla proportions (Figure S7B). On leaves collected at T0, phyla abundances were similar on NB- and H_2O-treated plants, and the Cyanobacteria abundance was greater on LAM-treated plants in comparison to H_2O-treated plants. At T1, abundances of Proteobacteria and Firmicutes were lower and greater on NB-treated plants in comparison to H_2O-treated plants, respectively. Moreover, abundances of Proteobacteria decreased and those of Firmicutes increased from T0 to T1, and lower abundance of Actinobacteria and Cyanobacteria was detected at T0 with respect to T1 on LAM-treated plants.

Distribution of Fungal Phyla, Family and Genera among Experiments, Treatments and Time Points

Ascomycota was the most common fungal phylum detected, and only 2.3% of total fungal OTU (24 out of 1051) was attributed to Basidiomycota and Zygomycota. Of fungal reads, 85.5% was attributed to taxa at the family level (796 OTU), and 89.2% was assigned to a fungal genus (768 OTU); 34, 53 and 87 different fungal families, genera and species were identified in total, respectively (Table S7). The proportions of the 10, 7 and 15 dominant families (Figure S9), genera (Figure S10) and species (Figure S11) were homogeneous between the two experiments, and they were only slightly affected by treatments and time points.

The relative abundance of Arthrodermataceae decreased on UNT, LAM- and NB-treated plants in comparison to H_2O-treated plants at T0 in Exp 1, while comparable abundances were observed for the other dominant families among treatments (Figure S9A). At T1, the presence of Agaricaceae was greater on LAM-treated plants in

comparison to H_2O-treated plants and comparable abundances were observed for all fungal families between NB- and H_2O-treated plants. Considering the proportions of fungal families at the two time points, abundances of Agaricaceae increased on H_2O- and LAM-treated plants from T0 to T1, while those of Arthrodermataceae decreased on H_2O-treated plants from T0 to T1. In Exp 2, H_2O treatment partially influenced family proportions at T0, and the relative abundance of Microascaceae and Erysiphaceae decreased compared to UNT plants (Figure S9B). Relative abundances of fungal families were comparable on NB- and H_2O-treated plants, and the presence of Apiosporaceae increased on LAM-treated plants at T0 and T1. Considering proportions of fungal families at two different time points, only the abundance of Onygenaceae and Arthrodermataceae changed on NB- and LAM-treated plants from T0 to T1, respectively.

In Exp 1, relative abundances of dominant fungal genera were generally comparable on H_2O-treated and UNT plants, only the presence of *Peniciullium* spp. was decreased by H_2O treatment (Figure S10A). Percentages of fungal genera were comparable for H_2O-, NB- and LAM-treated plants at T0, while the presence of *Hansfordia* spp. was greater on LAM-treated plants in comparison to H_2O- and NB-treated plants at T1. Relative abundance of bacterial genera was comparable on H_2O-, NB- and LAM-treated plants at the two time points, except for an increase in *Hanfordia* levels on LAM-treated plants from T0 to T1. In Exp 2, H_2O application did not affect genera proportions at T0 (Figure S10B). Relative abundances of fungal genera were comparable on NB- and H_2O-treated plants at T0 and T1. Comparing H_2O- and LAM-treated plants, the presence of *Aspergillus* decreased at T0, whereas that of *Arthrinium* spp. increased at T0 and T1. Moreover, abundances of *Chrysosporium* spp. and *Hansfordia* spp. increased on NB- and LAM-treated leaves from T0 to T1, respectively.

2. Supplementary Figures

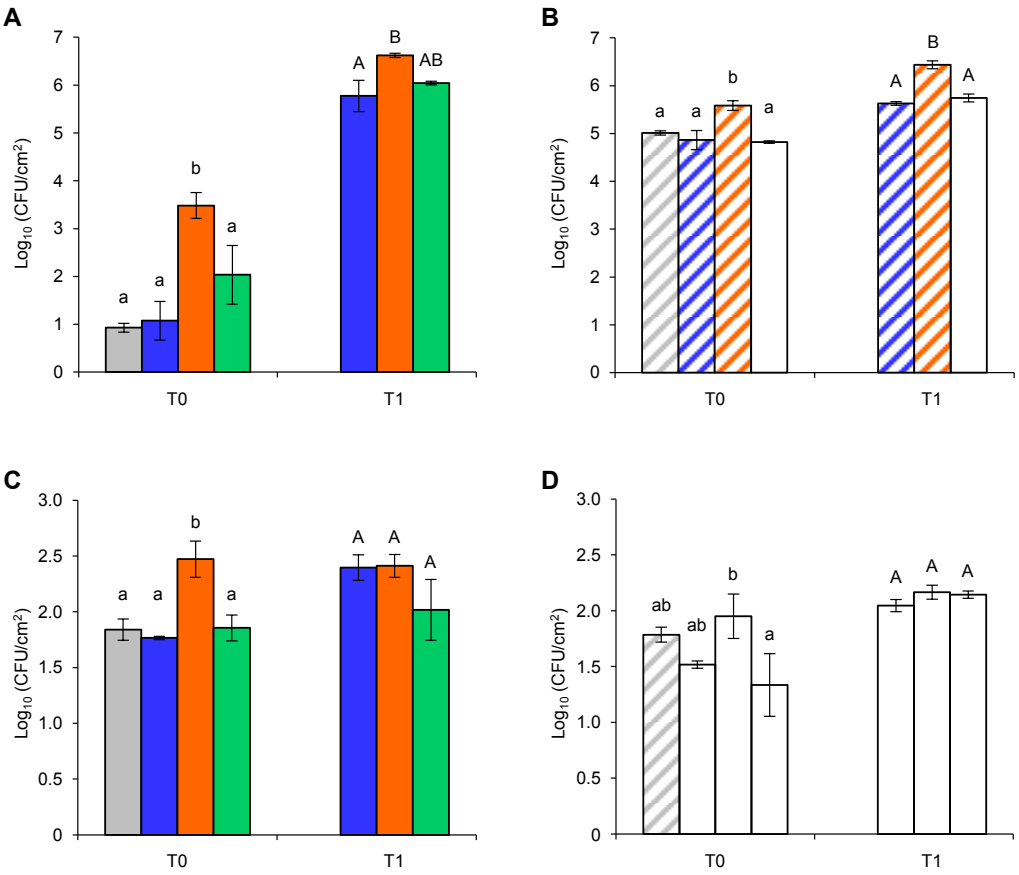

FIGURE S1 | Assessment of culturable microorganisms of grapevine leaves. Colony forming units (CFU) of bacteria (A, B) and fungi (C, D) per unit of grapevine leaf area (cm^2) were assessed for untreated plants (grey), and plants treated with water (blue), nutrient broth (orange) or laminarin (green) collected just before (T0) and one day after (T1) *Plasmopara viticola* in the experiment 1 (A, C) and experiment 2 (B, D), by plating method on selective media. Mean Log_{10} (CFU/cm^2) values and standard errors from three replicates (each as a pool of two plants) are presented for each sample. Different lowercase and uppercase letters indicate significant differences at T0 and T1 according to Fisher's test (α = 0.05), respectively.

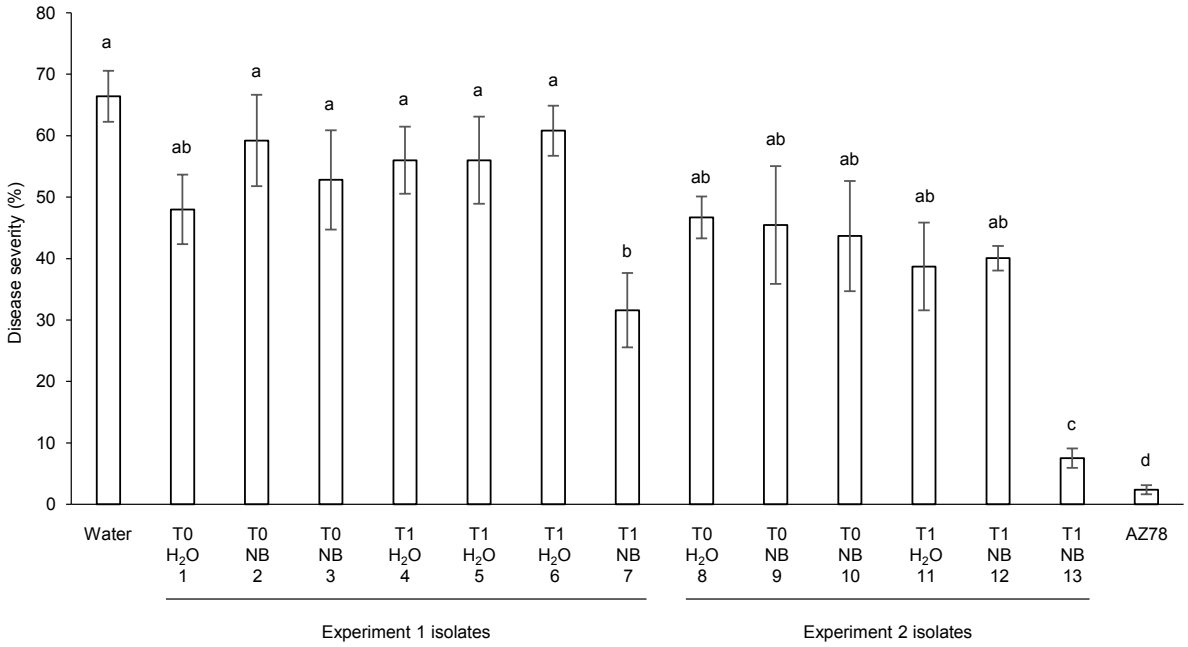

FIGURE S2 | Effect of culturable bacterial isolates against *Plasmopara viticola*. Seven and six bacterial isolates were randomly selected for experiment 1 and experiment 2, respectively, and tested against *P. viticola* on surface-sterilized leaf disks. Bacterial codes indicate time points (T0 or T1) and treatments (H_2O or NB) of leaf samples from where each bacteria was isolated, and a progressive numerical code assigned to the representative isolates. The pure *P. viticola* suspension (water) and the biocontrol strain *Lysobacter capsici* AZ78 (AZ78) were used as controls and disease severity was assessed as percentage of disc area covered by *P. viticola* sporulation seven days after inoculation. Mean severity and standard error values of five replicates (five dishes with five leaf disks each) of one representative experiment are reported for each treatment. Different letters indicate significant differences according to Fisher's test (α = 0.05). Isolates with the greatest efficacy corresponded to a *Pseudomonas* sp. (T1_NB_7 of Exp 1) and an *Enterobacter* sp. (T1_NB_13 of Exp 2).

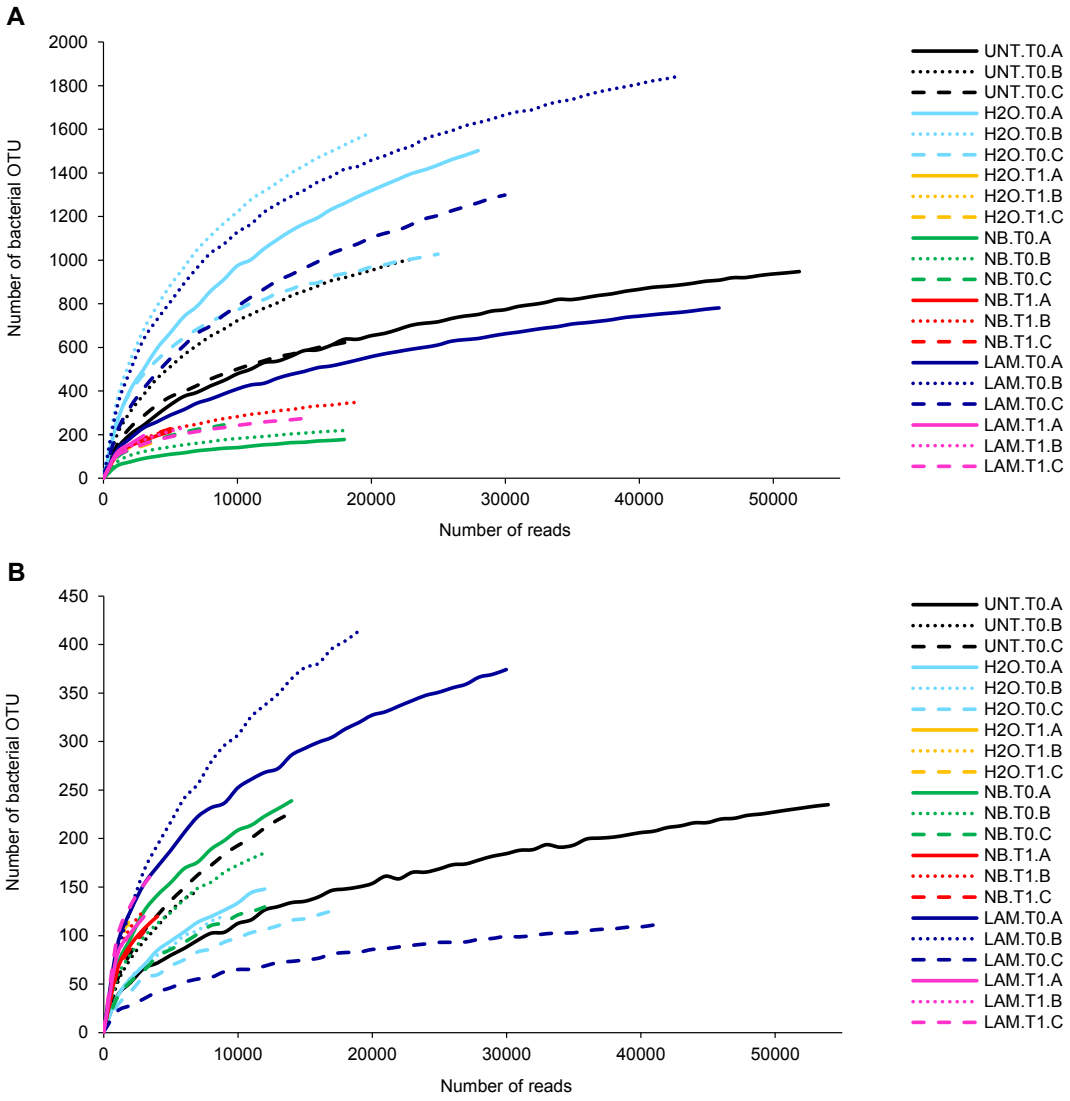

FIGURE S3 | Rarefaction curves of bacterial communities identified on grapevine leaves in the experiment 1 (A) and experiment 2 (B). Curves were obtained by random resampling without replacement using QIIME, for samples collected from untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation. Three replicates (each as a pool of two plants) were analyzed for each treatment and each time point (replicate A: solid lines; B: dotted lines; C: dashed lines).

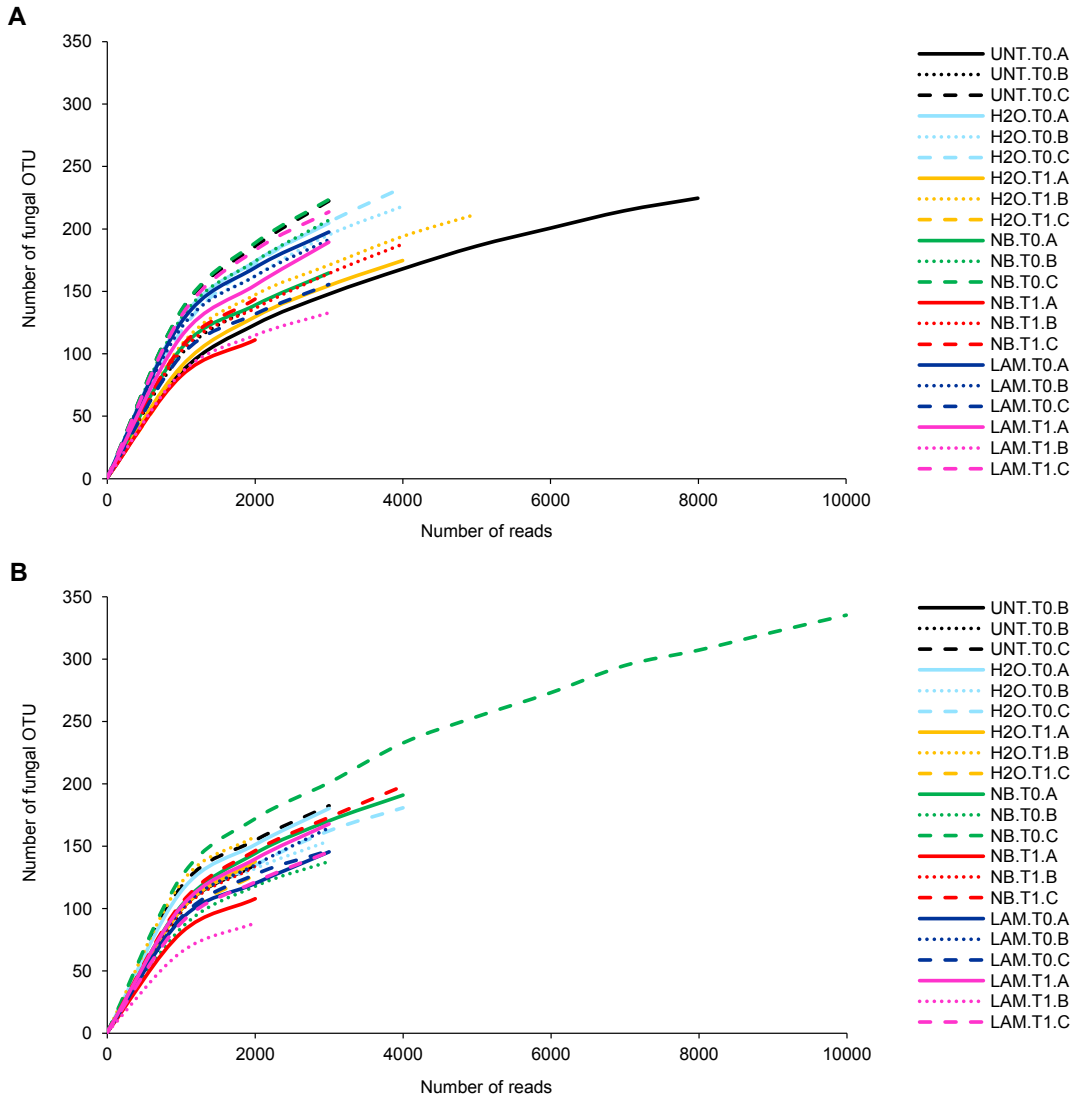

FIGURE S4 | Rarefaction curves of fungal communities identified on grapevine leaves in experiment 1 (A) and experiment 2 (B). Curves were obtained by random resampling without replacement with QIIME, for samples collected from untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation. Three replicates (each as a pool of two plants) were analyzed for each treatment and time point (replicate A: solid lines; B: dotted lines; C: dashed lines).

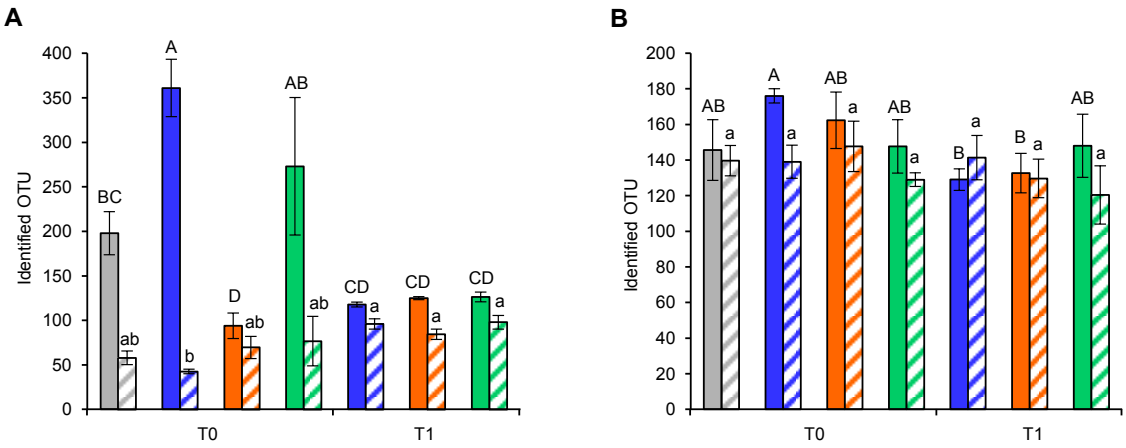

FIGURE S5 | Richness of bacterial (A) and fungal (B) populations on grapevine leaves. Operational taxonomic units (OTU) were determined for untreated plants (grey), and plants treated with water (blue), nutrient broth (orange) or laminarin (green) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation, and normalized to the lowest number of quality filtered reads in experiment 1 (solid bars) and experiment 2 (striped bars). Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. Different uppercase and lowercase letters indicate significant differences of experiment 1 and experiment 2 according to Fisher's test (α = 0.05), respectively.

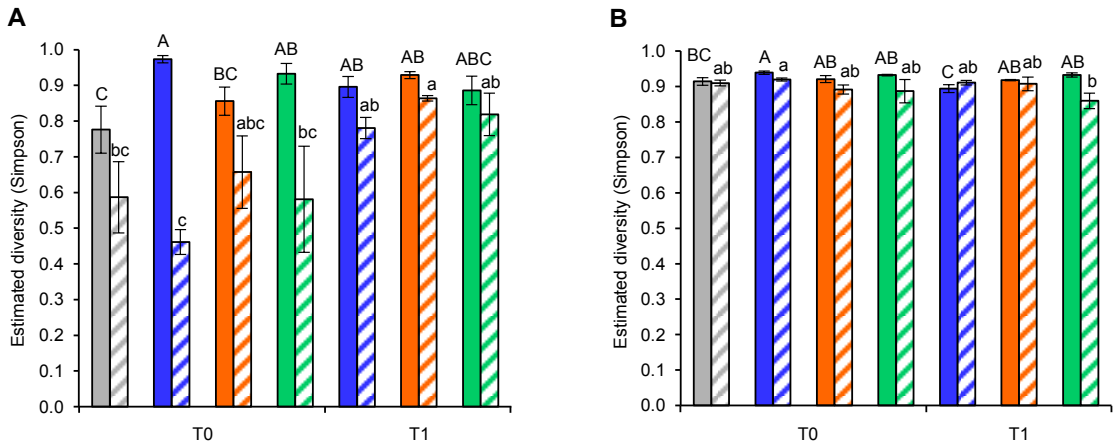

FIGURE S6 | Diversity of bacterial (A) and fungal (B) populations on grapevine leaves. The Simpson index was determined for untreated plants (grey), and plants treated with water (blue), nutrient broth (orange) and laminarin (green) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation, and normalized to the lowest number of quality filtered reads in experiment 1 (solid bars) and experiment 2 (striped bars). Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. Different uppercase and lowercase letters indicate significant differences of experiment 1 and experiment 2 according to Fisher's test (α = 0.05), respectively.

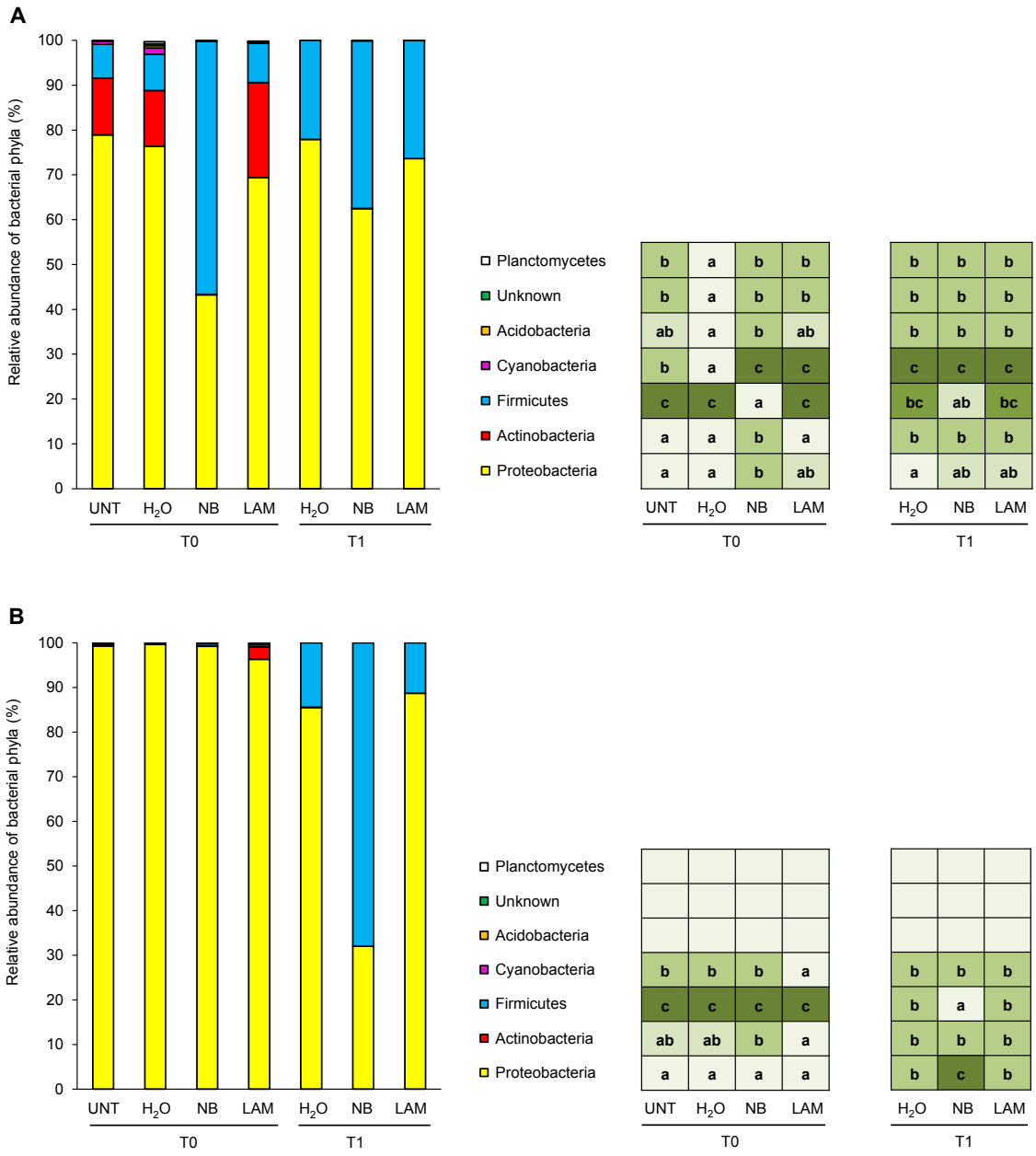

FIGURE S7 | Relative abundance of the dominant (more than 0.4 % of relative abundance in at least one sample) bacterial phyla on grapevine leaves. Percentages of relative abundance were determined for leaves of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in the experiment 1 (A) and experiment 2 (B). Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. For each taxon, the intensity of the color gradient and letters reported in the table indicate significant differences among treatments and time points according to Fisher's test ($\alpha = 0.05$).

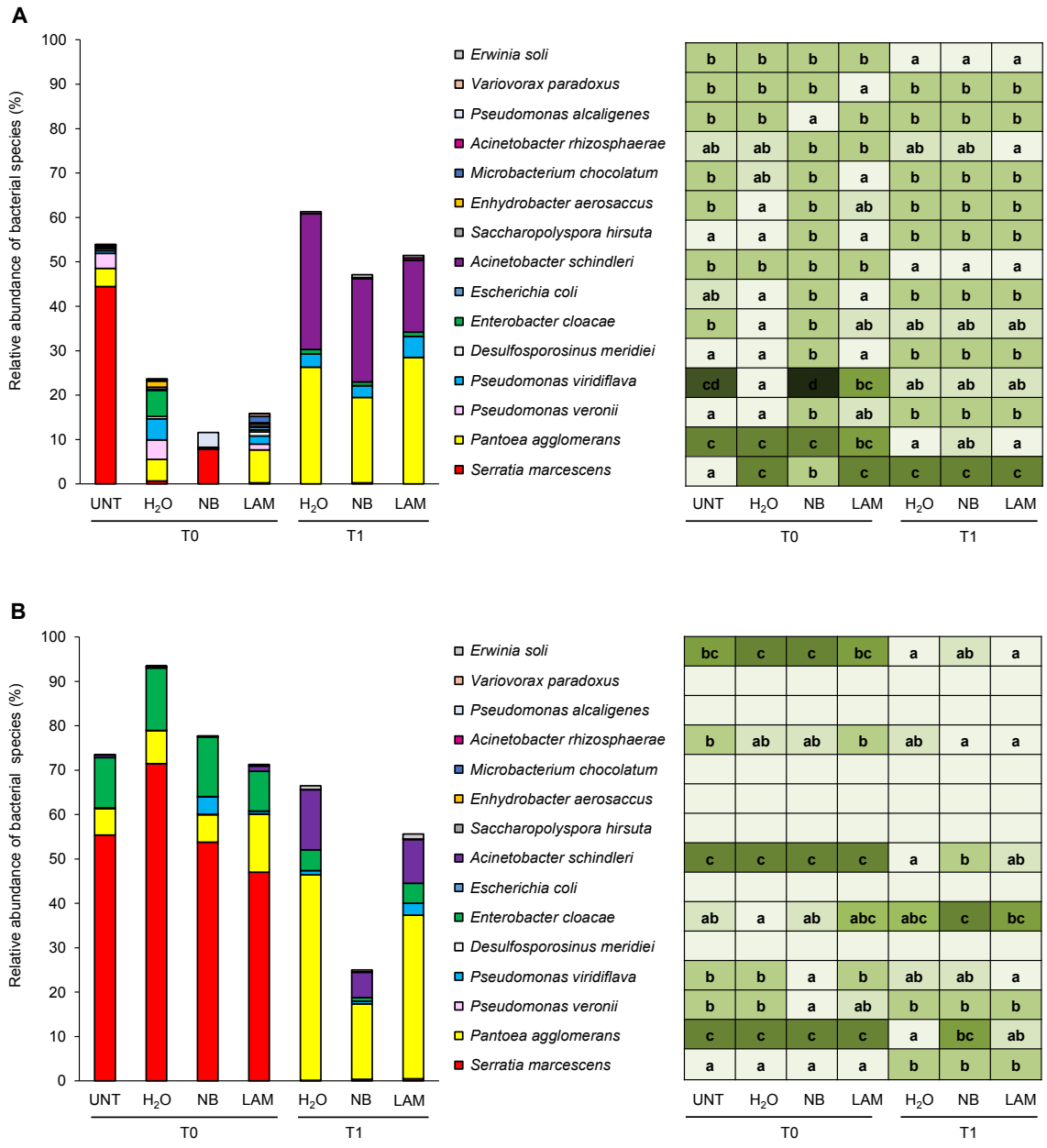

FIGURE S8 | Relative abundance of the dominant (more than 0.5 % of relative abundance in at least one sample) bacterial species on grapevine leaves. Percentages of relative abundance were determined for leaves of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in the experiment 1 (A) and experiment 2 (B). Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. For each taxon, the intensity of the color gradient and letters reported in the table indicate significant differences among treatments and time points according to Fisher's test (α = 0.05).

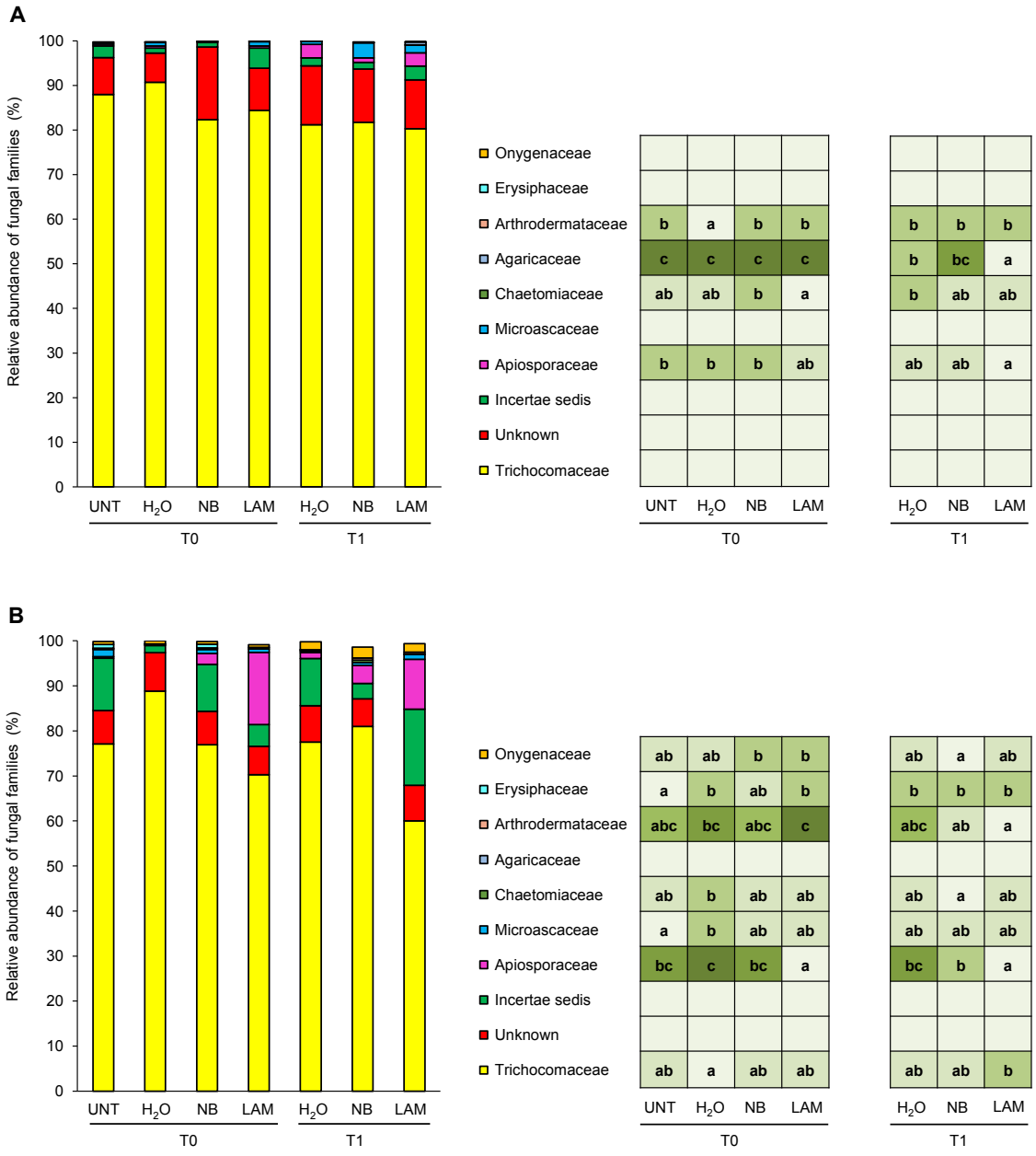

FIGURE S9 | Relative abundance of the dominant (more than 0.5 % of relative abundance in at least one sample) fungal families on grapevine leaves. Percentages of relative abundance were determined for leaves of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in the experiment 1 (A) and experiment 2 (B). Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. For each taxon, the intensity of the color gradient and letters reported in the table indicate significant differences among treatments and time points according to Fisher's test ($\alpha = 0.05$).

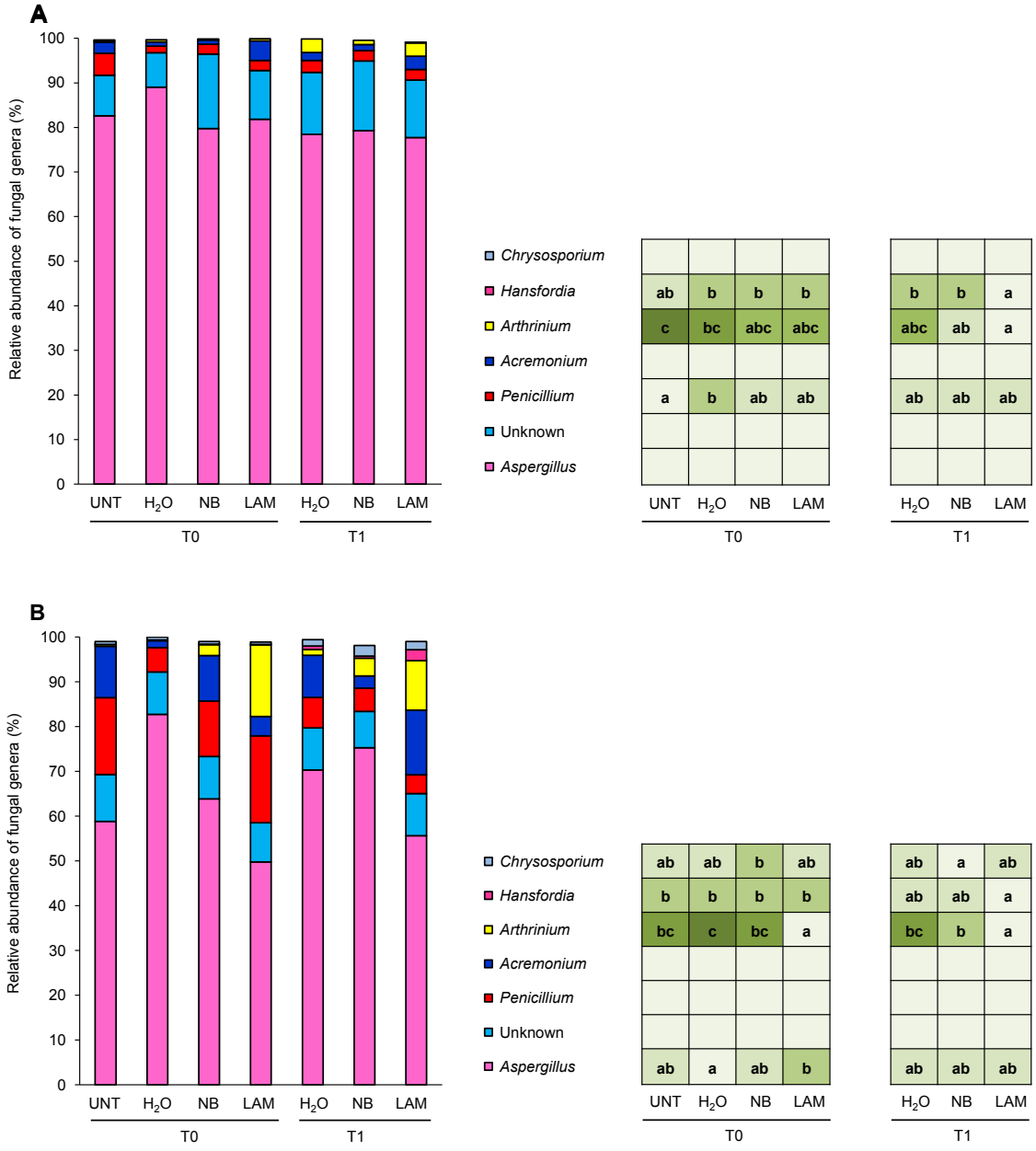

FIGURE S10 | Relative abundance of the dominant (more than 2 % of relative abundance in at least one sample) fungal genera on grapevine leaves. Percentages of relative abundance were determined for leaves of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in the experiment 1 (A) and experiment 2 (B). Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. For each taxon, the intensity of the color gradient and letters reported in the table indicate significant differences among treatments and time points according to Fisher's test ($\alpha = 0.05$).

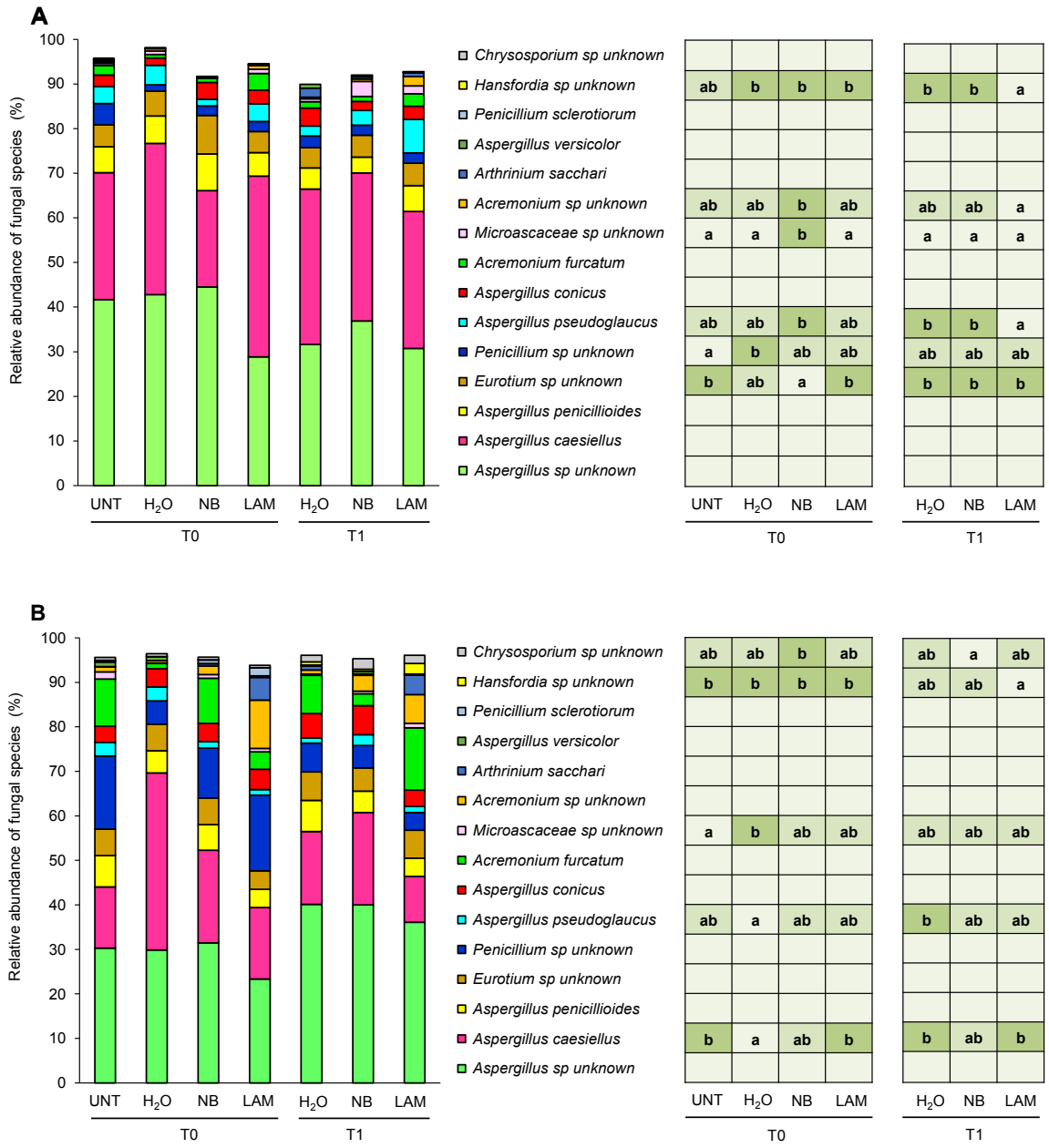

FIGURE S11 | Relative abundance of the dominant (more than 1 % of relative abundance in at least one sample) fungal species on grapevine leaves. Percentages of relative abundance were determined for leaves of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in the experiment 1 (A) and experiment 2 (B). Mean and standard error values of three replicates (each as a pool of two plants) were analyzed for each treatment and time point. For each taxon, the intensity of the color gradient and letters reported in the table indicate significant differences among treatments and time points according to Fisher's test (α = 0.05).

3. Supplementary Tables

TABLE S1 | Primer sequences for quantitative real-time PCR expression analysis of grapevine defence-related genes.

Gene abbreviation	**Gene name**	**NCBI accession number**	**Grapevine gene** (http://genomes.cribi.unipd.it/grape/)	**Primer sequencece forward**	**Primer sequencece reverse**
PR-1	Pathogenesis related protein 1	AJ536326	VIT_203s0088g00700	ACTTGTGGGTGGGGGAGAA	TGTTGCATTGAACCCTAGCG
PR-2	Pathogenesis related protein 2	AJ277900	VIT_208s0007g06060	GTTATTTCAGAGAGTGGTTGGC	AACATGGCAAACACGTAAGTCT
PR-4	Pathogenesis related protein 4	CF074510	VIT_214s0081g00030	CAGGCAACGGTGAGAATAGT	ACCACAGTCCACAAACTCGTA
CHIT-3	Acidic endochitinase 3	NM_001281119.1	VIT_216s0050g02220	GTCCATTCCCAGATAAGTTCCT	CAGAAGGTTATTGGTGTTGCC
OSM-1	Osmotin 1	XM_002282928.2	VIT_202s0025g04310	CGCTGCGCTAAAGACTACC	AAAAACCTTGAGTAATCTGTAGCA
OSM-2	Osmotin 2	AB372569.1	VIT_202s0025g04280	CGCTGCGCTAAAGACTACC	AAAAACCTTGAGTAATCTGTAGCA
Act	Actin	XM_010659103.1	VIT_212s0178g00200	ATTCCTCACCATCATCAGCA	GACCCCCTCCTACTAAAACT

References of primer pairs are: *PR-1*, *PR-2* and *PR-4* (Perazzolli et al. 2011 Biol Control 58: 74-82); *CHIT-3*, *OSM-1* and *OSM-2* (Perazzolli et al. 2012 BMC Genomics 13: 660)

TABLE S2 | Biocontrol features of culturable bacteria. Percentage (%) of culturable bacteria with proteolytic activity, siderophore production and antagonistic activity against *Phytophthora infestans* were assessed for 60 and 37 representative bacterial isolates of experiment 1 and experiment 2, respectively. Representative bacterial isolates were selected visually based on colony morphological features from cultures originating from plants treated with water (H_2O) or nutrient broth (NB), and collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation.

	Culturable bacteria - Experiment 1			**Culturable bacteria - Experiment 1**		
	Protease activity	**Siderophore production**	**Antagonism to *P. infestans***	**Protease activity**	**Siderophore production**	**Antagonism to *P. infestans***
H_2O T0	66.7	71.4	66.7	33.3	43.3	66.7
NB T0	68.8	81.3	46.7	50.0	55.0	66.7
H_2O T1	43.8	43.8	33.3	33.3	43.3	16.7
NB T1	23.1	61.5	33.3	30.0	40.0	16.7

TABLE S3 | Fusion primers for pyrosequencing. Sequences of primer pairs used for amplification and sequencing of the 16S rRNA gene (V6-V8 region) and ITS fragment (ITS3-f and ITS4-r) are reported, including the multiplex identifier (MID) codes for each DNA sample.

BACTERIA	Primer A	Key	MID	Template specific forward	MID Code	Full primer sequence	Sample Code
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACGAGTGCGT	ATGCAACGCGAAGAACCT	MID-1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTATGCAACGCGAAGAACCT	UNT.T0.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACGCTCGACA	ATGCAACGCGAAGAACCT	MID-2	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACAATGCAACGCGAAGAACCT	UNT.T0.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ATAGAGTACT	ATGCAACGCGAAGAACCT	MID-33	CCATCTCATCCCTGCGTGTCTCCGACTCAGATAGAGTACTATGCAACGCGAAGAACCT	UNT.T0.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TGATACGTCT	ATGCAACGCGAAGAACCT	MID-11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTATGCAACGCGAAGAACCT	H2O.T0.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CATAGTAGTG	ATGCAACGCGAAGAACCT	MID-13	CCATCTCATCCCTGCGTGTCTCCGACTCAGCATAGTAGTGATGCAACGCGAAGAACCT	H2O.T0.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CGTAGACTAG	ATGCAACGCGAAGAACCT	MID-21	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTAGACTAGATGCAACGCGAAGAACCT	H2O.T0.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	AGACTATACT	ATGCAACGCGAAGAACCT	MID-30	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACTATACTATGCAACGCGAAGAACCT	NB.T0.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TGTACTACTC	ATGCAACGCGAAGAACCT	MID-19	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCATGCAACGCGAAGAACCT	NB.T0.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACGACTACAG	ATGCAACGCGAAGAACCT	MID-20	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACTACAGATGCAACGCGAAGAACCT	NB.T0.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TAGAGACGAG	ATGCAACGCGAAGAACCT	MID-24	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGACGAGATGCAACGCGAAGAACCT	LAM.T0.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCGTCGCTCG	ATGCAACGCGAAGAACCT	MID-25	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGTCGCTCGATGCAACGCGAAGAACCT	LAM.T0.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CAGTAGACGT	ATGCAACGCGAAGAACCT	MID-35	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGTAGACGTATGCAACGCGAAGAACCT	LAM.T0.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ATACGACGTA	ATGCAACGCGAAGAACCT	MID-15	CCATCTCATCCCTGCGTGTCTCCGACTCAGATACGACGTAATGCAACGCGAAGAACCT	H2O.T1.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCACGTACTA	ATGCAACGCGAAGAACCT	MID-16	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTAATGCAACGCGAAGAACCT	H2O.T1.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TACTCTCGTG	ATGCAACGCGAAGAACCT	MID-23	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCTCGTGATGCAACGCGAAGAACCT	H2O.T1.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	AGCGTCGTCT	ATGCAACGCGAAGAACCT	MID-31	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCGTCGTCTATGCAACGCGAAGAACCT	NB.T1.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	AGTACGCTAT	ATGCAACGCGAAGAACCT	MID-32	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTACGCTATATGCAACGCGAAGAACCT	NB.T1.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCTCTATGCG	ATGCAACGCGAAGAACCT	MID-10	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGATGCAACGCGAAGAACCT	NB.T1.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACGCGAGTAT	ATGCAACGCGAAGAACCT	MID-27	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCGAGTATATGCAACGCGAAGAACCT	LAM.T1.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACTACTATGT	ATGCAACGCGAAGAACCT	MID-28	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACTATGTATGCAACGCGAAGAACCT	LAM.T1.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACTGTACAGT	ATGCAACGCGAAGAACCT	MID-29	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTACAGTATGCAACGCGAAGAACCT	LAM.T1.C
	Primer B	**Key**	**MID**	**Template specific reverse**		**Full primer sequence**	
	CCTATCCCCTGTGTGCCTTGGCAGTC	TCAG		TAGCGATTCCGACTTCA		CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTAGCGATTCCGACTTCA	

FUNGI	Primer A	Key	MID	Template specific forward	MID Code	Full primer sequence	Sample Code
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACGAGTGCGT	GCATCGATGAAGAACGC	MID-1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTGCATCGATGAAGAACGC	UNT.T0.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACGCTCGACA	GCATCGATGAAGAACGC	MID-2	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACAGCATCGATGAAGAACGC	UNT.T0.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ATAGAGTACT	GCATCGATGAAGAACGC	MID-33	CCATCTCATCCCTGCGTGTCTCCGACTCAGATAGAGTACTGCATCGATGAAGAACGC	UNT.T0.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TGATACGTCT	GCATCGATGAAGAACGC	MID-11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTGCATCGATGAAGAACGC	H2O.T0.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CATAGTAGTG	GCATCGATGAAGAACGC	MID-13	CCATCTCATCCCTGCGTGTCTCCGACTCAGCATAGTAGTGGCATCGATGAAGAACGC	H2O.T0.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CGTAGACTAG	GCATCGATGAAGAACGC	MID-21	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTAGACTAGGCATCGATGAAGAACGC	H2O.T0.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	AGACTATACT	GCATCGATGAAGAACGC	MID-30	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACTATACTGCATCGATGAAGAACGC	NB.T0.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TGTACTACTC	GCATCGATGAAGAACGC	MID-19	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCGCATCGATGAAGAACGC	NB.T0.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACGACTACAG	GCATCGATGAAGAACGC	MID-20	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACTACAGGCATCGATGAAGAACGC	NB.T0.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TAGAGACGAG	GCATCGATGAAGAACGC	MID-24	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGACGAGGCATCGATGAAGAACGC	LAM.T0.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCGTCGCTCG	GCATCGATGAAGAACGC	MID-25	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGTCGCTCGGCATCGATGAAGAACGC	LAM.T0.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CAGTAGACGT	GCATCGATGAAGAACGC	MID-35	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGTAGACGTGCATCGATGAAGAACGC	LAM.T0.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ATACGACGTA	GCATCGATGAAGAACGC	MID-15	CCATCTCATCCCTGCGTGTCTCCGACTCAGATACGACGTAGCATCGATGAAGAACGC	H2O.T1.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCACGTACTA	GCATCGATGAAGAACGC	MID-16	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTAGCATCGATGAAGAACGC	H2O.T1.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TACTCTCGTG	GCATCGATGAAGAACGC	MID-23	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCTCGTGGCATCGATGAAGAACGC	H2O.T1.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	AGCGTCGTCT	GCATCGATGAAGAACGC	MID-31	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCGTCGTCTGCATCGATGAAGAACGC	NB.T1.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	AGTACGCTAT	GCATCGATGAAGAACGC	MID-32	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTACGCTATGCATCGATGAAGAACGC	NB.T1.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCTCTATGCG	GCATCGATGAAGAACGC	MID-10	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGGCATCGATGAAGAACGC	NB.T1.C
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACGCGAGTAT	GCATCGATGAAGAACGC	MID-27	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCGAGTATGCATCGATGAAGAACGC	LAM.T1.A
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACTACTATGT	GCATCGATGAAGAACGC	MID-28	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACTATGTGCATCGATGAAGAACGC	LAM.T1.B
	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	ACTGTACAGT	GCATCGATGAAGAACGC	MID-29	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTACAGTGCATCGATGAAGAACGC	LAM.T1.C
	Primer B	**Key**	**MID**	**Template specific reverse**		**Full primer sequence**	
	CCTATCCCCTGTGTGCCTTGGCAGTC	TCAG		CCTCCGCTTATTGATATGC		CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCTCCGCTTATTGATATGC	

TABLE S4 | Pyrosequencing results of phyllosphere bacteria. Number of total quality filtered reads, coverage (Good's coverage), richness (Chao1) and diversity (Simpson) estimators were calculated for bacteria collected from grapevine leaves in two independent experiments (experiment 1 and experiment 2). Operational taxonomic units (OTU) were assessed before and after the normalization to the lowest number of quality filtered reads (1415 in H_2O_T1_A, Exp 2). The 16S rRNA were amplified from DNA extracted from leaf-washing suspensions of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation. Data of three replicates (named from A to C), mean and standard error values of three replicates are reported for each sample.

Sample	Replicate	Bacteria - EXPERIMENT 1						Bacteria - EXPERIMENT 2					
		Total filtered reads	Good's coverage	Chao1	Simpson	Identified OTUs	Identified OTUs in 1415 reads	Filtered reads	Good's coverage	Chao1	Simpson	Identified OTUs	Identified OTUs in 1415 reads
UNT	A	52719	0.93	420	0.69	953	165	54709	0.99	65	0.78	236	44
	B	23569	0.89	613	0.91	1014	245	7626	0.96	253	0.51	154	71
	C	19509	0.92	377	0.73	642	184	14096	0.97	157	0.46	228	59
	Mean Value	**31932.33**	**0.91**	**470.29**	**0.78**	**869.67**	**198.00**	**25477.00**	**0.97**	**158.34**	**0.59**	**206.00**	**58.00**
	Std Error	**10459.21**	**0.01**	**72.50**	**0.07**	**115.19**	**24.13**	**14734.85**	**0.01**	**54.21**	**0.10**	**26.10**	**7.81**
H2O.T0	A	28603	0.86	668	0.95	1513	315	12322	0.98	180	0.53	150	48
	B	20667	0.83	842	0.99	1601	423	9301	0.98	90	0.44	122	40
	C	25472	0.87	591	0.98	1034	345	17429	0.98	90	0.42	127	40
	Mean Value	**24914.00**	**0.85**	**700.24**	**0.97**	**1382.67**	**361.00**	**13017.33**	**0.98**	**120.00**	**0.46**	**133.00**	**42.67**
	Std Error	**2307.85**	**0.01**	**74.09**	**0.01**	**176.17**	**32.19**	**2371.97**	**0.00**	**30.00**	**0.03**	**8.62**	**2.67**
NB.T0	A	18471	0.97	117	0.78	180	75	14338	0.96	203	0.84	241	92
	B	18786	0.98	116	0.87	222	85	12088	0.98	116	0.65	186	68
	C	9046	0.96	175	0.92	246	122	12832	0.98	82	0.49	134	49
	Mean Value	**15434.33**	**0.97**	**135.98**	**0.86**	**216.00**	**94.00**	**13086.00**	**0.97**	**133.33**	**0.66**	**187.00**	**69.67**
	Std Error	**3195.46**	**0.00**	**19.51**	**0.04**	**19.29**	**14.29**	**661.82**	**0.01**	**35.99**	**0.10**	**30.89**	**12.44**
LAM.T0	A	46839	0.95	260	0.87	787	144	30484	0.96	194	0.81	376	118
	B	43368	0.82	905	0.96	1849	411	19448	0.96	249	0.62	418	88
	C	30558	0.89	648	0.96	1309	264	41675	0.99	30	0.31	111	24
	Mean Value	**40255.00**	**0.88**	**604.17**	**0.93**	**1315.00**	**273.00**	**30535.67**	**0.97**	**157.78**	**0.58**	**301.67**	**76.67**
	Std Error	**4950.95**	**0.04**	**187.66**	**0.03**	**306.59**	**77.21**	**6416.43**	**0.01**	**65.62**	**0.15**	**96.10**	**27.72**
H2O.T1	A	2703	0.97	212	0.84	162	114	1415	0.97	148	0.74	85	85
	B	2705	0.97	176	0.91	158	123	2709	0.98	133	0.76	130	98
	C	4517	0.97	180	0.94	178	116	2216	0.97	177	0.84	123	105
	Mean Value	**3308.33**	**0.97**	**189.44**	**0.90**	**166.00**	**117.67**	**2113.33**	**0.97**	**152.69**	**0.78**	**112.67**	**96.00**
	Std Error	**604.33**	**0.00**	**11.45**	**0.03**	**6.11**	**2.73**	**377.06**	**0.00**	**12.94**	**0.03**	**13.98**	**5.86**
NB.T1	A	5817	0.96	195	0.91	226	128	4735	0.98	120	0.86	126	77
	B	19429	0.96	195	0.95	351	125	3952	0.97	127	0.88	140	96
	C	5109	0.96	183	0.92	229	122	3698	0.98	130	0.86	112	80
	Mean Value	**10118.33**	**0.96**	**191.00**	**0.93**	**268.67**	**125.00**	**4128.33**	**0.97**	**125.32**	**0.86**	**126.00**	**84.33**
	Std Error	**4659.82**	**0.00**	**3.87**	**0.01**	**41.18**	**1.73**	**312.07**	**0.00**	**3.01**	**0.01**	**8.08**	**5.90**
LAM.T1	A	3692	0.96	237	0.89	214	126	3178	0.98	128	0.75	122	88
	B	6629	0.95	249	0.82	242	136	2046	0.98	136	0.76	102	93
	C	15692	0.97	198	0.95	279	117	4614	0.97	164	0.94	180	113
	Mean Value	**8671.00**	**0.96**	**227.72**	**0.89**	**245.00**	**126.33**	**3279.33**	**0.97**	**142.90**	**0.82**	**134.67**	**98.00**
	Std Error	**3611.43**	**0.00**	**15.49**	**0.04**	**18.82**	**5.49**	**743.05**	**0.00**	**11.04**	**0.06**	**23.39**	**7.64**

TABLE S5 | Pyrosequencing results of phyllosphere fungi. Number of total quality filtered reads, coverage (Good's coverage), richness (Chao1) and diversity (Simpson) estimators were calculated for fungi collected from grapevine leaves in two independent experiments (experiment 1 and experiment 2). Operational taxonomic units (OTU) were assessed before and after normalization to the lowest number of quality filtered reads (1979 in H_2O_T1_C, Exp 1). The ITS fragments were amplified from DNA extracted from leaf-washing suspensions of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation. Data for three replicates (named A to C), mean and standard error values of three replicates are reported for each sample.

Sample	Replicate	Fungi - EXPERIMENT 1						Fungi - EXPERIMENT 2					
		Total filtered reads	Good's coverage	Chao1	Simpson	Identified OTUs	Identified OTUs in 1979 reads	Total filtered reads	Good's coverage	Chao1	Simpson	Identified OTUs	Identified OTUs in 1979 reads
UNT	A	8652	0.97	216	0.90	233	123	2136	0.97	203	0.93	139	136
	B	2652	0.97	203	0.91	156	135	1982	0.98	174	0.90	128	127
	C	3268	0.96	283	0.93	230	179	3815	0.96	291	0.90	203	156
	Mean Value	**4857.33**	**0.97**	**233.85**	**0.91**	**206.33**	**145.67**	**2644.33**	**0.97**	**222.34**	**0.91**	**156.67**	**139.67**
	Std Error	**1905.65**	**0.00**	**24.83**	**0.01**	**25.18**	**17.02**	**587.02**	**0.00**	**35.07**	**0.01**	**23.38**	**8.57**
H2O.T0	A	3501	0.96	275	0.95	216	181	3460	0.96	280	0.91	190	157
	B	4059	0.96	257	0.93	219	168	3287	0.98	180	0.93	159	126
	C	4169	0.96	304	0.93	237	179	4009	0.97	202	0.92	181	134
	Mean Value	**3909.67**	**0.96**	**278.36**	**0.94**	**224.00**	**176.00**	**3585.33**	**0.97**	**220.66**	**0.92**	**176.67**	**139.00**
	Std Error	**206.79**	**0.00**	**13.61**	**0.00**	**6.56**	**4.04**	**217.64**	**0.00**	**30.24**	**0.00**	**9.21**	**9.29**
NB.T0	A	3802	0.97	204	0.91	182	131	4685	0.97	205	0.88	204	140
	B	3165	0.96	278	0.91	212	174	3790	0.97	199	0.87	151	128
	C	3956	0.96	291	0.94	248	182	10174	0.96	319	0.92	338	175
	Mean Value	**3641.00**	**0.97**	**257.46**	**0.92**	**214.00**	**162.33**	**6216.33**	**0.97**	**240.94**	**0.89**	**231.00**	**147.67**
	Std Error	**242.12**	**0.00**	**27.23**	**0.01**	**19.08**	**15.84**	**1995.63**	**0.00**	**39.02**	**0.01**	**55.64**	**14.10**
LAM.T0	A	3692	0.97	238	0.94	214	167	3093	0.97	206	0.83	147	123
	B	3275	0.97	244	0.93	197	158	3193	0.97	231	0.94	169	136
	C	3362	0.98	184	0.93	162	118	3746	0.97	191	0.89	160	128
	Mean Value	**3443.00**	**0.97**	**222.04**	**0.93**	**191.00**	**147.67**	**3344.00**	**0.97**	**209.06**	**0.89**	**158.67**	**129.00**
	Std Error	**127.01**	**0.00**	**19.09**	**0.00**	**15.31**	**15.06**	**203.06**	**0.00**	**11.51**	**0.03**	**6.39**	**3.79**
H2O.T1	A	4546	0.97	208	0.91	186	125	2813	0.97	195	0.90	160	136
	B	5323	0.97	221	0.87	216	141	2686	0.96	311	0.91	181	165
	C	1979	0.97	170	0.90	121	121	2407	0.97	212	0.92	138	123
	Mean Value	**3949.33**	**0.97**	**199.70**	**0.89**	**174.33**	**129.00**	**2635.33**	**0.97**	**239.46**	**0.91**	**159.67**	**141.33**
	Std Error	**1010.38**	**0.00**	**15.38**	**0.01**	**28.04**	**6.11**	**119.91**	**0.00**	**36.23**	**0.01**	**12.41**	**12.41**
NB.T1	A	2216	0.98	139	0.92	115	111	2174	0.98	155	0.87	111	108
	B	4437	0.97	234	0.92	196	139	2914	0.97	194	0.94	158	139
	C	2932	0.97	243	0.92	173	148	4332	0.97	210	0.92	206	142
	Mean Value	**3195.00**	**0.97**	**205.11**	**0.92**	**161.33**	**132.67**	**3140.00**	**0.97**	**186.13**	**0.91**	**158.33**	**129.67**
	Std Error	**654.49**	**0.00**	**33.23**	**0.00**	**24.10**	**11.14**	**633.13**	**0.00**	**16.26**	**0.02**	**27.42**	**10.87**
LAM.T1	A	3214	0.96	263	0.93	195	153	3353	0.97	244	0.90	175	146
	B	3155	0.98	178	0.93	136	115	2950	0.98	244	0.83	109	90
	C	3187	0.96	251	0.94	218	176	3860	0.97	189	0.85	160	125
	Mean Value	**3185.33**	**0.97**	**230.54**	**0.93**	**183.00**	**148.00**	**3387.67**	**0.97**	**225.70**	**0.86**	**148.00**	**120.33**
	Std Error	**17.05**	**0.00**	**26.65**	**0.01**	**24.42**	**17.79**	**263.27**	**0.00**	**18.27**	**0.02**	**19.97**	**16.33**

TABLE S6 | Bacterial operational taxonomic units (OTU) identified on grapevine leaves. Read counts are reported for each OTU identified using the GreenGenes database at 97% of sequence similarity for each replicate (named from A to C) of leaf samples collected from untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in two independent experiments (experiment 1 and experiment 2). Taxonomy indicates kingdom (K), phylum (P), class (C), order (O), family (F), genus (G), and species (S) of identified OTU.

TABLE S7 | Fungal operational taxonomic units (OTU) identified on grapevine leaves. Read counts are reported for each OTU identified using the GreenGenes database at 97% of sequence similarity for each replicate (named from A to C) of leaf samples collected from untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) and laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in two independent experiments (experiment 1 and experiment 2). Taxonomy indicates kingdom (K), phylum (P), class (C), order (O), family (F), genus (G), and species (S) of identified OTU.

Available online at: https://www.frontiersin.org/article/10.3389/fpls.2016.01053

TABLE S8 | Beta-diversity analysis of bacterial communities. PERMANOVA and Permutation tests were calculated with an ADONIS function and a Bray-Curtis dissimilarity matrix for bacterial data of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in two independent experiments (experiment 1 and experiment 2). Beta-diversity analysis was carried out for T0 samples, for T0 and T1 samples excluding UNT, and for T1 samples. Treatments considered by each permutation pairwise comparison test are indicated by the symbol ×.

T0 treated samples							
	PERMANOVA		CAP PERMUTATION TEST		PERMUTATION PAIRWISE COMPARISON		
	P-value	Significance	P-value	Significance	Pairwise comparisons	P-value	Significance
Treatment	0.0001	***	0.002	**	UNT x H2O	0.0001	***
Experiment	0.0001	***	0.001	***	H2O x NB	0.0062	**
					UNT x NB	0.0044	**
					H2O x LAM	0.0788	NS
					UNT x LAM	0.1536	NS
					NB x LAM	0.0037	**

T0 + T1 treated samples							
	PERMANOVA		CAP PERMUTATION TEST		PERMUTATION PAIRWISE COMPARISON		
	P-value	Significance	P-value	Significance	Pairwise comparisons	P-value	Significance
Treatment	0.0077	**	0.011	**	H2O x NB	0.0094	**
Time	0.0001	***	0.001	***	H2O x LAM	0.2334	NS
Experiment	0.0001	***	0.001	***	NB x LAM	0.0062	**
Treatment x Time	0.0329	**	0.028	**			

T1 treated samples							
	PERMANOVA		CAP PERMUTATION TEST		PERMUTATION PAIRWISE COMPARISON		
	P-value	Significance	P-value	Significance	Pairwise comparisons	P-value	Significance
Treatment	0.0354	**	0.195		H2O x NB	0.4528	NS
Experiment	0.0354	**	0.001	***	H2O x LAM	0.5759	NS
					NB x LAM	0.6483	NS

Significance	
P value	Code
> 0.05	not significative (NS)
< 0.05	*
< 0.01	**
< 0.001	***

TABLE S9 | Beta-diversity analysis obtained for fungal communities. PERMANOVA and Permutation tests were calculated with an ADONIS function and a Bray-Curtis dissimilarity matrix for fungal data of untreated plants (UNT), and plants treated with water (H_2O), nutrient broth (NB) or laminarin (LAM) collected just before (T0) and one day after (T1) *Plasmopara viticola* inoculation in two independent experiments (experiment 1 and experiment 2). Beta-diversity analysis was carried out for T0 samples, for T0 and T1 samples excluding UNT, and for T1 samples. Treatments considered by each permutation pairwise comparison test are indicated by the symbol ×.

T0 treated samples				
	PERMANOVA		CAP PERMUTATION TEST	
	P-value	Significance	P-value	Significance
Treatment	0.5314	NS	0.559	NS
Experiment	0.5314	NS	0.001	**

T0 + T1 treated samples				
	PERMANOVA		CAP PERMUTATION TEST	
	P-value	Significance	P-value	Significance
Treatment	0.8605	NS	0.851	NS
Time	0.1265	NS	0.117	NS
Experiment	0.3478	NS	0.001	**
Treatment x Time	0.2472	NS	0.245	NS

T1 treated samples				
	PERMANOVA		CAP PERMUTATION TEST	
	P-value	Significance	P-value	Significance
Treatment	0.4453	NS	0.567	
Experiment	0.4453	NS	0.001	**

Significance	
P value	Code
> 0.05	not significative (NS)
< 0.05	*
< 0.01	**
< 0.001	***

CHAPTER 3.

The effect of hydrolysis and protein source on the efficacy of protein hydrolysates as plant resistance inducers against powdery mildew

Published on Journal of Bioprocessing & Biotechniques
Vol. 7, Issue 4, Art. 306
June 26, 2017
https://doi.org/10.4172/2155-9821.1000306

Journal of Bioprocessing & Biotechniques

Cappelletti et al., J Bioprocess Biotech 2017, 7:4

DOI: 10.4172/2155-9821.1000306

Research Article OMICS International

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

Martina Cappelletti[1,2], Michele Perazzolli[1*], Andrea Nesler[1,3], Oscar Giovannini[1] and Ilaria Pertot[1,4]

[1]*Department of Sustainable Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach, 38010 San Michele all'Adige, Italy*
[2]*DI4A, Department of Agrifood, Environmental and Animal Sciences, University of Udine, 33100 Udine, Italy*
[3]*Bi-PA - Biological Products for Agriculture, B-1840 Londerzeel, Belgium*
[4]*Centre Agriculture, Food and Environment, University of Trento, 38010 San Michele all'Adige, Italy*

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 H_2SO_4 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 Ca^{2+} 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

***Corresponding author:** Michele Perazzolli, Research and Innovation Centre, Department of Sustainable Ecosystems and Bioresources, Fondazione Edmund Mach, 38010 San Michele all'Adige, Italy, Tel: +390461615411; E-mail: michele.perazzolli@fmach.it

Received June 16, 2017; **Accepted** June 24, 2017; **Published** August 26, 2017

Citation: Cappelletti M, Perazzolli M, Nesler A, Giovannini O, Pertot I (2017) The Effect of Hydrolysis and Protein Source on the Efficacy of Protein Hydrolysates as Plant Resistance Inducers against Powdery Mildew. J Bioprocess Biotech 7: 306. doi:10.4172/2155-9821.1000306

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 (H_2SO_4), 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 H_2SO_4 and neutralised it with KOH, leading to the production of potassium sulphate (K_2SO_4), 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:

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

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*-phthaldialdehyde (OPA) method, as first described by Church et al. [49], which is based on the reaction between amino groups released during hydrolysis and *o*-phthaldialdehyde, 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:

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

where serine-NH_2 is meqv serine NH_2/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:

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

where the values reported by Adler-Nissen [50] were used for ß (=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 (CH_3CN) 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 (1×10^5 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 K_2SO_4 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:

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

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 homogenously 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 K_2SO_4 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 ± 12.4% and 35 ± 8.8% respectively) and Flavourzyme (36.3 ± 5.4% and 31.1 ± 7.4%, respectively) at 1% and 50% as compared with H_2O-treated leaf disks (62.8 ± 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 ± 5.3% and 48.9 ± 1.4%, respectively) and Flavourzyme (30.6 ± 4.7% and 36.1 ± 5.7%, respectively), and with rapeseed enzymatic hydrolysates produced with Alcalase (30.6 ± 4.5% and 37.8 ± 4.4%, respectively) and Flavourzyme (32.8 ± 6.3% and 34.4 ± 4.1%, respectively) at 1% and 50% E/S ratio, as compared with H_2O-treated leaf disks (52.2 ± 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 ± 4.6%, whereas the additional effect of hydrolysed peptides and free amino acids ranged from 11.5 ± 2.2% to 17.5 ± 3.9%, from 0.8 ± 4.4% to 18.5 ± 2.5% and from 10.5 ± 2.8% to 28.9 ± 2.1% for soybean, rapeseed and guar 6N A and 6N B hydrolysates, respectively. More specifically, the levels of

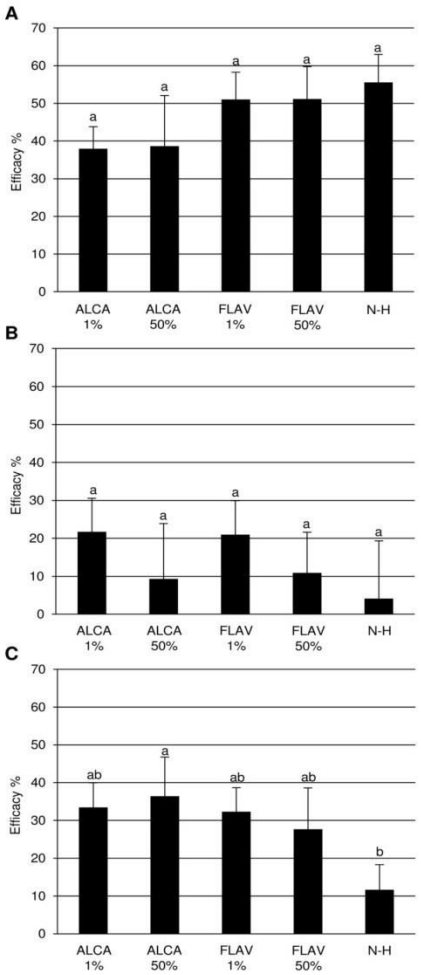

Figure 1: Effect of soybean (A), rapeseed (B) and guar (C) enzymatic hydrolysates against courgette powdery mildew under greenhouse conditions. Efficacy against powdery mildew (percentage) was evaluated on courgette plants treated with hydrolysed and non-hydrolysed (N-H) protein sources as compared to water-treated plants. Enzymatic hydrolysates were obtained using Alcalase (ALCA) and Flavourzyme (FLAV) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) and 50% of the protein content (ALCA 50% and FLAV 50%, respectively). An *F*-test revealed non-significant differences between two independent experiments ($p>0.05$), and data were pooled. The mean efficacy and standard error values of eight replicates (potted plants) in two experiments are presented for each treatment. Different letters indicate significant differences between treatments according to Fisher's LSD test.

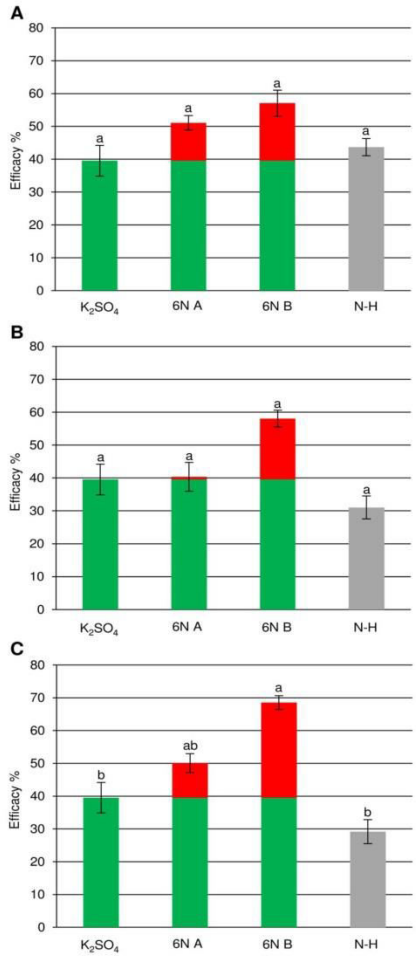

Figure 2: Effect of soybean (A), rapeseed (B) and guar (C) acid hydrolysates against courgette powdery mildew under greenhouse conditions. Efficacy against powdery mildew (percentage) was evaluated on courgette plants treated with hydrolysed and non-hydrolysed (N-H) protein sources or with 0.11 M K_2SO_4 as compared to water-treated plants. Acid hydrolysates were obtained by incubation of the protein source with 6 N H_2SO_4 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 K_2SO_4 and hydrolysed proteins to the efficacy in disease reduction was visually presented using green and red bars respectively.

efficacy considered without the contribution of K_2SO_4 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 H_2SO_4, 6N B. The percentage of conidia germination on guar 6N A- and 6N B-treated leaf disks (12.8 ± 2.9% and 18.3 ± 2.8%, respectively) was comparable (ANOVA, $p>0.05$; Fisher's LSD test) to that on K_2SO_4-treated leaves (20 ± 5.4%), and significantly lower as compared with H_2O-treated leaf disks (52.2 ± 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 (R^2 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.

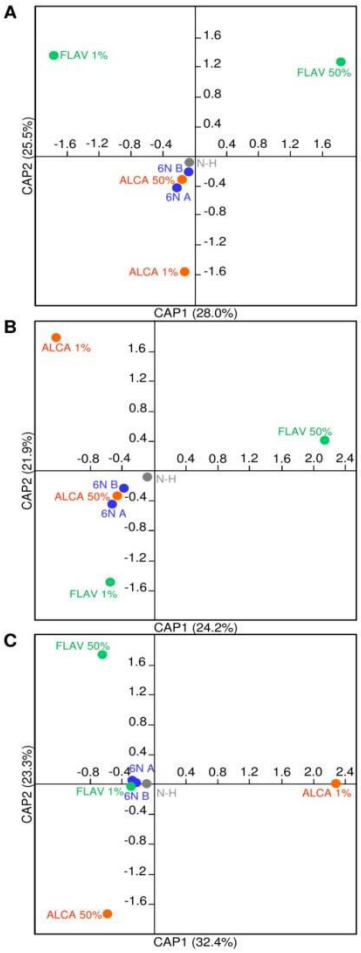

Figure 3: Principal component analysis (PCA) of hydrolysed samples according to their amino acid composition. PCA was obtained for soybean (A), rapeseed (C) and guar (C) samples using data for non-hydrolysed samples (N-H; grey), acid hydrolysates (blue) obtained by incubation with 6 N H_2SO_4 at 121°C 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.

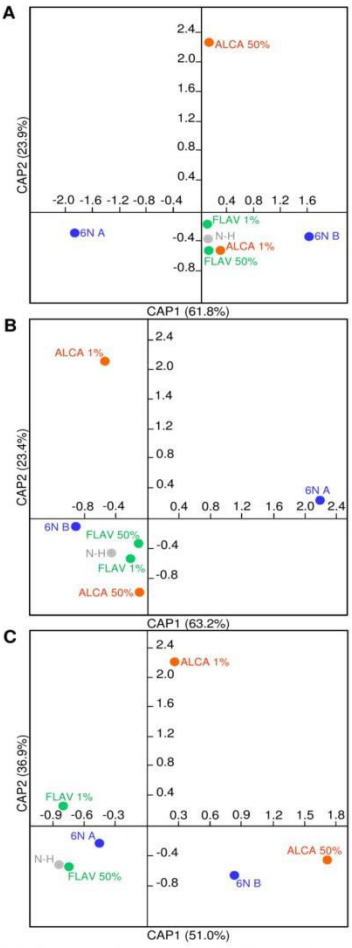

Figure 4: Principal component analysis (PCA) of hydrolysed samples according to their peptide composition. PCA was obtained for soybean (A), rapeseed (B) and guar (C) samples using data for non-hydrolysed samples (N-H; grey), acid hydrolysates (blue) obtained by incubation with 6 N H_2SO_4 at 121°C for 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	EWDSAGAGAGGGGAPGTDFVSCVGK	-0.348	0.028	-0.082	NS
2	GGTGGAQLPGRDGMLVAYAPALVAAAASVVPGAVEGLR	-0.042	NS	0.729	0.000
3	HEGSPGAAEGQGADQQGGGLAVAAAGEPDGDGDGGVRR	-0.068	NS	-0.707	0.000
4	ISASGVVVAPPPPPPPPPPPPPHLK	-	-	0.071	NS
5	TNHRFSEIEIDMISLNVIEVFPAIQQSLI	-	-	0.561	0.001
6	QKIGGGGGGGGGGGGGGGGPPK	0.108	NS	-	-
7	IGGGGGGGGGGGGGGGGPPK	0.108	NS	-	-
8	TTTMALAGAAAGMGKGNGLSSSSMHSVAR	0.108	NS	-	-
9	FYGSGEGGMPGGMPGAGGPGGFPGAGGPAGGHGGDDGPTVEEVD	0.170	NS	-	-
10	EGAGAGAAAATTGGAAAAR	0.170	NS	-	-
11	MGGGHHDMGGMAMAPPPAAAAAAAHGGNK	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 (R^2 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 (R^2 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 H_2SO_4 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 (GGTGGAQLPGRDGMLVAYAPALVAAAASVVPGAVEGLR) and Peptide 5 (TNHRFSEIEIDMISLNVIEVFPAIQQSLI) of guar increased after acid hydrolysis and correlated with increased efficacy against powdery mildew. Thus, similar peptides found in the 6N A acid sample (EMGGKGGGGGGGGGGGGGGPG) and the 6N B acid sample (DGGGGGGSGAGAVVG and TNHRFSEIEIDMISLNVIEVFPAIQQSLI) 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 6 N 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.

Acknowledgements

We thank Franca Valentini for providing technical support with the courgette plants, and Carmela Sicher and Livia Zanotelli for helping with the conidia germination experiments.

Funding

This project has received funding from the European Union´s Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 324416 (project INNOVA, theme FP7-PEOPLE-2012-IAPP) and from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement no. 722642 (project INTERFUTURE).

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.

References

1. Fantke P, Friedrich R, Jolliet O (2012) Health impact and damage cost assessment of pesticides in Europe. Environ Int 49: 9-17.
2. Hillocks RJ (2012) Farming with fewer pesticides: EU pesticide review and resulting challenges for UK agriculture. Crop Prot 31: 85-93.
3. Skevas T, Lansink A, Stefanou SE (2013) Designing the emerging EU pesticide policy: a literature review. Njas Wagen J Life Sc 64-65: 95-103.
4. Delaunois B, Farace G, Jeandet P, Clement C, Baillieul F, et al. (2014) Elicitors as alternative strategy to pesticides in grapevine? Current knowledge on their mode of action from controlled conditions to vineyard. Environ Sci Pollut R 21: 4837-4846.
5. Jones JDG, Dangl JL (2006) The plant immune system. Nature 444: 323-329.
6. Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60: 379-406.
7. Wu S, Shan L, He P (2014) Microbial signature-triggered plant defense responses and early signaling mechanisms. Plant Sci 228: 118-126.
8. Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5: 308-316.
9. Walters DR, Ratsep J, Havis ND (2013) Controlling crop diseases using induced resistance: challenges for the future. J Exp Bot 64: 1263-1280.
10. Van Wees SCM, Van der Ent S, Pieterse CMJ (2008) Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol 11: 443-448.
11. Albert M (2013) Peptides as triggers of plant defence. J Exp Bot 64: 5269-5279.
12. Yamaguchi Y, Huffaker A (2011) Endogenous peptide elicitors in higher plants. Curr Opin Plant Biol 14: 351-357.
13. Calvo P, Nelson L, Kloepper JW (2014) Agricultural uses of plant biostimulants. Plant Soil 383: 3-41.
14. Colla G, Nardi S, Cardarelli M, Ertani A, Lucini L, et al. (2015) Protein hydrolysates as biostimulants in horticulture. Sci Hortic 196: 28-38.
15. Sinha R, Radha C, Prakash J, Kaul P (2007) Whey protein hydrolysate: functional properties, nutritional quality and utilization in beverage formulation. Food Chem 101: 1484-1491.
16. Panyam D, Kilara A (1996) Enhancing the functionality of food proteins by enzymatic modification. Trends Food Sci Tech 7: 120-125.
17. Mahmoud MI (1994) Physicochemical and functional-properties of protein hydrolysates in nutritional products. Food Technol 48: 89-95.
18. Chabanon G, Chevalot I, Framboisier X, Chenu S, Marc I (2007) Hydrolysis of rapeseed protein isolates: kinetics, characterization and functional properties of hydrolysates. Process Biochem 42: 1419-1428.
19. Adler-Nissen J (1982) Limited enzymic degradation of proteins: a new approach in the industrial application of hydrolases. J Chem Technol Biotechnol 32: 138-156.
20. Cheison SC, Zhang SB, Wang Z, Xu SY (2009) Comparison of a modified spectrophotometric and the pH-stat methods for determination of the degree of hydrolysis of whey proteins hydrolysed in a tangential-flow filter membrane reactor. Food Res Int 42: 91-97.
21. Stiegler JC, Richardson MD, Karcher DE, Roberts TL, Norman RJ (2013) Foliar absorption of various inorganic and organic nitrogen sources by creeping bentgrass. Crop Sci 53: 1148-1152.
22. du Jardin P (2015) Plant biostimulants: definition, concept, main categories and regulation. Sci Hortic 196: 3-14.
23. Colla G, Rouphael Y, Canaguier R, Svecova E, Cardarelli M (2014) Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. Front Plant Sci 5: 448.
24. Lachhab N, Sanzani SM, Adrian M, Chiltz A, Balacey S, et al. (2014) Soybean and casein hydrolysates induce grapevine immune responses and resistance against Plasmopara viticola. Front Plant Sci 5: 716.
25. Lachhab N, Sanzani SM, Bahouaoui MA, Boselli M, Ippolito A (2016) Effect of some protein hydrolysates against gray mould of table and wine grapes. Eur J Plant Pathol 144: 821-830.
26. Nesler A, Perazzolli M, Puopolo G, Giovannini O, Elad Y, et al. (2015) A complex protein derivative acts as biogenic elicitor of grapevine resistance against powdery mildew under field conditions. Front Plant Sci 6: 715.
27. Cappelletti M, Perazzolli M, Antonielli L, Nesler A, Torboli E, et al. (2016) Leaf treatments with a protein-based resistance inducer partially modify phyllosphere microbial communities of grapevine. Front Plant Sci 7: 1053.
28. Pecha J, Fuerst T, Kolomaznik K, Friebrova V, Svoboda P (2012) Protein biostimulant foliar uptake modeling: the impact of climatic conditions. AIChE J 58: 2010-2019.
29. Lisiecka J, Knaflewski M, Spizewski T, Frąszczak B, Kałuzewicz A, et al. (2011) The effect of animal protein hydrolysate on quantity and quality of strawberry daughter plants cv. 'Elsanta'. Acta Sci Pol-Hortoru 10: 31-40.
30. Cerdan M, Sanchez-Sanchez A, Oliver M, Juarez M, Sanchez-Andreu JJ (2009) Effect of foliar and root applications of amino acids on iron uptake by tomato plants. Acta Hortic 830: 481-488.
31. Oaks A, Aslam M, Boesel I (1977) Ammonium and amino-acids as regulators of nitrate reductase in corn roots. Plant Physiol 59: 391-394.
32. Ryan CA, Pearce G, Scheer J, Moura DS (2002) Polypeptide hormones. Plant Cell 14: S251-S264.
33. Day L (2013) Proteins from land plants - Potential resources for human nutrition and food security. Trends Food Sci Tech 32: 25-42.
34. Nidhina N, Muthukumar SP (2015) Antinutritional factors and functionality of protein-rich fractions of industrial guar meal as affected by heat processing. Food Chem 173: 920-926.
35. Wickramasuriya SS, Yi YJ, Yoo J, Kang NK, Heo JM (2015) A review of canola meal as an alternative feed ingredient for ducks. J Anim Sci Technol 57: 1-9.
36. Grasser LA, Fadel JG, Garnett I, Depeters EJ (1995) Quantity and economic importance of nine selected by-products used in california dairy rations. J Dairy Sci 78: 962-971.
37. Perez-Garcia A, Romero D, Fernandez-Ortuno D, Lopez-Ruiz F, De Vicente A, et al. (2009) The powdery mildew fungus Podosphaera fusca (synonym Podosphaera xanthii), a constant threat to cucurbits. Mol Plant Pathol 10: 153-160.
38. Mucharromah E, Kuc J (1991) Oxalate and phosphates induce systemic resistance against diseases caused by fungi, bacteria and viruses in cucumber. Crop Prot 10: 265-270.
39. Reuveni M, Agapov V, Reuveni R (1993) Induction of systemic resistance to powdery mildew and growth increase in cucumber by phosphates. Biol Agric Hortic 9: 305-315.
40. Reuveni R, Dor G, Raviv M, Reuveni M, Tuzun S (2000) Systemic resistance against Sphaerotheca fuliginea in cucumber plants exposed to phosphate in hydroponics system, and its control by foliar spray of mono-potassium phosphate. Crop Prot 19: 355-361.
41. van Aubel G, Buonatesta R, Van Cutsem P (2014) COS-OGA: A novel oligosaccharidic elicitor that protects grapes and cucumbers against powdery mildew. Crop Protection 65: 129-137.
42. He R, Girgih AT, Malomo SA, Ju X, Aluko RE (2013) Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. J Funct Foods 5: 219-227.
43. Nadtochii L, Zabodalova L, Domoroshchenkova M (2015) Development of cheese product with hydrolyzed soybean emulsion. Agron Res 13: 1010-1019.
44. Peña-Ramos EA, Xiong YL (2002) Antioxidant activity of soy protein hydrolysates in a liposomal system. J Food Sci 67: 2952-2956.
45. Merz M, Eisele T, Berends P, Appel D, Rabe S, et al. (2015) Flavourzyme, an enzyme preparation with industrial relevance: automated nine-step purification and partial characterization of eight enzymes. J Agric Food Chem 63: 5682-5693.

46. Aaslyng MD, Elmore JS, Mottram DS (1998) Comparison of the aroma characteristics of acid-hydrolyzed and enzyme-hydrolyzed vegetable proteins produced from soy. J Agric Food Chem 46: 5225-5231.
47. Zorb C, Senbayram M, Peiter E (2014) Potassium in agriculture - status and perspectives. J Plant Physiol 171: 656-669.
48. Nielsen PM, Petersen D, Dambmann C (2001) Improved method for determining food protein degree of hydrolysis. J Food Sci 66: 642-646.
49. Church FC, Swaisgood HE, Porter DH, Catignani GL (1983) Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J Dairy Sci 66: 1219-1227.
50. Adler-Nissen J (1986) Enzymic hydrolysis of food proteins. Elsevier Applied Science Publishers, New York.
51. Simonian MH (2004) Spectrophotometric determination of protein concentration. Current Protocols in Toxicology, Mahin D Maines, Appendix 3: 1-7.
52. EPPO (1990) EPPO Bullettin: Guideline for the biological evaluation of fungicides: powdery mildews on cucurbits and other vegetables 20: 451-463.
53. Romero D, De Vicente A, Olmos JL, Dávila JC, Pérez-Garcia A (2007) Effect of lipopeptides of antagonistic strains of Bacillus subtilis on the morphology and ultrastructure of the cucurbit fungal pathogen Podosphaera fusca. J Appl Microbiol 103: 969-976.
54. Peries OS (1962) Studies on strawberry mildew, caused by Sphaerotheca macularis (Wallr. ex Fries) Jaczewski. Ann Appl Biol 50: 211-224.
55. Pertot I, Fiamingo F, Amsalem L, Maymon M, Freeman S, et al. (2007) Sensitivity of two Podosphaera aphanis populations to disease control agents. J Plant Pathol 89: 85-96.
56. Jamdar SN, Rajalakshmi V, Pednekar MD, Juan F, Yardi V, et al. (2010) Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. Food Chem 121: 178-184.
57. Spellman D, McEvoy E, O'Cuinn G, FitzGerald RJ (2003) Proteinase and exopeptidase hydrolysis of whey protein: comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. Int Dairy J 13: 447-453.
58. Pearce G, Yamaguchi Y, Barona G, Ryan CA (2010) A subtilisin-like protein from soybean contains an embedded, cryptic signal that activates defense-related genes. Proc Natl Acad Sci U S A 107: 14921-14925.
59. Huffaker A, Pearce G, Ryan CA (2006) An endogenous peptide signal in Arabidopsis activates components of the innate immune response. Proc Natl Acad Sci USA 103: 10098-10103.
60. Bhattacharya R, Koramutla MK, Negi M, Pearce G, Ryan CA (2013) Hydroxyproline-rich glycopeptide signals in potato elicit signalling associated with defense against insects and pathogens. Plant Sci 207: 88-97.
61. Igarashi D, Tsuda K, Katagiri F (2012) The peptide growth factor, phytosulfokine, attenuates pattern-triggered immunity. Plant J 71: 194-204.
62. Schmelz EA, LeClere S, Carroll MJ, Alborn HT, Teal PEA (2007) Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory. Plant Physiol 144: 793-805.
63. Yamaguchi Y, Barona G, Ryan CA, Pearce G (2011) GmPep914, an eight-amino acid peptide isolated from soybean leaves, activates defense-related genes. Plant Physiol 156: 932-942.
64. Scheideler M, Schlaich NL, Fellenberg K, Beissbarth T, Hauser NC, et al. (2002) Monitoring the switch from housekeeping to pathogen defense metabolism in Arabidopsis thaliana using cDNA arrays. J Biol Chem 277: 10555-10561.
65. Ward JL, Forcat S, Beckmann M, Bennett M, Miller SJ, et al. (2010) The metabolic transition during disease following infection of Arabidopsis thaliana by Pseudomonas syringae pv. tomato. Plant J 63: 443-457.
66. Liu G, Ji Y, Bhuiyan NH, Pilot G, Selvaraj G, et al. (2010) Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in Arabidopsis. Plant Cell 22: 3845-3863.
67. Cecchini NM, Monteoliva MI, Alvarez ME (2011) Proline dehydrogenase contributes to pathogen defense in Arabidopsis. Plant Physiol 155: 1947-1959.
68. Kadotani N, Akagi A, Takatsuji H, Miwa T, Igarashi D (2016) Exogenous proteinogenic amino acids induce systemic resistance in rice. Bmc Plant Biology 16: 60.
69. Hasabi V, Askari H, Alavi SM, Zamanizadeh H (2014) Effect of amino acid application on induced resistance against citrus canker disease in lime plants. J Plant Prot Res 54: 144-149.
70. Lattanzio V, Lattanzio VMT, Cardinali A (2006) Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. In: Imperato F (ed) Phytochemistry: Advances in research 23-67.
71. Nicholson RL, Hammerschmidt R (1992) Phenolic-compounds and their role in disease resistance. Annu Rev Phytopathol 30: 369-389.
72. Shah J (2005) Lipids, lipases, and lipid-modifying enzymes in plant disease resistance. Annu Rev Phytopathol 43: 229-260.
73. Hoffmann-Benning S (2015) Transport and function of lipids in the plant phloem.
74. Trouvelot S, Héloir MC, Poinssot B, Gauthier A, Paris F, et al. (2014) Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays. Front Plant Sci 5: 592.

Citation: Cappelletti M, Perazzolli M, Nesler A, Giovannini O, Pertot I (2017) The Effect of Hydrolysis and Protein Source on the Efficacy of Protein Hydrolysates as Plant Resistance Inducers against Powdery Mildew. J Bioprocess Biotech 7: 306. doi:10.4172/2155-9821.1000306

Supplementary Material

The effect of hydrolysis and protein source on the efficacy of protein hydrolysates as plant resistance inducers against powdery mildew

Martina Cappelletti[1,2], Michele Perazzolli[1,*], Andrea Nesler[1,3], Oscar Giovannini[1] and Ilaria Pertot[1,4]

[1] Department of Sustainable Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach, 38010 San Michele all'Adige, Italy; [2] DI4A, Department of Agrifood, Environmental and Animal Sciences, University of Udine, 33100 Udine, Italy; [3] Bi-PA - Biological Products for Agriculture, B-1840 Londerzeel, Belgium, [4] Centre Agriculture, Food and Environment, University of Trento, 38010 San Michele all'Adige, Italy

1. **Supplementary Figures**
2. **Supplementary Tables**

1. Supplementary Figures

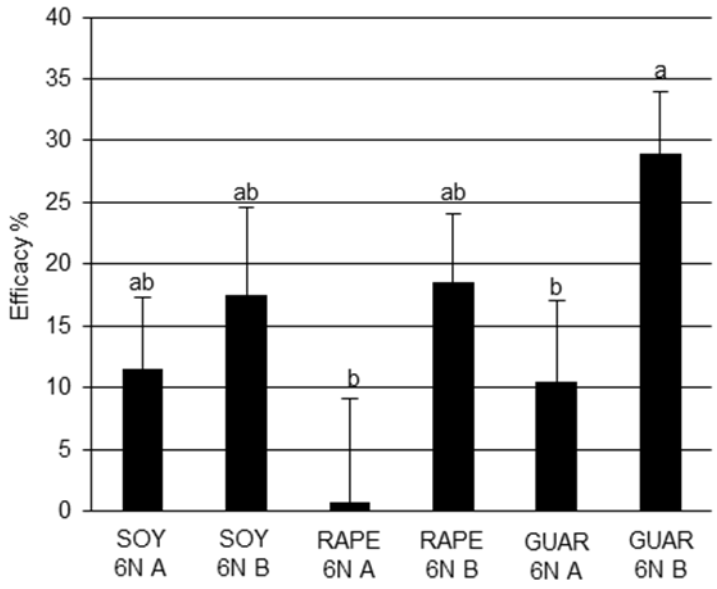

Figure S1: Efficacy of soybean (SOY), rapeseed (RAPE) and guar (GUAR) acid hydrolysates against courgette powdery under greenhouse conditions. Efficacy against powdery mildew was evaluated on courgette plants treated with hydrolysed protein sources and with 0.11 M K_2SO_4 as compared to water-treated plants. Acid hydrolysates were obtained by incubation of the protein source with 6 N H_2SO_4 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. The mean effect of 0.11 M K_2SO_4 (39.5 ± 4.6%) was subtracted to efficacy data, mean 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 ($p \leq 0.05$).

2. Supplementary Tables

Table S1: Peptide composition of soybean samples. Peptides were analysed by liquid chromatography coupled with mass spectrometry for the non-hydrolysed sample (N-H), for acid hydrolysates obtained with 6 N H2SO4 at 121°C for 15 min (6N A) or at 100°C for 8 h (6N B), and for enzymatic hydrolysates obtained with Alcalase (ALCA) and Flavourzyme (FLAV) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) or 50% (ALCA 50% and FLAV 50%, respectively) of the protein content. Peptides identified in hydrolysed samples were aligned with those detected in the N-H sample, and quantitation ratios were calculated for similar peptides (sequence identity equal to or higher than 70%) based on peptide peak areas. For each peptide, the amino acid sequence is reported, together with the identification number of the correspondent protein, the E-value of the alignment against Glycine max protein database (http://plant.thegpm.org/tandem/thegpm_tandem.html), the coverage (%) and the molecular weight (MW) of the protein.

Table S2: Peptide composition data of rapeseed samples. Peptides were analysed by liquid chromatography coupled with mass spectrometry for the non-hydrolysed sample (N-H), for acid hydrolysates obtained with 6 N H_2SO_4 at 121°C for 15 min (6N A) or at 100°C for 8 h (6N B), and for enzymatic hydrolysates obtained with Alcalase (ALCA) and Flavourzyme (FLAV) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) or 50% (ALCA 50% and FLAV 50%, respectively) of the protein content. Peptides identified in hydrolysed samples were aligned with those detected in the N-H sample, and quantitation ratios were calculated for similar peptides (sequence identity equal to or higher than 70%) based on peptide peak areas. For each peptide, the amino acid sequence is reported, together with the identification number of the correspondent protein, the *E*-value of the alignment against *Brassica napus* protein database (http://plant.thegpm.org/tandem/thegpm_tandem.html), the coverage (%) and the molecular weight (MW) of the protein.

Table S3: Peptide composition data of guar samples. Peptides were analysed by liquid chromatography coupled with mass spectrometry for the non-hydrolysed sample (N-H), for acid hydrolysates obtained with 6 N H_2SO_4 at 121°C for 15 min (6N A) or at 100°C for 8 h (6N B), and for enzymatic hydrolysates obtained with Alcalase (ALCA) and Flavourzyme (FLAV) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) or 50% (ALCA 50% and FLAV 50%, respectively) of the protein content. Peptides identified in hydrolysed samples were aligned with those detected in the N-H sample, and quantitation ratios were calculated for similar peptides (sequence identity equal to or higher than 70%) based on peptide peak areas. For each peptide, the amino acid sequence is reported, together with the identification number of the correspondent protein, the *E*-value of the alignment against the Viridiplantae protein database (http://plant.thegpm.org/tandem/thegpm_tandem.html), the coverage (%) and the molecular weight (MW) of the protein.

Available online at: https://doi.org/10.4172/2155-9821.1000306

Table S4: Free amino acid composition of soybean samples. The concentration of each amino acid (µg/ml) was assessed by liquid chromatography coupled with mass spectrometry for the non-hydrolysed sample (N-H), for acid hydrolysates obtained with 6 N H_2SO_4 at 121°C for 15 min (6N A) or at 100°C for 8 h (6N B), and for enzymatic hydrolysates obtained with Alcalase (ALCA) and Flavourzyme (FLAV) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) or 50% (ALCA 50% and FLAV 50%, respectively) of the protein content. The mean and standard deviation (Std Dev) values of two independent analysis are reported for each amino acid and sample.

AMINO ACID	N-H		6N A		6N B		ALCA1%		ALCA 50%		FLAV 1%		FLAV 50 %	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
L-Glicine	18.03	3.61	168.88	33.78	620.38	124.08	1845.05	369.01	118.24	23.65	164.43	32.89	2845.42	569.08
L-Alanine	33.03	6.61	509.63	101.93	755.59	151.12	300.98	60.20	428.29	85.66	340.30	68.06	929.45	185.89
L-Proline	0.00	0.00	322.25	64.45	15.03	3.01	146.76	29.35	163.73	32.75	0.00	0.00	599.83	119.97
L-Threonine	11.19	2.24	223.74	44.75	1177.78	235.56	231.60	46.32	520.67	104.13	336.64	67.33	712.74	142.55
L-Leucine/Isoleucine	0.89	0.18	240.93	48.19	122.49	24.50	211.22	42.24	5839.39	1167.88	530.76	106.15	88.62	17.72
L-Histidine	0.00	0.00	4.09	0.82	0.00	0.00	989.83	197.97	372.00	74.40	0.00	0.00	344.00	68.80
L-Aspartic acid	0.00	0.00	178.42	35.68	0.00	0.00	3134.10	626.82	383.93	76.79	5.39	1.08	0.00	0.00
L-Arginine	192.99	38.60	87.42	17.48	0.00	0.00	0.00	0.00	2390.78	478.16	238.12	47.62	0.00	0.00
DL-Homophenylalanine	0.10	0.02	76.86	15.37	12.20	2.44	0.05	0.01	2.44	0.49	1.10	0.22	10.74	2.15
L-Aspartic acid	0.05	0.01	70.76	14.15	2.32	0.46	0.49	0.10	3.05	0.61	0.78	0.16	18.54	3.71
L-Cysteine	0.14	0.03	12.20	2.44	7.20	1.44	1.34	0.27	5.73	1.15	1.83	0.37	3.66	0.73
L-Glutamic acid	0.05	0.01	90.28	18.06	12.08	2.42	1.90	0.38	3.54	0.71	0.07	0.01	14.40	2.88
L-Glutamine	0.01	0.00	21.96	4.39	3.29	0.66	2.34	0.47	9.27	1.85	0.90	0.18	8.30	1.66
L-Lysine	0.01	0.00	114.68	22.94	7.32	1.46	2.42	0.48	0.37	0.07	1.12	0.22	10.25	2.05
L-Methionine	0.05	0.01	25.62	5.12	8.17	1.63	1.81	0.36	11.47	2.29	0.93	0.19	8.54	1.71
L-Phenylalanine	0.23	0.05	85.40	17.08	1.59	0.32	1.63	0.33	7.93	1.59	1.56	0.31	7.32	1.46
L-Serine	0.09	0.02	98.82	19.76	7.69	1.54	0.24	0.05	9.27	1.85	0.32	0.06	19.76	3.95
L-Valine	0.04	0.01	53.68	10.74	2.07	0.41	1.90	0.38	11.96	2.39	1.24	0.25	10.74	2.15
Glycated L-Lysine	85.55	17.11	3772.72	754.54	710.36	142.07	103.30	20.66	614.01	122.80	110.10	22.02	728.52	145.70
Glycated-L-Arginine	0.00	0.00	247.13	49.43	12587.35	2517.47	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S5: Free amino acid composition of rapeseed samples. The concentration of each amino acid (µg/ml) was assessed by liquid chromatography coupled with mass spectrometry for the non-hydrolysed sample (N-H), for acid hydrolysates obtained with 6 N H_2SO_4 at 121°C for 15 min (6N A) or at 100°C for 8 h (6N B), and for enzymatic hydrolysates obtained with Alcalase (ALCA) and Flavourzyme (FLAV) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) or 50% (ALCA 50% and FLAV 50%, respectively) of the protein content. The mean and standard deviation (Std Dev) values of two independent analysis are reported for each amino acid and sample.

AMINO ACID	N-H		6N A		6N B		ALCA1%		ALCA 50%		FLAV 1%		FLAV 50 %	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
L-Glicine	0.05	0.01	14869.04	2973.81	343.93	68.79	361.53	72.31	114.19	22.84	968.82	19.38	1424.73	28.49
L-Alanine	12.25	2.45	322.79	64.56	3993.81	798.76	29.86	2.99	752.27	150.45	395.50	7.91	444.32	8.89
L-Proline	0.00	0.00	260.26	52.05	2850.19	570.04	225.95	22.59	118.58	23.72	1903.44	38.07	0.00	0.00
L-Threonine	2.02	0.00	493.55	98.71	2007.00	401.40	536.87	53.69	208.29	41.66	346.90	6.94	499.96	10.00
L-Leucine/Isoleucine	7.15	0.00	0.00	0.00	0.00	0.00	10439.60	1043.96	142.07	28.41	409.76	8.20	856.49	17.13
L-Histidine	0.00	0.00	0.00	0.00	0.00	0.00	97.65	9.77	5154.83	1030.97	0.00	0.00	0.00	0.00
L-Aspartic acid	1.17	0.23	7729.63	1545.93	51.38	10.28	4.66	0.47	5554.91	1110.98	0.00	0.00	0.00	0.00
L-Arginine	0.00	0.00	0.00	0.00	0.00	0.00	32.18	3.22	745.65	149.13	611.41	12.23	0.00	0.00
DL-Homophenylalanine	0.01	0.00	4.06	0.81	8.64	1.73	3.82	0.38	3.64	0.73	21.44	0.43	4.43	0.09
L-Aspartic acid	0.04	0.01	21.59	4.32	12.45	2.49	3.50	0.35	2.84	0.57	5.59	0.11	3.73	0.07
L-Cysteine	0.08	0.02	21.84	4.37	19.05	3.81	1.17	0.12	0.33	0.07	8.39	0.17	3.26	0.07
L-Glutamic acid	0.03	0.01	18.54	3.71	4.06	0.81	0.33	0.03	2.98	0.60	13.28	0.27	5.83	0.12
L-Glutamine	0.01	0.00	21.59	4.32	12.19	2.44	0.70	0.07	2.42	0.48	21.90	0.44	6.06	0.12
L-Lysine	0.01	0.00	1.78	0.36	20.57	4.11	1.26	0.13	0.23	0.05	16.31	0.33	20.51	0.41
L-Methionine	0.05	0.01	6.60	1.32	4.83	0.97	1.72	0.17	3.45	0.69	2.80	0.06	8.85	0.18
L-Phenylalanine	0.09	0.02	12.19	2.44	17.78	3.56	1.44	0.14	3.12	0.62	10.02	0.20	12.58	0.25
L-Serine	0.10	0.02	11.43	2.29	17.27	3.45	2.61	0.26	2.28	0.46	9.55	0.19	1.63	0.03
L-Valine	0.02	0.00	1.52	0.30	8.89	1.78	4.15	0.41	2.28	0.46	16.78	0.34	7.69	0.15
Glycated L-Lysine	4.47	0.89	887.28	177.46	1096.21	219.24	230.98	23.10	174.45	34.89	823.16	16.46	969.08	19.38
Glycated-L-Arginine	0.00	0.00	473.29	94.66	12115.92	2423.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S6: Free amino acid composition of guar samples. The concentration of each amino acid (µg/ml) was assessed by liquid chromatography coupled with mass spectrometry for the non-hydrolysed sample (N-H), for acid hydrolysates obtained with 6 N H_2SO_4 at 121°C for 15 min (6N A) or at 100°C for 8 h (6N B), and for enzymatic hydrolysates obtained with Alcalase (ALCA) and Flavourzyme (FLAV) at a dosage of 1% (ALCA 1% and FLAV 1%, respectively) or 50% (ALCA 50% and FLAV 50%, respectively) of the protein content. The mean and standard deviation (Std Dev) values of two independent analysis are reported for each amino acid and sample.

AMINO ACID	N-H		6N A		6N B		ALCA1%		ALCA 50%		FLAV 1%		FLAV 50 %	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
L-Glicine	7.28	1.46	1759.07	351.81	957.41	191.48	765.78	153.16	2795.26	559.05	19.35	3.87	680.86	136.17
L-Alanine	10.35	2.07	1143.61	228.72	645.22	129.04	411.33	82.27	2167.66	433.53	131.55	26.31	324.94	64.99
L-Proline	65.27	13.05	742.66	148.53	7592.92	1518.58	0.00	0.00	8387.26	1677.45	63.75	12.75	77.28	15.46
L-Threonine	5.36	1.07	2363.64	472.73	1017.80	203.56	938.78	187.76	2748.91	549.78	21.55	4.31	501.69	100.34
L-Leucine/Isoleucine	10.33	2.07	1043.69	208.74	369.67	73.93	10060.75	2012.15	0.00	0.00	0.00	0.00	7.20	1.44
L-Histidine	0.00	0.00	571.86	114.37	1216.54	243.31	683.07	136.61	5337.43	1067.49	68.85	13.77	0.00	0.00
L-Aspartic acid	237.44	47.49	2968.91	593.78	646.90	129.38	0.00	0.00	461.81	92.36	3.41	0.68	1101.03	220.21
L-Arginine	2.64	0.53	0.00	0.00	628.77	125.75	1322.86	264.57	3057.03	611.41	223.34	44.67	150.90	30.18
DL-Homophenylalanine	0.00	0.00	6.86	1.37	8.64	1.73	4.83	0.97	4.39	0.88	1.90	0.38	2.78	0.56
L-Aspartic acid	0.00	0.00	2.29	0.46	8.64	1.73	2.10	0.42	28.79	5.76	2.88	0.58	1.22	0.24
L-Cysteine	0.00	0.00	6.86	1.37	3.30	0.66	1.66	0.33	5.37	1.07	4.00	0.80	1.07	0.21
L-Glutamic acid	0.00	0.00	10.41	2.08	13.97	2.79	1.51	0.30	1.46	0.29	3.66	0.73	0.10	0.02
L-Glutamine	0.00	0.00	7.62	1.52	24.38	4.88	3.61	0.72	4.39	0.88	3.17	0.63	1.56	0.31
L-Lysine	0.00	0.00	5.59	1.12	23.37	4.67	3.90	0.78	24.89	4.98	2.20	0.44	3.71	0.74
L-Methionine	0.00	0.00	21.59	4.32	0.25	0.05	2.39	0.48	32.21	6.44	4.64	0.93	3.76	0.75
L-Phenylalanine	0.00	0.00	15.49	3.10	18.29	3.66	4.49	0.90	6.34	1.27	4.59	0.92	0.39	0.08
L-Serine	0.00	0.00	16.76	3.35	22.86	4.57	1.17	0.23	40.02	8.00	3.42	0.68	1.27	0.25
L-Valine	0.00	0.00	1.78	0.36	22.86	4.57	2.68	0.54	6.83	1.37	2.54	0.51	4.49	0.90
Glycated L-Lysine	9.51	1.90	1219.26	243.85	915.24	183.05	201.41	40.28	1866.21	373.24	217.05	43.41	190.25	38.05
Glycated-L-Arginine	0.00	0.00	0.00	0.00	2694.90	538.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S7: Pearson's correlation between efficacy against powdery mildew and free amino acid concentrations of soybean enzymatic and acid hydrolysates. 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 soybean meal, correlation (R^2 value) and significant (*p*-value ≤ 0.05) or non-significant (NS) values were calculated. The concentration of each amino acid was assessed by liquid chromatography coupled with mass spectrometry.

	Enzymatic hydrolysates		Acid hydrolysates	
Amino acid	**R^2 value**	***p*- value**	**R^2 value**	***p*- value**
L-Glicine	-0.031	NS	0.067	NS
L-Alanine	-0.015	NS	0.017	NS
L-Proline	-0.003	NS	-0.109	NS
L-Threonine	-0.061	NS	0.073	NS
L-Leucine/Isoleucine	-0.17	NS	-0.082	NS
L-Histidine	-0.234	NS	-0.11	NS
L-Aspartic acid	-0.206	NS	-0.11	NS
L-Arginine	-0.15	NS	-0.032	NS
DL-Homophenylalanine	0.062	NS	-0.105	NS
L-Aspartic acid	0.062	NS	-0.109	NS
L-Cysteine	-0.143	NS	-0.074	NS
L-Glutamic acid	0.024	NS	-0.106	NS
L-Glutamine	-0.122	NS	-0.105	NS
L-Lysine	0.051	NS	-0.108	NS
L-Methionine	-0.123	NS	-0.097	NS
L-Phenylalanine	-0.101	NS	-0.109	NS
L-Serine	0.009	NS	-0.108	NS
L-Valine	-0.102	NS	-0.109	NS
Glycated L-Lysine	-0.047	NS	-0.105	NS
Glycated L-Arginine	-	-	0.084	NS

Table S8: Pearson's correlation between efficacy against powdery mildew and free amino acid concentrations of rapeseed enzymatic and acid hydrolysates. 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 rapeseed meal, correlation (R^2 value) and significant (*p*-value ≤ 0.05) or non-significant (NS) values were calculated. The concentration of each amino acid was assessed by liquid chromatography coupled with mass spectrometry.

	Enzymatic hydrolysates		Acid hydrolysates	
Amino acid	**R^2 value**	***p*- value**	**R^2 value**	***p*- value**
L-Glicine	0.072	NS	0.06	NS
L-Alanine	-0.024	NS	0.436	0.013
L-Proline	0.133	NS	0.44	0.012
L-Threonine	0.156	NS	0.486	0.005
L-Leucine/Isoleucine	0.133	NS	-0.517	0.002
L-Histidine	-0.061	NS	-	-
L-Aspartic acid	-0.063	NS	0.052	NS
L-Arginine	0.034	NS	-	-
DL-Homophenylalanine	0.137	NS	0.538	0.001
L-Aspartic acid	0.172	NS	0.359	0.044
L-Cysteine	0.126	NS	0.484	0.005
L-Glutamic acid	0.097	NS	0.157	NS
L-Glutamine	0.111	NS	0.353	0.048
L-Lysine	0.057	NS	0.439	0.012
L-Methionine	-0.003	NS	0.432	0.014
L-Phenylalanine	0.053	NS	0.555	0.001
L-Serine	0.15	NS	0.555	0.001
L-Valine	0.14	NS	0.464	0.008
Glycated L-Lysine	0.08	NS	0.548	0.001
Glycated L-Arginine	-	-	0.424	0.015

CHAPTER 4.
Discussion

In order to reduce the massive use of chemical fungicides and develop new harmless strategies to be included in organic pest management programs, the objectives of my doctoral project were to analyse the current regulatory procedures required for the registration of biopesticide in agriculture, and to provide new insight on the production and mechanism of action of protein-based products against crop diseases. Amino acids and peptides contained in protein-based products may act as biostimulants because they enhance the abiotic stress tolerance (Colla et al., 2014), and morover they could potentially be recognised by plants as MAMPs (Jones & Dangl, 2006) or DAMPs (Wu et al., 2014), and exert a bioactive role by triggering plant defence mechanisms (Albert, 2013), as recently reported for soybean and casein hydrolysates in grapevine (Lachhab et al., 2014; Lachhab et al., 2016). For this reason, they can be also considered as plant protection products and therefore they must be authorised according to the current regulations. In spite of their natural origin and the absence of toxicity and eco-toxicity, the EU regulation does not encourage their registration, which follows the same procedure and timelines as any other synthetic chemical pesticide. To support the registration process by characterizing the mechanism of action of these compounds, we focused on a protein derivative (NB), which previously showed activity against grapevine powdery mildew in vineyards across seasons through the activation of defence-related genes (Nesler et al., 2015). Beyond its properties as a resistance inducer, we hypothesized that NB might also act as nutritional source and affect the composition and dynamics of phyllosphere microbial populations, which in turn might contribute to resistance induction and/or display direct biocontrol properties against the pathogen. Hence, the first part of our research aimed to investigate the efficacy of NB against grapevine downy mildew and to assess whether its mode of action was related only to the induction of resistance, or eventually also to an indirect effect caused by its application on leaf microbial communities. Our results indicated that under axenic conditions, NB strongly reduced downy mildew symptoms and induced the expression of five defence-related genes (*PR-2*, *PR-4*, *OSM-1*, *OSM-2* and *CHIT-3*), suggesting that it was effective against *P. viticola* through the induction of grapevine resistance. The expression of *PR-1* (a marker gene of SA, that regulates the pathways of SAR) was not modulated by NB application under axenic conditions. Preventive foliar treatment with NB reduced downy mildew symptoms under greenhouse conditions, through the induction of all defence-related genes tested, including *PR-1*. Accordingly, the expression profiles of *in vitro*-grown plants partially differed from those of greenhouse-grown plants. Although

different expression levels between axenic and greenhouse conditions could be linked to different growing conditions of the plants, they could also be associated with the modifications occurred in the leaf microbiota after the application of NB on greenhouse plants, and these changes may partially affect the hormone-mediated signalling pathways involved, providing a contribution to the resistance induction. However, a higher efficacy of NB was observed under axenic conditions with respect to greenhouse conditions, and this can be explained by two reasons. We profiled that in greenhouse plants are naturally exposed to a wide variety of microorganisms, and resistance mechanisms might be already partially activated. In addition, some components of protein-derived products can be metabolized by the phyllosphere microorganisms, thus reducing the properties and efficacy of the originally applied product (Colla et al., 2015). Although NB induced a lower expression of some defence-related genes (*CHIT-3*, *OSM-1*, *OSM-2* and *PR-4*) in comparison with the LAM treatment, it presented a higher efficacy against downy mildew. These results suggest that multiple mechanisms of action are involved in the biocontrol role of NB, and additional biotic factors, such as the phyllosphere microbiota, could influence the efficacy against downy mildew. The metabarcoding analysis allowed the dissection of compositions and modifications of the bacterial and fungal populations residing on the grapevine phyllosphere after the treatments tested. Even though plants originated from the same nursery stock, and were grown under the same controlled conditions, significant differences among microbial populations were found between the two greenhouse experiments. Indeed, the plant phyllosphere act as an open system, and the structure of its microbial community reflects immigration, survival and growth of microbial colonists, which in turn is influenced by numerous environmental factors, in addition to the leaf physicochemical properties (Whipps et al., 2008). Subsequently, changes occurred in leaf microbial communities after the treatments were influenced by the composition of the originally residing microbiota. However, bacterial community structure was globally affected by time points and by NB treatment in both experiments, while no effect was noticed from LAM treatment. More specifically, NB act as nutritional substrate, changing proportions of some leaf microbial taxa that may be related to the biological control of plant pathogens, by competition or parasitism activity. For example, NB application increased abundances of the Exiguobacteraceae family and the *Exiguobacterium* genus as compared with H_2O treatment at T0 in Exp 1 and T1 in Exp 2, respectively. Interactions between *Exiguobacterium acetylicum* and two other bacteria (namely *Microbacterium* spp. and *Pantoea agglomerans*) have been reported

to contribute to the suppression of the wheat root disease caused by *Rhizoctonia solani* (Barnett et al., 2006), indicating potential biocontrol properties of some *Exiguobacterium* strains. In Exp 2, the NB treatment increased the proportion of the Pseudomonadaceae family and the *Pseudomonas* genus as compared with UNT plants and H_2O-treated plants at T0, and *P. viridiflava* and *P. veronii* levels showed the same trend. Some species of this genera are known as active resistance inducers (van Wees et al., 2008) and biocontrol agents, for their ability to produce proteases (Elad, 2000), siderophores (van Wees et al., 2008) and antimicrobial metabolites (Ligon et al., 2000). Specifically for grapevine plants, members of *Pseudomonas* have been demonstrated to effectively control *Botrytis cinerea* infections by induction of resistance mechanisms (Trotel-Aziz et al., 2008). Finally, the NB treatment increased also the proportion of *P. alcaligenes* at T0 in Exp 1, and this species has been reported as biocontrol agent against *Fusarium oxysporum* (Akhtar et al., 2010). In Exp 2, the Enterobacteriaceae family accounted for the majority of bacterial OTU at T0, and its abundance was affected by NB at both time points. The most dominant species was *Serratia marcescens*, which significantly increased by NB with respect to H_2O-treated plants at T0 in Exp 1. *S. marcescens* was reported as biocontrol agent against the soil-borne fungus *Magnaporthe poae* (Kobayashi et al., 1995) and the rice pathogen *Magnaporthe oryzae* (Jaiganesh et al., 2007), thanks to its chitinolytic activity. A strain of *Lysobacter capsici* reduced downy mildew symptoms in grapevine (Puopolo et al., 2014), and the abundance of the *Lysobacter* genus on grapevine leaves increased as a result of NB treatment at T0 in Exp 2. On the other hand, the structure of fungal communities was similar in Exp 1 and Exp 2, and they were not globally affected by NB and LAM treatment, with a few exceptions. For example, the NB treatment modified the abundances of some specific fungal taxa, such as the *Alternaria* genus at T1 in Exp 1, and *A. alternata* was able to control *P. viticola* on leaf disks (Musetti et al., 2006). Relative abundances of *Trichoderma* spp. and *Aureobasidium* spp. were increased by the NB treatment at T1 in Exp 2. A strain of *Trichoderma harzianum* induces grapevine resistance (Perazzolli et al., 2011), and an isolate of *Aureobasidium pullulans* partially protects against downy mildew (Harm et al., 2011). However, fungal communities on grapevine leaves were more stable compared to bacterial ones, due to the preference of bacteria for protein and amino acids as nutritional source (Vorholt, 2012), or probably to the longer generation time of fungi, that actually did not allow to appreciate modifications within the short time of the experiment (four days). Although culturable microorganisms represent a limited fraction of the total, they are the most likely to be

influenced by NB, which is a laboratory microbiological medium. The increase of culturable microorganisms on NB-treated plants confirmed that the protein derivative had a nutritional role that affected mainly bacteria. However, *in vitro* assays suggested the absence of positive selection of potential biocontrol agents against oomycetes, since the NB treatment did not affect proportions of bacterial isolates with proteolytic activity, siderophore production and antagonistic activity against *P. infestans*. Nevertheless, two isolates from NB-treated plants showed a significant biocontrol activity against *P. viticola* on leaf disks, and these sequences were finally identified as *Pseudomonas* spp. and *Enterobacter* spp. Summarizing, the preventive foliar application of NB on grapevine plants under greenhouse conditions partially increased the number of culturable bacteria, and altered the structures and composition of the residing phyllosphere microbiota. These changes may contribute to pathogen control resulting from competition for space, parasitism or from other biocontrol strategies, resistance induction included.

In practice, NB is an expensive microbiological medium, hard to be employed as biopesticides in pest management programs. Moreover animal-based products may create concerns about food safety, and cannot be used on the edible parts of organic crops (EC, 2014). For this reason, we focused our attention on plant-derived protein hydrolysates. Among other things, they contain organic compounds, such as phenols, lipids and carbohydrates (Colla et al., 2015), 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 (Nicholson & Hammerschmidt, 1992; Lattanzio et al., 2006), and lipids (Shah, 2005; Hoffmann-Benning, 2015) and carbohydrates (Trouvelot et al., 2014) have been similarly demonstrated to be involved in plant immunity. Conversely, animal-derived protein hydrolysates lack carbohydrates, phenols and phytohormones (Colla et al., 2015), and repeated foliar applications can provoke phytotoxic effects on plant growth (Cerdan et al., 2009) that could be attributed to an unbalanced amino acid composition (Oaks et al., 1977) and a high salinity (Colla et al., 2014). Subsequently, we first optimized an experimental procedure to develop low-cost protein hydrolysates starting from plant agro-industrial by-products, namely soybean, rapeseed and guar meals. Later, we compared the effect of both acid and enzymatic hydrolysis in term of biocontrol efficacy against the powdery mildew of Cucurbitaceae (caused by the pathogen *P. xanthii*), investigating the potential contribution of amino acids and peptide fragments generated during the hydrolysis to the activation of plant resistance. Indeed,

the biocontrol properties of protein hydrolysates were previously proved to be affected by the original protein source, hydrolysis method and degree of hydrolysis (Cheison et al., 2009; Pecha et al., 2012), as well as by their biochemical characteristics (Chabanon et al., 2007; Jamdar et al., 2010). Our results showed that both enzymatic and acid methods significantly improved the efficacy of guar protein source against powdery mildew symptoms, in particular when the proteolytic process was carried out with the enzyme Alcalase at 50% E:S ratio, and with sulfuric acid 6N used at 100°C for 8 h, respectively. Conversely, the biocontrol activity of soybean and rapeseed was not improved by the tested hydrolysis processes. In agreement with previous findings (Spellman et al., 2003), our results confirmed that the functional properties of a protein derivative may be altered by hydrolysis and depends on DH, suggesting that high hydrolysis time and temperature conditions could increase the biocontrol activity of the guar protein source. Indeed, we found significant positive correlations between efficacy values and DH of guar acid hydrolysates. In other words, DH affects the physicochemical characteristics of protein hydrolysates, and could in turn affect their functionality (Mahmoud, 1994; Panyam & Kilara, 1996). Among other things, amino acid and peptide compositions were proved to modulate the biological activity of protein hydrolysates (Jamdar et al., 2010), depending on molecular size, structure and specific sequence (Chabanon et al., 2007). Our results revealed significant correlations for guar enzymatic and acid hydrolysates between efficacy values and the quantitation ratios of specific peptide sequences, suggesting their crucial role against the disease. Specifically, some of them may be responsible for plant defence activation against powdery mildew, by possibly mimicking the biological activity of endogenous natural DAMPs. Indeed, specific peptides of plant origin have been demonstrated to act as non-toxic signalling molecules for innate plant defence (Ryan et al., 2002). In particular, peptides deriving from cytosolic proteins, such as the active form of systemin (Pearce et al., 2010) and the AtPep1 peptide (Huffaker et al., 2006), were shown to be internal signals for plant defence mechanisms in the soybean and *A. thaliana* respectively. A similar function was demonstrated for peptides originating from secreted precursors, such as hydroxyproline-rich systemins in the potato (Bhattacharya et al., 2013) and phytosulphokines in *A. thaliana* (Igarashi et al., 2012). 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 (Schmelz et al., 2007) and other peptide fragments in the soybean (Pearce et al., 2010; Yamaguchi et al., 2011). All of them seem to be active as elicitors and can activate the expression of

typical defence marker genes (Albert, 2013). For guar acid hydrolysates, positive correlations were also found between efficacy values and amino acid concentrations, suggesting that they may contribute to efficacy against courgette 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 activation of amino acid metabolism during plant disease responses (Scheideler et al., 2002; Liu et al., 2010; Ward et al., 2010; Cecchini et al., 2011). Finally, we analysed the direct toxic effect of enzymatic and acid hydrolysates against conidial germination on leaf disks. Guar enzymatic hydrolysates did not affect conidia germination, suggesting a mode of action mainly based on the stimulation of plant resistance mechanisms, as observed for NB. On the contrary, treatments with acid hydrolysates affected the conidia germination, because the use of strong acids, such as sulfuric acid during hydrolysis, caused an increase in the salinity of protein hydrolysates (Colla et al., 2015), and the formation of potassium sulfate in guar acid hydrolysates contributed to disease control.

CHAPTER 5.
Conclusions

The current doctoral project presents an updated overview on the regulatory procedures for the registration of low-risk substances and biopesticide, and provides an innovative approach to reduce the use of chemical pesticides in integrated pest management programs, based on the preventive foliar application of protein-based products. This study shows a clear picture of the multiple mechanism of action displayed by the protein derivative NB, which acts like promising sustainable alternative for the control of grapevine downy and powdery mildew, considering its natural origin and the absence of negative effect on plant growth and yield, with minimal risks for grape production and quality (Nesler et al., 2015). As demonstrated for the control of powdery mildew (Nesler et al., 2015), the reduction of downy mildew symptoms is mainly based on the induction of resistance in grapevine, involving multiple signalling pathways. Furthermore, NB increased the number of culturable phyllosphere microorganisms, and changed proportions of some taxa that have previously been linked to the biological control of plant pathogens. Although the efficacy of NB in controlling grapevine downy mildew is mainly based on direct induction of grapevine resistance, modifying phyllosphere populations by increasing natural biocontrol agents with the application of selected nutritional factors can open new opportunities in terms of sustainable plant protection strategies. Aiming to avoid food safety concerns and high application costs, the current doctoral study focused on developing low-cost plant-derived protein hydrolysates, providing new insights into their role as biopesticides, beyond the biostimulant activity. Our results indicate the efficacy of guar protein hydrolysates against the powdery mildew of Cucurbitaceae, and two specific hydrolysis methods led to the formation of bioactive products. As previously reported, the biocontrol activity 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.

Thanks to the data produced in this doctoral project, it is possible to conceive the future development of protein-based products as low-risk active substances against phytopathogenic microorganisms. Preventive foliar application of protein-based products may offer considerable environmental and economic benefits, and they could activate systemic defence mechanisms. However, the economic advantages of using agricultural by-products (such as protein meals deriving from oil extraction) may be nullified, if expensive commercial enzymes need to be used in the hydrolysis process. Moreover, knowledge of the application of protein-based products to crops is far from being complete, and further studies are required in order to fully clarify the impacts of

phyllosphere microbial communities on the persistance of these compounds on plant leaves.

References

Adler-Nissen, J. 1982. Limited enzymic degradation of proteins: a new approach in the industrial application of hydrolases. *J Chem Technol Biotechnol,* 32: 138-156.

Agrios, G. N. (2005). *Plant Pathology* (Fifth ed.). San Diego: Academic Press.

Akhtar, M. S., Shakeel, U., & Siddiqui, Z. A. 2010. Biocontrol of *Fusarium* wilt by *Bacillus pumilus*, *Pseudomonas alcaligenes*, and *Rhizobium sp.* on lentil. *Turk J Biol,* 34 (1): 1-7.

Albert, M. 2013. Peptides as triggers of plant defence. *J Exp Bot,* 64: 5269-5279.

Allegre, M., Heloir, M.-C., Trouvelot, S., Daire, X., Pugin, A., et al. 2009. Are grapevine stomata involved in the elicitor-induced protection against downy mildew? *Mol Plant Microbe Interact,* 22 (8): 977-986.

Angeli, D., Micheli, S., Maurhofer, M., & Pertot, I. (2016). Enhancing the aggressiveness of the powdery mildew mycoparasite *Ampelomyces quisqualis*. In: S. Compant & F. Mathieu (Eds.), In *Biocontrol of major grapevine diseases: leading research.* pp. 113-116.

Armijo, G., Schlechter, R., Agurto, M., Munoz, D., Nunez, C., et al. 2016. Grapevine pathogenic microorganisms: understanding infection strategies and host response scenarios. *Front Plant Sci,* 7: 382.

Atkinson, N. J., & Urwin, P. E. 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. *J Exp Bot,* 63 (10): 3523-3543.

Aziz, A., Heyraud, A., & Lambert, B. 2004. Oligogalacturonide signal transduction, induction of defense-related responses and protection of grapevine against *Botrytis cinerea*. *Planta,* 218 (5): 767-774.

Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bezier, A., et al. 2003. Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. *Mol Plant Microbe Interact,* 16 (12): 1118-1128.

Aziz, A., Trotel-Aziz, P., Dhuicq, L., Jeandet, P., Couderchet, M., et al. 2006. Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew. *Phytopathol,* 96 (11): 1188-1194.

Baglieri, A., Cadili, V., Mozzetti Monterumici, C., Gennari, M., Tabasso, S., et al. 2014. Fertilization of bean plants with tomato plants hydrolysates. Effect on biomass production, chlorophyll content and N assimilation. *Sci Hortic,* 176: 194-199.

Banani, H., Roatti, B., Ezzahi, B., Giovannini, O., Gessler, G., et al. 2014. Characterization of resistance mechanisms activated by *Trichoderma harzianum* T39 and benzothiadiazole to downy mildew in different grapevine cultivars. *Plant Pathol,* 63 (2): 334-343.

Barnett, S. J., Roget, D. K., & Ryder, M. H. 2006. Suppression of *Rhizoctonia solani* AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and *Microbacteria*. *Soil Res,* 44 (4): 331-342.

Bartels, S., & Boller, T. 2015. Quo vadis, Pep? Plant elicitor peptides at the crossroads of immunity, stress, and development. *J Exp Bot,* 66 (17): 5183-5193.

Barzman, M. S., Bertschinger, L., Dachbrodt-Saaydeh, S., Graf, B., Jensen, J. E., et al. (2014). Integrated Pest Management policy, research and implementation: european initiatives. In: R. Peshin & D. Pimentel (Eds.), In *Integrated Pest Management: experiences with implementation, global overview.* Vol. 4, pp. 415-428. Dordrecht: Springer Netherlands.

Berg, G., Grube, M., Schloter, M., & Smalla, K. 2014. Unraveling the plant microbiome: looking back and future perspectives. *Front Microbiol,* 4 (5): 48.

Berlec, A. 2012. Novel techniques and findings in the study of plant microbiota: search for plant probiotics. *Plant Sci,* 193: 96-102.

Bhattacharya, R., Koramutla, M. K., Negi, M., Pearce, G., & Ryan, C. A. 2013. Hydroxyproline-rich glycopeptide signals in potato elicit signalling associated with defense against insects and pathogens. *Plant Sci,* 207: 88-97.

Boller, T., & Felix, G. 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol,* 60: 379-406.

Boubakri, H., Chong, J., Poutaraud, A., Schmitt, C., Bertsch, C., et al. 2013. Riboflavin (vitamin B2) induces defence responses and resistance to *Plasmopara viticola* in grapevine. *Eur J Plant Pathol,* 136 (4): 837-855.

Boubakri, H., Wahab, M. A., Chong, J., Bertsch, C., Mliki, A., et al. 2012. Thiamine induced resistance to *Plasmopara viticola* in grapevine and elicited host-defense responses, including HR like-cell death. *Plant Physiol Biochem,* 57: 120-133.

Braverman, M., Nelson, W., Torla, B., Mendelshon, M., Matten, S., et al. (2003). *Understanding the biopesticide registration process at EPA*. Retrieved from: http://ir4.rutgers.edu/biopesticides/RWP/PowerPoint/Understanding%20the%20Registration%20Process%20at%20EPA.pdf

Brunner, F., Rosahl, S., Lee, J., Rudd, J. J., Geiler, C., et al. 2002. Pep-13, a plant defense-inducing pathogen-associated pattern from *Phytophthora* transglutaminases. *EMBO J,* 21 (24): 6681-6688.

Burruano, S. 2000. The life-cycle of *Plasmopara viticola*, cause of downy mildew of vine. *Mycologist,* 14 (4): 179-182.

Caillot, S., Rat, S., Tavernier, M. L., Michaud, P., Kovensky, J., et al. 2012. Native and sulfated oligoglucuronans as elicitors of defence-related responses inducing protection against *Botrytis cinerea* of *Vitis vinifera*. *Carbohydr Polym,* 87 (2): 1728-1736.

Calvo, P., Nelson, L., & Kloepper, J. W. 2014. Agricultural uses of plant biostimulants. *Plant Soil,* 383: 3-41.

Cecchini, N. M., Monteoliva, M. I., & Alvarez, M. E. 2011. Proline dehydrogenase contributes to pathogen defense in *Arabidopsis*. *Plant Physiol,* 155: 1947-1959.

Cerdan, M., Sanchez-Sanchez, A., Jorda, J. D., Juarez, M., & Sanchez-Andreu, J. 2013. Effect of commercial amino acids on iron nutrition of tomato plants grown under lime-induced iron deficiency. *J Plant Nutr Soil Sci,* 176 (6): 859-866.

Cerdan, M., Sanchez-Sanchez, A., Oliver, M., Juarez, M., & Sanchez-Andreu, J. J. 2009. Effect of foliar and root applications of amino acids on iron uptake by tomato plants. *Acta Hortic,* 830: 481-488.

Chabanon, G., Chevalot, I., Framboisier, X., Chenu, S., & Marc, I. 2007. Hydrolysis of rapeseed protein isolates: kinetics, characterization and functional properties of hydrolysates. *Process Biochem,* 42: 1419-1428.

Chandler, D., Bailey, A. S., Tatchell, G. M., Davidson, G., Greaves, J., et al. 2011. The development, regulation and use of biopesticides for integrated pest management. *Philos Trans R Soc Lond B Biol Sci,* 366 (1573): 1987-1998.

Chang, X. L., & Nick, P. 2012. Defence signalling triggered by Flg22 and harpin is integrated into a different stilbene output in *Vitis* cells. *PLoS ONE,* 7 (7): e40446.

Cheison, S. C., Zhang, S. B., Wang, Z., & Xu, S. Y. 2009. Comparison of a modified spectrophotometric and the pH-stat methods for determination of the degree of hydrolysis of whey proteins hydrolysed in a tangential-flow filter membrane reactor. *Food Res Int,* 42 (1): 91-97.

Chen, C. C. 1964. The absorption and mobility of root and foliar applied calcium, sulfur, zinc and iron by tomato seedlings as influenced by gibberellin treatments. *Bot Bull Acad Sinica,* 5: 17-25.

Chen, Y. Y., Fu, X. M., Mei, X., Zhou, Y., Du, B., et al. 2016. Characterization of functional proteases from flowers of tea (*Camellia sinensis*) plants. *J Funct Foods,* 25: 149-159.

Chisholm, S. T., Coaker, G., Day, B., & Staskawicz, B. J. 2006. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell,* 124 (4): 803-814.

Choi, H. W., & Klessig, D. F. 2016. DAMPs, MAMPs, and NAMPs in plant innate immunity. *Bmc Plant Biol,* 16 (1): 232.

Clemente, A. 2000. Enzymatic protein hydrolysates in human nutrition. *Trends Food Sci Technol,* 11 (7): 254-262.

Cohen, Y. 2002. β-aminobutyric acid-induced resistance against plant pathogens. *Plant Dis,* 86 (5): 448-457.

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., et al. 2015. Protein hydrolysates as biostimulants in horticulture. *Sci Hortic,* 196: 28-38.

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., & Cardarelli, M. 2014. Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front Plant Sci,* 5:448.

Conrath, U., Beckers, G. J. M., Flors, V., Garcia-Agustin, P., Jakab, G., et al. 2006. Priming: getting ready for battle. *Molec Plant Microb Interact,* 19 (10): 1062-1071.

Copeland, J. K., Yuan, L. J., Layeghifard, M., Wang, P. W., & Guttman, D. S. 2015. Seasonal community succession of the phyllosphere microbiome. *Molec Plant Microb Interact,* 28 (3): 274-285.

Czaja, K., Goralczyk, K., Strucinski, P., Hernik, A., Korcz, W., et al. 2015. Biopesticides - towards increased consumer safety in the European Union. *Pest Manag Sci,* 71 (1): 3-6.

Dagostin, S., Scharer, H. J., Pertot, I., & Tamm, L. 2011. Are there alternatives to copper for controlling grapevine downy mildew in organic viticulture? *Crop Prot,* 30 (7): 776-788.

Delaunois, B., Farace, G., Jeandet, P., Clement, C., Baillieul, F., et al. 2014. Elicitors as alternative strategy to pesticides in grapevine? Current knowledge on their mode of action from controlled conditions to vineyard. *Environ Sci Pollut Res Int.,* 21 (7): 4837-4846.

Delledonne, M., Xia, Y. J., Dixon, R. A., & Lamb, C. 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature,* 394 (6693): 585-588.

Dercks, W., & Creasy, L. L. 1989. Influence of fosetyl-Al on phytoalexin accumulation in the *Plasmopara viticola* grapevine interaction. *Physiol Mol Plant Pathol,* 34 (3): 203-213.

DeWit, P. 1997. Pathogen avirulence and plant resistance: a key role for recognition. *Trends Plant Sci,* 2 (12): 452-458.

Dhiman, K., Gupta, A., Sharma, D. K., Gill, N. S., & Goyal, A. 2012. A review on the medicinally important plants of the family Cucurbitaceae. *Asian J Clin Nutr,* 4 (1): 16-26.

du Jardin, P. 2015. Plant biostimulants: definition, concept, main categories and regulation. *Sci Hortic,* 196: 3-14.

Dufour, M. C., Lambert, C., Bouscaut, J., Merillon, J. M., & Corio-Costet, M. F. 2013. Benzothiadiazole-primed defence responses and enhanced differential expression of defence genes in *Vitis vinifera* infected with biotrophic pathogens *Erysiphe necator* and *Plasmopara viticola*. *Plant Pathol,* 62 (2): 370-382.

Durrant, W. E., & Dong, X. 2004. Systemic acquired resistance. *Annu Rev Phytopathol,* 42: 185-209.

EBIC. (2017). *Promoting the biostimulant industry and the role of plant biostimulants in making agriculture more sustainable*. Retrieved from: http://www.biostimulants.eu/

EC. (2009). Regulation (EU) No. 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC.

EC. (2013a). Reglulation (EU) No. 283/2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No. 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market (pp. 1-84).

EC. (2013b). Regulation (EU) No. 284/2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No. 1107/2009 of the European Parliament and the Council concerning the placing of plant protection products on the market (pp. 85-152).

EC. (2014). Commission Implementing Regulation (EU) No. 354/2014 of 8 April 2014 amending and correcting Regulation (EC) N.o 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No. 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control.

EFSA. (2017). *Pesticide evaluations: overview and procedure*. Retrieved from: http://www.efsa.europa.eu/en/applications/pesticides

Elad, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot,* 19 (8-10): 709-714.

EPA. (1991). Code of Rederal Regulation No. 40, Part 158 - Data Requirements for pesticides.

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., et al. 2009. Biostimulant activity of two protein hydrolyzates in the growth and nitrogen metabolism of maize seedlings. *J Plant Nutr Soil Sci,* 172 (2): 237-244.

Ertani, A., Pizzeghello, D., Francioso, O., Sambo, P., Sanchez-Cortes, S., et al. 2014. *Capsicum chinensi*s L. growth and nutraceutical properties are enhanced by biostimulants in a long-term period: chemical and metabolomic approaches. *Front Plant Sci,* 5: 375.

Ertani, A., Schiavon, M., Muscolo, A., & Nardi, S. 2013. Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants. *Plant Soil,* 364: 145-158.

EU. (2017). *Pesticides*. Retrieved from: https://ec.europa.eu/food/plant/pesticides_en

FAO. (2004). *The state of food insecurity in the world*. Retrieved from: http://www.fao.org/docrep/007/y5650e/y5650e00.htm

FAO. (2014). *Faostat*. Retrieved from: www.fao.org/faostat/en

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., et al. 2002. NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and *Arabidopsis*. *Plant J,* 32 (3): 375-390.

Fernández, V., & Brown, P. H. 2013. From plant surface to plant metabolism: the uncertain fate of foliar-applied nutrients. *Front Plant Sci,* 4: 289.

Ferreira, R. B., Monteiro, S. S., Picarra-Pereira, M. A., & Teixeira, A. R. 2004. Engineering grapevine for increased resistance to fungal pathogens without compromising wine stability. *Trends Biotechnol,* 22 (4): 168-173.

Fofana, B., McNally, D. J., Labbé, C., Boulanger, R., Benhamou, N., et al. 2002. Milsana-induced resistance in powdery mildew-infected cucumber plants correlates with the induction of chalcone synthase and chalcone isomerase. *Physiol Mol Plant Pathol,* 61 (2): 121-132.

Garcia-Brugger, A., Lamotte, O., Vandelle, E., Bourque, S., Lecourieux, D., et al. 2006. Early signaling events induced by elicitors of plant defenses. *Molec Plant Microb Interact,* 19 (7): 711-724.

Gessler, C., Pertot, I., & Perazzolli, M. 2011. *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathol Mediterr,* 50 (1): 3-44.

Gibbs, B. F., Zougman, A., Masse, R., & Mulligan, C. 2004. Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *Food Res Int,* 37 (2): 123-131.

Gilardi, G., Baudino, M., Garibaldi, A., & Gullino, M. L. 2012. Efficacy of biocontrol agents and natural compounds against powdery mildew of zucchini. *Phytoparasitica,* 40 (2): 147-155.

Godard, S., Slacanin, I., Viret, O., & Gindro, K. 2009. Induction of defence mechanisms in grapevine leaves by emodin- and anthraquinone-rich plant extracts and their conferred resistance to downy mildew. *Plant Physiol Biochem,* 47 (9): 827-837.

Gohre, V., & Robatzek, S. 2008. Breaking the barriers: microbial effector molecules subvert plant immunity. *Annu Rev Phytopathol,* 46: 189-215.

Hamiduzzaman, M. M., Jakab, G., Barnavon, L., Neuhaus, J. M., & Mauch-Mani, B. 2005. *ß*-aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. *Mol Plant Microbe Interact,* 18 (8): 819-829.

Hamza, A., Mohamed, A., & Derbalah., A. 2017. Chemical inducers for resistance induction against powdery mildew of cucumber under greenhouse conditions. *Acta Phytopathol Entomol Hung,* 52 (1): 49-60.

Handford, C. E., Elliott, C. T., & Campbell, K. 2015. A review of the global pesticide legislation and the scale of challenge in reaching the global harmonization of food safety standards. *Integr Environ Assess Manag,* 11 (4): 525-536.

Harborne, J. B. 1999. The comparative biochemistry of phytoalexin induction in plants. *Biochem Syst Ecol,* 27 (4): 335-367.

Harm, A., Kassemeyer, H.-H., Seibicke, T., & Regner, F. 2011. Evaluation of chemical and natural resistance inducers against downy mildew (*Plasmopara viticola*) in grapevine. *Am J Enol Vitic,* 62 (2): 184-192.

He, S. Y., Huang, H.-C., & Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* harpinPss: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell,* 73 (7): 1255-1266.

Hoffmann-Benning, S. (2015). *Transport and function of lipids in the plant phloem*. Retrieved from: http://lipidlibrary.aocs.org/Biochemistry/content.cfm?ItemNumber=41357

Huber, L. (2016). *Biopesticides and the EU regulatory process*. Retrieved from: http://www.scc-gmbh.de/images/documents/Agrow-Biopesticides-2016-SCC-Scientific.pdf

Huffaker, A., Dafoe, N. J., & Schmelz, E. A. 2011. *Zm*Pep1, an ortholog of *Arabidopsis* elicitor peptide 1, regulates maize innate immunity and enhances disease resistance. *Plant Physiol,* 155 (3): 1325-1338.

Huffaker, A., Pearce, G., & Ryan, C. A. 2006. An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response. *Proc Natl Acad Sci U S A,* 103: 10098-10103.

Ielciu, I., Frederich, M., Tits, M., Angenot, L., Paltinean, R., et al. 2016. *Bryonia alba* l. and *Ecballium elaterium* (l.) A. Rich. - two related species of the cucurbitaceae family with important pharmaceutical potential. *Farmacia,* 64 (3): 323-332.

Igarashi, D., Tsuda, K., & Katagiri, F. 2012. The peptide growth factor, phytosulfokine, attenuates pattern-triggered immunity. *Plant J,* 71: 194-204.

Iriti, M., Vitalini, S., Di Tommaso, G., D'Amico, S., Borgo, M., et al. 2011. New chitosan formulation prevents grapevine powdery mildew infection and improves polyphenol content and free radical scavenging activity of grape and wine. *Aust J Grape Wine Res,* 17 (2): 263-269.

Ito, Y., Nakanomyo, I., Motose, H., Iwamoto, K., Sawa, S., et al. 2006. Dodeca-CLE peptides as suppressors of plant stem cell differentiation. *Science,* 313 (5788): 842-845.

Jaiganesh, V., Eswaran, A., Balabaskar, P., & Kannan, C. 2007. Antagonistic activity of *Serratia marcescens* against *Pyricularia oryzae*. *Not Bot Horti Agrobo,* 35 (2): 48-54.

Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., Yardi, V., et al. 2010. Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chem,* 121: 178-184.

Jaulneau, V., Lafitte, C., Corio-Costet, M. F., Stadnik, M. J., Salamagne, S., et al. 2011. An *Ulva armoricana* extract protects plants against three powdery mildew pathogens. *Eur J Plant Pathol,* 131 (3): 393-401.

Jaulneau, V., Lafitte, C., Jacquet, C., Fournier, S., Salamagne, S., et al. 2010. Ulvan, a sulfated polysaccharide from green algae, activates plant immunity through the jasmonic acid signaling pathway. *J Biomed Biotechnol,* 2010: 1-11.

Jeewanthi, R. K. C., Lee, N. K., & Paik, H. D. 2015. Improved functional characteristics of whey protein hydrolysates in food industry. *Korean J Food Sci An,* 35 (3): 350-359.

Jonak, C., Ökrész, L., Bögre, L., & Hirt, H. 2002. Complexity, cross talk and integration of plant MAP Kinase signalling. *Curr Opin Plant Biol,* 5 (5): 415-424.

Jones, J. D. G., & Dangl, J. L. 2006. The plant immune system. *Nature,* 444 (7117): 323-329.

Kamoun, S. 2001. Nonhost resistance to *Phytophthora*: novel prospects for a classical problem. *Curr Opin Plant Biol,* 4 (4): 295-300.

Kim, M. G., da Cunha, L., McFall, A. J., Belkhadir, Y., DebRoy, S., et al. 2005. Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in *Arabidopsis*. *Cell,* 121 (5): 749-759.

Kloepper, J. W., Ryu, C. M., & Zhang, S. A. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus spp*. *Phytopathol,* 94 (11): 1259-1266.

Kobayashi, D. Y., Guglielmoni, M., & Clarke, B. B. 1995. Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turfgrass. *Soil Biol Biochem,* 27 (11): 1479-1487.

Kocyan, A., Zhang, L. B., Schaefer, H., & Renner, S. S. 2007. A multi-locus chloroplast phylogeny for the Cucurbitaceae and its implications for character evolution and classification. *Mol Phylogenet Evol,* 44 (2): 553-577.

Kondo, T., Sawa, S., Kinoshita, A., Mizuno, S., Kakimoto, T., et al. 2006. A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. *Science,* 313 (5788): 845-848.

Kumar, S., Teotia, U. V. S., & Sanghi, A. 2013. Antioxidative property of cow milk caseinates hydrolyzed with different proteases. *Int J Pharm Sci,* 5: 975–1491.

Kunkel, B. N., & Brooks, D. M. 2002. Cross talk between signaling pathways in pathogen defense. *Curr Opin Plant Biol,* 5 (4): 325-331.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., et al. 2004. The N Terminus of bacterial Elongation Factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell,* 16 (12): 3496-3507.

Lachhab, N., Sanzani, S. M., Adrian, M., Chiltz, A., Balacey, S., et al. 2014. Soybean and casein hydrolysates induce grapevine immune responses and resistance against *Plasmopara viticola*. *Front Plant Sci,* 5: 716.

Lachhab, N., Sanzani, S. M., Bahouaoui, M. A., Boselli, M., & Ippolito, A. 2016. Effect of some protein hydrolysates against gray mould of table and wine grapes. *Eur J Plant Pathol,* 144: 821-830.

Lachhab, N., Sanzani, S. M., Fallanaj, F., Youssef, K., Nigro, F., et al. (2015). Protein hydrolysates as resistance inducers for controlling green mould of citrus fruit (1065 ed., pp. 1593-1598): International Society for Horticultural Science (ISHS), Leuven, Belgium.

Laquitaine, L., Gomes, E., Francois, J., Marchive, C., Pascal, S., et al. 2006. Molecular basis of ergosterol-induced protection of grape against *Botrytis cinerea*: induction of type I LTP promoter activity, WRKY, and stilbene synthase gene expression. *Molec Plant Microb Interact,* 19 (10): 1103-1112.

Lattanzio, V., Lattanzio, V. M. T., & Cardinali, A. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. In: F. Imperato (Ed.), In *Phytochemistry: Advances in research.* pp. 23-67.

Leahy, J., Mendelsohn, M., Kough, J., Jones, R., & Berckes, N. (2014). Biopesticide oversight and registration at the U.S. Environmental Protection Agency. In *Biopesticides: state of the art and future opportunities.* Washington DC: ACS Symposium Series, American Chemical Society.

Lebeda, A., Křístková, E., Sedláková, B., McCreight, J. D., & Coffey, M. D. 2016. Cucurbit powdery mildews: methodology for objective determination and denomination of races. *Eur J Plant Pathol,* 144 (2): 399-410.

Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R., & Pugin, A. 2002. Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell,* 14 (10): 2627-2641.

Leveau, J. H. J., & Lindow, S. E. 2001. Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proc Natl Acad Sci U S A,* 98 (6): 3446-3453.

Levitan, L. 2000. “How to” and “why”:: assessing the enviro–social impacts of pesticides. *Crop Prot,* 19 (8): 629-636.

Ligon, J. M., Hill, D. S., Hammer, P. E., Torkewitz, N. R., Hofmann, D., et al. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Manag Sci,* 56 (8): 688-695.

Lindow, S. E., & Brandl, M. T. 2003. Microbiology of the phyllosphere. *Appl Environ Microbiol,* 69 (4): 1875-1883.

Lisiecka, J., Knaflewski, M., Spizewski, T., Frąszczak, B., Kałuzewicz, A., et al. 2011. The effect of animal protein hydrolysate on quantity and quality of strawberry daughter plants cv. 'Elsanta'. *Acta Sci Pol Hortoru* 10 (1): 31-40.

Liu, G., Ji, Y., Bhuiyan, N. H., Pilot, G., Selvaraj, G., et al. 2010. Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in *Arabidopsis*. *Plant Cell,* 22 (11): 3845-3863.

Liu, X. Q., Ko, K. Y., Kim, S. H., & Lee, K. S. 2007. Effect of amino acid fertilization on nitrate assimilation of leafy radish and soil chemical properties in high nitrate soil. *Commun Soil Sci Plant Anal,* 39 (1-2): 269-281.

Looser, R., Froescheis, O., Cailliet, G. M., Jarman, W. M., & Ballschmiter, K. 2000. The deep-sea as a final global sink of semivolatile persistent organic pollutants? Part II: organochlorine pesticides in surface and deep-sea dwelling fish of the North and South Atlantic and the Monterey Bay Canyon (California). *Chemosphere,* 40 (6): 661-670.

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., et al. 2015. The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci Hortic,* 182: 124-133.

Ma, Y., Zhao, Y., Walker, R. K., & Berkowitz, G. A. 2013. Molecular steps in the immune signaling pathway evoked by plant elicitor peptides: Ca^{2+}-dependent protein kinases, nitric oxide, and reactive oxygen species are downstream from the early Ca^{2+} signal. *Plant Physiol,* 163 (3): 1459-1471.

Mahmoud, M. I. 1994. Physicochemical and functional-properties of protein hydrolysates in nutritional products. *Food Technol,* 48: 89-95.

Maini, P. 2006. The experience of the first biostimulant, based on aminoacids and peptides: a short retrospective review on the laboratory researches and the practical results. *Fertilitas Agrorum,* 1 (1): 29-43.

McGrath, M. T. 2001. Fungicide resistance in cucurbit powdery mildew: experiences and challenges. *Plant Dis,* 85 (3): 236-245.

Miller, R. N. G., Alves, G. S. C., & Van Sluys, M. A. 2017. Plant immunity: unravelling the complexity of plant responses to biotic stresses. *Ann Bot,* 119 (5): 681-687.

Mishina, T. E., & Zeier, J. 2007. Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in *Arabidopsis*. *Plant J,* 50 (3): 500-513.

Moe, L. A. 2013. Amino acids in the rhizosphere: from plants to microbes. *Am J Bot,* 100: 1692-1705.

Mohamed, N., Lherminier, J., Farmer, M. J., Fromentin, J., Beno, N., et al. 2007. Defense responses in grapevine leaves against *Botrytis cinerea* induced by application of a *Pythium oligandrum* strain or its elicitin, oligandrin, to roots. *Phytopathol,* 97 (5): 611-620.

Mucharromah, E., & Kuc, J. 1991. Oxalate and phosphates induce systemic resistance against diseases caused by fungi, bacteria and viruses in cucumber. *Crop Prot,* 10 (4): 265-270.

Mueller, T., & Ruppel, S. 2014. Progress in cultivation-independent phyllosphere microbiology. *FEMS Microbiol Ecol,* 87 (1): 2-17.

Musetti, R., Vecchione, A., Stringher, L., Borselli, S., Zulini, L., et al. 2006. Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. *Phytopathol,* 96 (7): 689-698.

Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., et al. 2004. The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. *Plant Physiol,* 135 (2): 1113-1128.

Nesler, A., Perazzolli, M., Puopolo, G., Giovannini, O., & Pertot, I. 2015. A complex protein hydrolysate acts as biogenic elicitor of plant resistance against powdery mildew. *Front Plant Sci,* 6: 715.

Newton, A. C., Gravouil, C., & Fountaine, J. M. 2010. Managing the ecology of foliar pathogens: ecological tolerance in crops. *Ann Appl Biol,* 157 (3): 343-359.

Nicholson, R. L., & Hammerschmidt, R. 1992. Phenolic-compounds and their role in disease resistance. *Annu Rev Phytopathol,* 30: 369-389.

Nuñez-Palenius, H. G., Hopkins, D., & Cantliffe, D. J. 2006. Powdery mildew of cucurbits in Florida. *IFAS Extensions - University of Florida,* HS1067: 1-9.

Oaks, A., Aslam, M., & Boesel, I. 1977. Ammonium and amino-acids as regulators of nitrate reductase in corn roots. *Plant Physiol,* 59: 391-394.

Pal, K. K., & McSpadden Gardener, B. 2006. Biological control of plant pathogens. *Plant Health Instr,* 10: 1094-1117.

Panyam, D., & Kilara, A. 1996. Enhancing the functionality of food proteins by enzymatic modification. *Trends Food Sci Tech,* 7: 120-125.

Paris, H. S. 2016. Germplasm enhancement of *Cucurbita pepo* (pumpkin, squash, gourd: Cucurbitaceae): progress and challenges. *Euphytica,* 208 (3): 415-438.

Parrado, J., Bautista, J., Romero, E. J., Garcia-Martinez, A. M., Friaza, V., et al. 2008. Production of a carob enzymatic extract: potential use as a biofertilizer. *Bioresour Technol,* 99 (7): 2312-2318.

Pearce, G., Strydom, D., Johnson, S., & Ryan, C. A. 1991. A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science,* 253 (5022): 895-897.

Pearce, G., Yamaguchi, Y., Barona, G., & Ryan, C. A. 2010. A subtilisin-like protein from soybean contains an embedded, cryptic signal that activates defense-related genes. *Proc Natl Acad Sci U S A,* 107: 14921-14925.

Pearce, G., Yamaguchi, Y., Munske, G., & Ryan, C. A. 2008. Structure-activity studies of *At*Pep1, a plant peptide signal involved in the innate immune response. *Peptides,* 29 (12): 2083-2089.

Pecha, J., Fuerst, T., Kolomaznik, K., Friebrova, V., & Svoboda, P. 2012. Protein biostimulant foliar uptake modeling: the impact of climatic conditions. *AIChE J,* 58: 2010-2019.

Penuelas, J., & Terradas, J. 2014. The foliar microbiome. *Trends Plant Sci,* 19 (5): 278-280.

Perazzolli, M., Dagostin, S., Ferrari, A., Elad, Y., & Pertot, I. 2008. Induction of systemic resistance against *Plasmopara viticola* in grapevine by *Trichoderma harzianum* T39 and benzothiadiazole. *Biol Control,* 47 (2): 228-234.

Perazzolli, M., Moretto, M., Fontana, P., Ferrarini, A., Velasco, R., et al. 2012. Downy mildew resistance induced by *Trichoderma harzianum* T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. *BMC Genomics,* 13 (1): 660.

Perazzolli, M., Roatti, B., Bozza, E., & Pertot, I. 2011. *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. *Biol Control,* 58 (1): 74-82.

Perez-Garcia, A., Romero, D., Fernandez-Ortuno, D., Lopez-Ruiz, F., De Vicente, A., et al. 2009. The powdery mildew fungus *Podosphaera fusca* (synonym *Podosphaera xanthii*), a constant threat to cucurbits. *Mol Plant Pathol,* 10 (2): 153-160.

Petrozza, A., Santaniello, A., Summerer, S., Di Tommaso, G., Di Tommaso, D., et al. 2014. Physiological responses to Megafol (R) treatments in tomato plants under drought stress: a phenomic and molecular approach. *Sci Hortic,* 174: 185-192.

Phelan, M., Aherne, A., FitzGerald, R. J., & O'Brien, N. M. 2009. Casein-derived bioactive peptides: biological effects, industrial uses, safety aspects and regulatory status. *Int Dairy J,* 19 (11): 643-654.

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S., & Van Wees, S. C. M. 2009. Networking by small-molecule hormones in plant immunity. *Nat Chem Biol,* 5 (5): 308-316.

Pieterse, C. M. J., & van Loon, L. C. 1999. Salicylic acid-independent plant defence pathways. *Trends Plant Sci,* 4 (2): 52-58.

Pieterse, C. M. J., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., et al. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell,* 10 (9): 1571-1580.

Pieterse, C. M. J., vanWees, S. C. M., Hoffland, E., vanPelt, J. A., & vanLoon, L. C. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell,* 8 (8): 1225-1237.

Pimentel, D., & Greiner, A. (1997). Environmental and socio-economic costs of pesticide use. In: D. Pimentel (Ed.), In *Techniques for reducing pesticide use: economic and environmental benefits.* pp. 51-78. Chichester: John Wiley & Sons.

Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., et al. 2014. Unravelling the diversity of grapevine microbiome. *PLoS ONE,* 9 (1): e85622.

Puopolo, G., Giovannini, O., & Pertot, I. 2014. *Lysobacter capsici* AZ78 can be combined with copper to effectively control *Plasmopara viticola* on grapevine. *Microbiol Res,* 169 (7-8): 633-642.

Qiao, F., Chang, X.-L., & Nick, P. 2010. The cytoskeleton enhances gene expression in the response to the harpin elicitor in grapevine. *J Exp Bot,* 61 (14): 4021-4031.

Rastogi, G., Coaker, G. L., & Leveau, J. H. J. 2013. New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. *FEMS Microbiol Lett,* 348 (1): 1-10.

Repka, V. 2001. Elicitor-stimulated induction of defense mechanisms and defense gene activation in grapevine cell suspension cultures. *Biol Plant,* 44 (4): 555-565.

Reuveni, M., Agapov, V., & Reuveni, R. 1993. Induction of systemic resistance to powdery mildew and growth increase in cucumber by phosphates. *Biol Agric Hortic,* 9 (4): 305-315.

Reuveni, M., & Reuveni, R. 2000. Prior inoculation with non-pathogenic fungi induces systemic resistance to powdery mildew on cucumber plants. *Eur J Plant Pathol,* 106 (7): 633-638.

Reuveni, R., Dor, G., Raviv, M., Reuveni, M., & Tuzun, S. 2000. Systemic resistance against *Sphaerotheca fuliginea* in cucumber plants exposed to phosphate in hydroponics system, and its control by foliar spray of mono-potassium phosphate. *Crop Prot,* 19 (5): 355-361.

Ritpitakphong, U., Falquet, L., Vimoltust, A., Berger, A., Metraux, J. P., et al. 2016. The microbiome of the leaf surface of *Arabidopsis* protects against a fungal pathogen. *New Phytol,* 210 (3): 1033-1043.

Roberti, R., Galletti, S., Burzi, P. L., Righini, H., Cetrullo, S., et al. 2015. Induction of defence responses in zucchini (*Cucurbita pepo*) by *Anabaena* sp water extract. *Biol Control,* 82: 61-68.

Romero, D., de Vicente, A., Olmos, J. L., Davila, J. C., & Perez-Garcia, A. 2007. Effect of lipopeptides of antagonistic strains of *Bacillus subtilis* on the morphology and

ultrastructure of the cucurbit fungal pathogen *Podosphaera fusca*. *J Appl Microbiol,* 103 (4): 969-976.

Romero, D., Pérez-García, A., Rivera, M. E., Cazorla, F. M., & de Vicente, A. 2004. Isolation and evaluation of antagonistic bacteria towards the cucurbit powdery mildew fungus *Podosphaera fusca*. *Appl Microbiol Biotechnol,* 64 (2): 263-269.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., et al. 1996. Systemic acquired resistance. *Plant Cell,* 8 (10): 1809-1819.

Ryan, C. A., Pearce, G., Scheer, J., & Moura, D. S. 2002. Polypeptide hormones. *Plant Cell,* 14: S251-S264.

Schaafsma, G. 2009. Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. *Eur J Clin Nutr,* 63 (10): 1161-1168.

Schaller, A. 2001. Bioactive peptides as signal molecules in plant defense, growth, and development. *Stud Nat Prod Chem,* 25: 367-411.

Schaller, A., & Ryan, C. A. 1996. Systemin – a polypeptide defense signal in plants. *Bioessays,* 18 (1): 27-33.

Scheideler, M., Schlaich, N. L., Fellenberg, K., Beissbarth, T., Hauser, N. C., et al. 2002. Monitoring the switch from housekeeping to pathogen defense metabolism in *Arabidopsis thaliana* using cDNA arrays. *J Biol Chem,* 277: 10555-10561.

Schiavon, M., Ertani, A., & Nardi, S. 2008. Effects of an alfalfa protein hydrolysate on the gene expression and activity of enzymes of the tricarboxylic acid (TCA) cycle and nitrogen metabolism in Zea mays L. *J Agric Food Chem,* 56 (24): 11800-11808.

Schmelz, E. A., LeClere, S., Carroll, M. J., Alborn, H. T., & Teal, P. E. A. 2007. Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory. *Plant Physiol,* 144: 793-805.

Schulz, R., Thiere, G., & Dabrowski, J. M. 2002. A combined microcosm and field approach to evaluate the aquatic toxicity of azinphosmethyl to stream communities. *Environ Toxicol Chem,* 21 (10): 2172-2178.

Shah, J. 2005. Lipids, lipases, and lipid-modifying enzymes in plant disease resistance. *Annu Rev Phytopathol,* 43: 229-260.

Sharpley, A. N., McDowell, R. W., & Kleinman, P. J. A. 2001. Phosphorus loss from land to water: integrating agricultural and environmental management. *Plant and Soil,* 237 (2): 287-307.

Shi, Z., Wang, F., Zhou, W., Zhang, P., & Fan, Y. J. 2007. Application of osthol induces a resistance response against powdery mildew in pumpkin leaves. *Int J Molec Sci,* 8 (9): 1001-1012.

Sinha, R., Radha, C., Prakash, J., & Kaul, P. 2007. Whey protein hydrolysate: functional properties, nutritional quality and utilization in beverage formulation. *Food Chem,* 101: 1484-1491.

Skevas, T., Lansink, A. G. J. M. O., & Stefanou, S. E. 2013. Designing the emerging EU pesticide policy: a literature review. *NJAS Wagening J Life Sci,* 64-65: 95-103.

Smalling, K. L., Fellers, G. M., Kleeman, P. M., & Kuivila, K. M. 2013. Accumulation of pesticides in pacific chorus frogs (Pseudacris regilla) from California's Sierra Nevada Mountains, USA. *Environ Toxicol Chem,* 32 (9): 2026-2034.

Spellman, D., McEvoy, E., O'Cuinn, G., & FitzGerald, R. J. 2003. Proteinase and exopeptidase hydrolysis of whey protein: comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *Int Dairy J,* 13: 447-453.

Spoel, S. H., & Dong, X. N. 2012. How do plants achieve immunity? Defence without specialized immune cells. *Nat Rev Immunol,* 12 (2): 89-100.

Stiegler, J. C., Richardson, M. D., Karcher, D. E., Roberts, T. L., & Norman, R. J. 2013. Foliar absorption of various inorganic and organic nitrogen sources by creeping bentgrass. *Crop Sci,* 53: 1148-1152.

Storck, V., Karpouzas, D. G., & Martin-Laurent, F. 2017. Towards a better pesticide policy for the European Union. *Sci Total Environ,* 575 (C): 1027-1033.

Subbarao, S., Aftab Hussain, I., & Ganesh, P. 2015. Biostimulant activity of protein hydrolysate: influence on plant growth and yield. *J Plant Sci Res,* 2 (2): 125.

Terral, J. F., Tabard, E., Bouby, L., Ivorra, S., Pastor, T., et al. 2010. Evolution and history of grapevine (*Vitis vinifera*) under domestication: new morphometric perspectives to understand seed domestication syndrome and reveal origins of ancient european cultivars. *Ann Bot,* 105 (3): 443-455.

Thuerig, B., Slaughter, A., Marouf, E., Held, M., Mauch-Mani, B., et al. 2011. Site-specific field resistance of grapevine to *Plasmopara viticola* correlates to altered gene expression and was not modulated by the application of organic amendments. *Eur J Plant Pathol,* 129 (2): 255-265.

Tilman, D. 1999. Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices. *Proc Natl Acad Sci U S A,* 96 (11): 5995-6000.

Trdà, L., Fernandez, O., Boutrot, F., Heloir, M. C., Kelloniemi, J., et al. 2014. The grapevine flagellin receptor VvFLS2 differentially recognizes flagellin-derived epitopes from the endophytic growth-promoting bacterium *Burkholderia phytofirmans* and plant pathogenic bacteria. *New Phytol,* 201 (4): 1371-1384.

Trotel-Aziz, P., Couderchet, M., Biagianti, S., & Aziz, A. 2008. Characterization of new bacterial biocontrol agents *Acinetobacter*, *Bacillus*, *Pantoea* and *Pseudomonas spp.* mediating grapevine resistance against *Botrytis cinerea*. *Environ Exp Bot,* 64 (1): 21-32.

Trotel-Aziz, P., Couderchet, M., Vernet, G., & Aziz, A. 2006. Chitosan stimulates defense reactions in grapevine leaves and inhibits development of Botrytis cinerea. *Eur J Plant Pathol,* 114 (4): 405-413.

Trouvelot, S., Héloir, M.-C., Poinssot, B., Gauthier, A., Paris, F., et al. 2014. Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays. *Front Plant Sci,* 5: 592.

Trouvelot, S., Varnier, A. L., Allegre, M., Mercier, L., Baillieul, F., et al. 2008. A beta-1,3 glucan sulfate induces resistance in grapevine against *Plasmopara viticola* through priming of defense responses, including HR-like cell death. *Mol Plant Microbe Interact,* 21 (2): 232-243.

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J. D., & Katagiri, F. 2008. Interplay between MAMP-triggered and SA-mediated defense responses. *Plant J,* 53 (5): 763-775.

van Aubel, G., Buonatesta, R., & Van Cutsem, P. 2014. COS-OGA: a novel oligosaccharidic elicitor that protects grapes and cucumbers against powdery mildew. *Crop Prot,* 65: 129-137.

van der Ent, S., Koornneef, A., Ton, J., & Pieterse, C. M. J. (2009). Induced Resistance – orchestrating defence mechanisms through crosstalk and priming. In *Annual Plant Reviews: Molecular Aspects of Plant Disease Resistance.* pp. 334-370: Wiley-Blackwell.

van Emden, H. F., & Service, M. W. (2004). *Pest and vector control*. Cambridge, UK: Cambridge University Press.

van Loon, L. C., Rep, M., & Pieterse, C. M. J. 2006. Significance of inducible defense-related proteins in infected plants. *Ann Rev Phytopathol,* 44: 135-162.

van Wees, S. C. M., de Swart, E. A. M., van Pelt, J. A., van Loon, L. C., & Pieterse, C. M. J. 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A,* 97 (15): 8711-8716.

van Wees, S. C. M., Van der Ent, S., & Pieterse, C. M. J. 2008. Plant immune responses triggered by beneficial microbes. *Curr Opin Plant Biol,* 11 (4): 443-448.

Vandenackerveken, G., Vossen, P., & Dewit, P. 1993. The avr9 race-specific elicitor of *Cladosporium fulvum* is processed by endogenous and plant proteases. *Plant Physiol,* 103 (1): 91-96.

Varnier, A. L., Sanchez, L., Vatsa, P., Boudesocque, L., Garcia-Brugger, A., et al. 2009. Bacterial rhamnolipids are novel MAMPs conferring resistance to *Botrytis cinerea* in grapevine. *Plant Cell Environ,* 32 (2): 178-193.

Villaverde, J. J., Sevilla-Moran, B., Sandin-Espana, P., Lopez-Goti, C., & Alonso-Prados, J. L. 2014. Biopesticides in the framework of the European Pesticide Regulation (EC) No. 1107/2009. *Pest Manag Sci,* 70 (1): 2-5.

Vorholt, J. A. 2012. Microbial life in the phyllosphere. *Nat Rev Microbiol,* 10 (12): 828-840.

Ward, J. L., Forcat, S., Beckmann, M., Bennett, M., Miller, S. J., et al. 2010. The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. tomato. *Plant J,* 63: 443-457.

Whipps, J. M., Hand, P., Pink, D., & Bending, G. D. 2008. Phyllosphere microbiology with special reference to diversity and plant genotype. *J Appl Microbiol,* 105 (6): 1744-1755.

Wiesel, L., Newton, A. C., Elliott, I., Booty, D., Gilroy, E. M., et al. 2014. Molecular effects of resistance elicitors from biological origin and their potential for crop protection. *Front Plant Sci,* 5: 655.

Wu, S., Shan, L., & He, P. 2014. Microbial signature-triggered plant defense responses and early signaling mechanisms. *Plant Sci,* 228: 118-126.

Yalpani, N., Silverman, P., Wilson, T. M., Kleier, D. A., & Raskin, I. 1991. Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell,* 3 (8): 809-818.

Yamaguchi, Y., Barona, G., Ryan, C. A., & Pearce, G. 2011. GmPep914, an eight-amino acid peptide isolated from soybean leaves, activates defense-related genes. *Plant Physiol,* 156 (2): 932-942.

Yamaguchi, Y., & Huffaker, A. 2011. Endogenous peptide elicitors in higher plants. *Curr Opin Plant Biol,* 14 (4): 351-357.

Zeighaminejad, R., Sharifi-Sirchi, G. R., Mohamadi, H., & Aminai, M. M. 2016. Induction of resistance against powdery mildew by Beta-aminobutyric acid in squash. *J Appl Bot Food Qual,* 89: 176-182.

Zhang, S., Mersha, Z., Vallad, G. E., & Huang, C. H. 2016. Management of powdery mildew in squash by plant and alga extract biopesticides. *Plant Pathol J,* 32 (6): 528-536.

Zhang, S. A., Vallad, G. E., White, T. L., & Huang, C. H. 2011. Evaluation of microbial products for management of powdery mildew on summer squash and cantaloupe in Florida. *Plant Dis,* 95 (4): 461-468.

Zhang, W. J., Jiang, F. B., & Ou, J. F. 2011. Global pesticide consumption and pollution: with China as a focus. *Proc Int Acad Ecol Environ Sci,* 1 (2): 125-144.

Zipfel, C. 2014. Plant pattern-recognition receptors. *Trends Immunol,* 35 (7): 345-351.

Funding

The current doctoral research has been carried out in agreement with Fondazione Edmund Mach (San Michele all'Adige, Trento, IT).

Specifically, the project published as "*Leaf treatments with a protein-based resistance inducer partially modify phyllosphere microbial communities of grapevine*" received funding from the EU´s Seventh Framework Programme for research, technological development and demonstration under grant agreements No. 289497 (project CO-FREE, theme KBBE.2011.1.2-06), No. 265865 (project PURE, theme FP7-KBBE-2010-4) and No. 324416 (project INNOVA, theme FP7-PEOPLE-2012-IAPP). Furthermore, the project published as "*The effect of hydrolysis and protein source on the efficacy of protein hydrolysates as plant resistance inducers against powdery mildew*" has received funding from the EU´s Seventh Framework Programme for research, technological development and demonstration under grant agreement No. 324416 (project INNOVA, theme FP7-PEOPLE-2012-IAPP) and from the EU's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 722642 (project INTERFUTURE).

Ringraziamenti

Ringrazio il mio Supervisor Prof.ssa **Ilaria Pertot** per la sua fiducia, per gli spunti di ragionamento e per aver ascoltato le mie difficoltà durante questi anni. Grazie per non avermi fatto pressione quando le cose non andavano nel verso giusto. Ringrazio il mio tutor Dott. **Michele Perazzolli** per il supporto al mio progetto e le sue indicazioni.

Ringrazio il Prof. **Giuseppe Colla** dell'Università della Tuscia e il Prof. **Aziz Aziz** de l'Université de Reims Champagne-Ardenne per aver accettato di revisionare la mia tesi di Dottorato. Un ringraziamento anche alla Dott.ssa **Marta Martini** ed al Dott. **Paolo Ermacora** dell'Università di Udine per il loro impegno come commissari interni durante la fase di pre-difesa.

Ringrazio il Prof. **Giuseppe Firrao** dell'Università di Udine per aver accettato, qualche mese fa, l'incarico di Coordinatore del Dottorato in Agraria, apportando cambiamenti significativi anche a supporto dei candidati che, come me, lavorano a tempo pieno alla Fondazione E. Mach di Trento e non possono essere in contatto costante con l'Università di Udine e la sua burocrazia.

Ringrazio il team del reparto Development di **Belchim Crop Protection** in Londerzeel, Belgio per avermi fornito, durante i 6 mesi di tirocinio, spunti interessanti per il mio progetto, ed avermi permesso di conoscere la loro realtà aziendale.

Grazie alla mia **famiglia** e a Gianpaolo per il fondamentale supporto economico che spesso ha accontentato anche i miei vizi, e grazie in particolare a Marisa per aver accolto i miei piagnistei da eterna studentessa. Grazie anche a Flavia ed alla sua famiglia per esserci sempre stati.

Grazie ai miei **colleghi FEM** per l'aiuto, gli scambi e le chiacchiere, in particolare a (in ordine casuale) Gabriella, Valentina, Lidia, Daniele, Selena, Elena, Giulia, Livia, Claudia, Francesca, Carmela, Luisa, Franca, Sara, Tiziana, Christian, Federico, Silvia. A tutti voi la mia gratitudine ed il mio affetto. Un pensiero speciale va ad Oscar, per l'aiuto negli esperimenti, e per le poche ma efficaci parole nei momenti più bui.
Non posso dimenticare Riccardo e la sua insostituibile presenza.

Grazie **Gianluca**, per come mi hai sostenuto e supportato durante questi anni, e per come hai affrontato assieme a me i momenti di soddisfazione e gioia, e pure quelli di frustrazione e smarrimento. So di averti chiesto molto impegno. Grazie per la tua infinita pazienza e generosità, e per avermi insegnato a vedere sempre il bicchiere mezzo pieno. Grazie per non avere mai smesso di crederci, nonostante i 600 km da riempire in ognuno di questi 1000 e più giorni passati.

Ad meliora et maiora semper!

Un sentito grazie a tutti!

Udine, Febbraio 2018