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Review Article

Proteomics of Plant Pathogenic Fungi

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Plant pathogenic fungi cause important yield losses in crops. In order to develop efficient and environmental friendly crop protection strategies, molecular studies of the fungal biological cycle, virulence factors, and interaction with its host are necessary. For that reason, several approaches have been performed using both classical genetic, cell biology, and biochemistry and the modern, holistic, and high-throughput, omic techniques. This work briefly overviews the tools available for studying Plant Pathogenic Fungi and is amply focused on MS-based Proteomics analysis, based on original papers published up to December 2009. At a methodological level, different steps in a proteomic workflow experiment are discussed. Separate sections are devoted to fungal descriptive (intracellular, subcellular, extracellular) and differential expression proteomics and interactomics. From the work published we can conclude that Proteomics, in combination with other techniques, constitutes a powerful tool for providing important information about pathogenicity and virulence factors, thus opening up new possibilities for crop disease diagnosis and crop protection.

1. Introduction: Plant Parasitic Fungi

Fungi form a large and heterogeneous eukaryotic group of living organisms characterized by their lack of photosynthetic pigment and their chitinous cell wall. It has been estimated that the fungal kingdom contains more than 1.5 million species, but only around 100,000 have so far been described, with yeast, mold, and mushroom being the most familiar [1]. Although the majority of fungal species are saprophytes, a number of them are parasitics, in order to complete their biological cycle, animals or plants, with around 15,000 of them causing disease in plants, the majority belonging to the Ascomycetes and Basidiomycetes [2] (Table 1). Within a fungal plant pathogen species, for example, in *Fusarium oxysporum*, up to 120 different formae specialis can be found based on specificity to host species belonging to a wide range of plant families [3].

According to the type of parasitism and infection strategy, fungi are classified as biotrophic (e.g., *Blumeria graminis*), necrotophic (e.g., *Botrytis cinerea*), or hemibiotrophic (e.g., *Colletotrichum destructivum*). While the former derives nutrients from dead cells, the latter takes nutrients from the plant but does not kill it [4]. Hemibiotrophes sequentially deploy a biotrophic and then a necrotrophic mode of nutrition. Necrotrophic species tend to attack a broad range of plant species; on the contrary, biotrophes usually exhibit a high degree of specialization for individual plant species. Most biotrophic fungi are obligatory parasites, surviving only limited saprophytic phases. Differently from necrotrophes, the cultivation of biotrophic fungi succeeds only in a few exceptions, for example, *Podosphaera fusuca* [5] or *B. graminis* (M. M. Corbitt, personal communication, adapted from [5]).

Fungal diseases are, in nature, more the exception than the rule. Thus, only a limited number of fungal species are able to penetrate and invade host tissues, avoiding recognition and plant defence responses, in order to obtain nutrients from them, causing disease and sometimes host death. In agriculture, annual crop losses due to pre- and post harvest fungal diseases exceed 200 billion euros, and, in the United Stated alone, over $600 million are annually spent on fungicides [6].

Table 1: Main plant pathogenic fungi causing disease in plants.

Phylum	Genus	Anamorphic stage	Hosts	Disease	Example
Chytridiomycota	Olpidium		cabbage	root diseases	*O. brassicae*
	Physoderma		corn	brown spot	*P. maydis*
			alfalfa	crown wart	*P. (= Urophlyctis) alfalfae*
	Synchytrium		potato	potato wart	*S. endobioticum*
Zygomycota	Rhizopus		fruits and vegetables	bread molds and soft rot	*R. oligosporus*
	Choanephora		squash	soft rot	*C. cucurbitarum*
	Mucor		fruits and vegetables	bread mold and storage rots	*M. indicus*
Ascomycota	Taphrina		peach plum oak	leaf curl leaf blister and so forth	*T. deformans*
	Galactomyces		citrus	sour rot	*G. candidum*
	Blumeria		cereals and grasses	powdery mildew	*B. graminis*[1]
	Erysiphe	Oidium	many herbaceous plants	powdery mildew	*E. pisi*
	Leveillula		tomato	powdery mildew	*L. taurica*
	Microsphaera		lilac	powdery mildew	*M. penicullata*
	Oidium		tomato	powdery mildew	*O. neolycopersici*
	Podosphaera		apple	powdery mildew	*P. leucotricha*
	Sphaerotheca		roses and peach	powdery mildew	*S. pannosa*
	Uncinula		grape	powdery mildew	*U. necator*
	Nectria		trees	twig and stem cankers	*N. galligena*
	Gibberella		corn and small grains	foot or stalk rot	*F. graminearum*[1]
		Fusarium	several plants	vascular wilts root rots stem rots seed infections	F. oxysporum[1]
	Claviceps		grain crops	ergot	*C. purpurea*
	Ceratocystis	Chalara	oak	oak wilt	*C. fagacearum*
			stone fruit and sweet potato	cankers and root rot	*C. fimbriata*
			pineapple	butt rot	*C. paradoxa*
	Monosporascus		cucurbits	root rot and collapse	*M.* cannonballus
	Glomerella		apple	anthracnoses and bitter rot	*G. cingulata*
		Colletotrichum	many plants	anthracnoses	*C. lindemuthianum*
	Phyllachora		grasses	leaf spots	*P. graminis*
	Ophiostoma	Sporothrix and Graphium	elm	Dutch elm disease	*O. novo*-ulm
	Diaporthe			citrus melanose	*D. citri*
				eggplant fruit rot	*D. vexans*
				soybean pod and stem rot	*D. phaseolorum*
	Gaeumannomyces		grain crops and grasses	take-all disease	*G. graminis*
	Magnaporthe		rice	rice blast	*M. grisea*[1]

Table 1: Continued.

Phylum	Genus	Anamorphic stage	Hosts	Disease	Example
	Cryphonectria		chestnut	blight disease	*C. parasitica*
	Leucostoma		peach and other trees	canker diseases	*L. persoonii*
	Hypoxylon		poplars	canker disease	*H. mammatum*
	Rosellinia		fruit trees and vines	root diseases	*R. necatrix*
	Xylaria		trees	tree cankers and wood decay	*X. longipes*
	Eutypa		fruit trees and vines	canker	*E. armeniacae*
	Mycosphaerella	Cercospora	Banana	Sigatoka disease	*M. musicola and M. fijiensis*
		Septoria	cereals and grasses	leaf spots	*M. graminicola*
			strawberry	leaf spot	*M. fragariae*
	Elsinoë		citrus trees	citrus scab	*E. fawcetti*
			grape	anthracnose	*E. ampelina*
			raspberry	anthracnose	*E. veneta*
	Capnodium		most plants	sooty molds	*C. elaeophilum*
	Cochliobolus	Bipolaris	grain crops and grasses	leaf spots and root rots	*C. carbonum and B. maydis*
		Curvularia	grasses	leaf spots	*C. lunata*[1]
	Pyrenophora	Drechslera	cereals and grasses	leaf spots	*P. graminea*
	Setosphaera		cereals and grasses	leaf spots	*S. turcica*
	Pleospora	Stemphylium	tomato	black mold rot	*P. lycopersici and S. solani*
	Leptosphaeria		cabbage	black leg and foot rot	*L. maculans*[1]
	Venturia		apple	apple scab	*V. inaequalis*
			pear	pear scab	*V. pyrina*
		Cladosporium	tomato	leaf mold	*C. fulvum*
			peach and almond	scab	*C. carpophilum*
	Guignardia	Phyllosticta	grapes	black rot	*G. bidwellii*
	Apiosporina		cherries and plums	black knot	*A. morbosa*
	Hypoderma		pines	needle cast	*H. desmazierii*
	Lophodermium		pines	needle cast	*L. pinastri*
	Rhabdocline		pines	Douglas fir needle cast	*R. weirii*
	Rhytisma		maple	tar spot of leaves	*R. acerinum*
	Monilinia		stone fruit	brown rot disease	*M. fruticola*
	Sclerotinia		vegetables	white mold	*S. sclerotiorum*[1]
	Stromatinia		gladiolus	corm rot	*S. gladioli*
	Pseudopeziza		alfalfa	leaf spot	*P. trifolii*
	Diplocarpon		quince and pear	black spot	*D. maculatum*
	Talaromyces	Penicillium	fruits	blue mold rot	*P. digitatum*
		Aspergillus	seeds	bread mold and seed decays	*A. niger*[1]
	Hypocrea	Verticillium	many plants	vascular wilts	*V. dahliae*[1]
	Lewia	Alternaria	many plants	leaf spots and blights	*A. alternata*
	Setosphaera	Exserohilum	grasses	leaf spots	*E. longirostratum*
	Botryosphaeria	Sphaeropsis	apple	black rot	*S. pyriputrescens*
	Botryotinia	Botrytis	many plants	gray mold rots	*B. cinerea*[1]

Table 1: Continued.

Phylum	Genus	Anamorphic stage	Hosts	Disease	Example
	Monilinia	Monilia	stone fruits	brown rot	*M. fruticola*
	Diplocarpon	Entomosporium	pear	leaf and fruit spot	*E. mespili*
	Greeneria	Melanconium	grape	bitter rot	*M. fuligineum*
Basidiomycota	Ustilago		corn	smut	*U. maydis*[1]
			oats	loose smuts	*U. avenae*
			barley	loose smuts	*U. nuda*
			wheat	loose smuts	*U. tritici*
	Tilletia		wheat	covered smut or bunt	*T. caries*
			wheat	Karnal bunt	*T. indica*
	Urocystis		onion	smut	*U. cepulae*
	Sporisorium		sorghum	covered kernel smut	*S. sorghi*
			sorghum	loose sorghum smut	*S. cruentum*
	Sphacelotheca		sorghum	head smut	*S. reiliana*
	Cronartium		pines	blister rust	*C. ribicola*
	Gymnosporangium		apple	cedar-apple rust	*G. juniperi-virginianae*
	Hemileia		coffee	rust	*H. vastatrix*
	Melampsora		flax	rust	*M. lini*
	Phakopsora		soybeans	rust	*P. pachyrrhizi*
	Puccinia		cereals	rust	*P. recondita*
	Uromyces		beans	rust	*U. appendiculatus*[1]
	Exobasidium		ornamentals	leaf flower and stem galls	*E. japonicum*
	Athelia		many plants	Southern blight	*A. rolfsii*
		Sclerotium	onions	white rot	*S. cepivorum*
	Thanatephorus	Rhizoctonia	many plants	root and stem rots damping-off and fruit rots	*T. cucumeris and R.solani*
	Typhula		turf grasses	snow mold	*T. incarnata*
	Armillaria		trees	root rots	*A. mellea*
	Crinipellis		cacao	witches'-broom	*C. perniciosus*
	Marasmius		turf grasses	fairy ring disease	*M. oreades*
	Pleurotus		trees	white rot on logs tree stumps and living trees	*P. ostreatus*[1]
	Pholiota		trees	brown wood rot	*P. squarrosa*
	Chondrostereum		trees	silver leaf disease	*C. purpureum*
	Corticium		turf grasses	red thread disease	*C. fuciforme*
	Heterobasidion		trees	root and butt rot	*H. annosum*
	Ganoderma		trees	root and basal stem rots	*G. boninense*
	Inonotus		trees	heart rot	*I. hispidus*
	Polyporus		trees	heart rot	*P. glomeratus*
	Postia		trees	wood and root rot	*P. fragilis*

[1] These phytopathogenic fungi are named in the text.

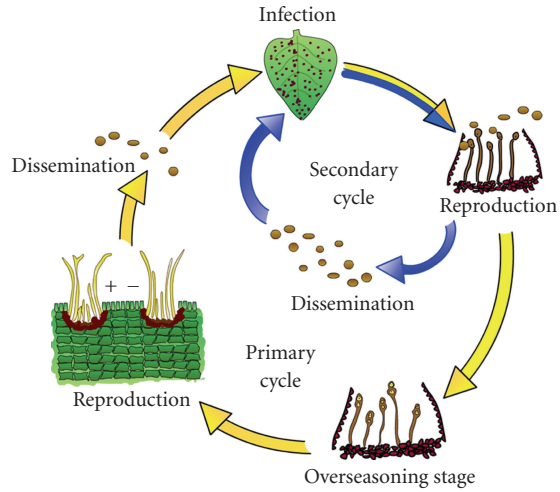

Figure 1: Diagram of monocyclic (yellow) and polycyclic (yellow and blue) fungi. In monocyclic diseases the fungus produces spores at the end of the season that serve as primary and only inoculum for the following year. The primary inoculum infects plants during the growth season and, at the end of the growth season, produces new spores in the infected tissues. These spores remain in the soil (overseasoning stage) and serve as the primary inoculum the following season. In polycyclic fungal pathogens, the primary inoculum often consists of the sexual (perfect) spore or, in fungi that lack the sexual stage, some other structures such as sclerotia, pseudosclerotia, or mycelium in infected tissue. This inoculum causes the primary infection and then large numbers of asexual spores (secondary inoculum) are produced at each infection site and these spores can themselves cause new (secondary) infections that produce more asexual spores for more infections.

Fungal pathogens have complicated life cycles, with both asexual and sexual reproduction, and stages involving the formation of different infective, vegetative, and reproductive structures [7]. The primary events in a disease cycle are establishment of infection, colonization (invasion), growth and reproduction, of the pathogen, dissemination of the pathogen, and survival of the pathogen in the absence of the host, that is, overwintering or oversummering (overseasoning) of the pathogen (Figure 1). However, the execution of each stage largely differs depending on the pathogen [8]. In polycyclic diseases there are several infection cycles within one, the so-called secondary cycles [3] (Figure 1).

The fungal plant interplay depends on mutual recognition, signalling, and the expression of pathogenicity and virulence factors, from the fungal side, and the existence of passive, preformed, or inducible defence mechanisms in the plant, resulting in compatible (susceptibility) or incompatible (nonhost, basal or host specific resistance) interactions. From a genetic point of view, and according to the gene-for-gene interaction hypothesis, proposed by Flor while studying flax rust [9], resistance results from the combination of a dominant avirulence (*Avr*) gene in the pathogen and a cognate resistance (*R*) gene in the host; the interaction of both gene products leads to the activation of host defence responses, such as the hypersensitive response, that arrests the growth of fungi. This hypothesis has been experimentally demonstrated for a number of pathosystems, mainly involving biotrophic fungi, with a number of avirulent genes identified [10].

A number of fungal mechanisms and molecules have been shown to contribute to fungal pathogenicity or virulence, understood as the capacity to cause damage in a host, in absolute or relative terms. Among them, cell wall degrading proteins, inhibitory proteins [11], and enzymes involved in the synthesis of toxins [12–15] are included. These virulence factors are typically involved in evolutionary arms races between plants and pathogens [16, 17].

Knowledge of the pathogenic cycle and that of virulence factors [18, 19] is crucial for designing effective crop protection strategies, including the development of resistant plant genotypes through classical plant breeding [20] or genetic engineering [21], fungicides [22], or the use of biological control strategies [23].

Studies of fungal pathogens and their interactions with plants have been performed using several approaches, from classical genetic, cell biology, and biochemistry [24–33], to the modern, holistic, and high-throughput omic techniques [34, 35] accompanied by proper bioinformatic tools [36]. In recent years, the study of fungal plant pathogens has been greatly promoted by the availability of their genomic sequences and resources for functional genomic analysis, including transcriptomics, proteomics, and metabolomics [37], which, in combination with targeted mutagenesis or transgenic studies, are unravelling molecular host-pathogen crosstalk, the complex mechanisms involving pathogenesis and host avoidance [38]. This review work makes an overview and summarizes the contribution of the most recent molecular techniques to the knowledge of phytopathogenic fungi biology and is mainly focused on the MS-based Proteomics approach.

2. From Structural to Functional Genomics

The importance of plant pathogenic fungi studies is underlined by the increasing number of fungal genome sequencing projects. Currently, over 40 fungal genomes

Table 2: Publicly available plant pathogenic fungal Genome sequences.

Phytopathogen Species[a]	URL
Ascomycota	
Dothydeomycetes	
Mycosphaerella fijensis (Banana black leaf streak)	http://genome.jgi-psf.org/Mycfi1/Mycfi1.home.html
Mycosphaerella graminicola (Wheat leaf blotch)	http://genome.jgi-psf.org/Mycgr1/Mycgr1.home.html
Pyrenophora tritici-repentis (Wheat disease)	http://www.broad.mit.edu/annotation/genome/pyrenophora_tritici_repentis/Home-html
Stagonospora nodorum (Wheat glume blotch)	http://www.broad.mit.edu/annotation/fungi/stagonospora_nodorum
	http://www.acnfp.murdoch.edu.au/Mission.htm
Eurotiomycetes	
Aspergillus flavus	http://www.aspergillusflavus.org/
Leotiomycetes	
Botrytis cinerea (Grape/other host grey rot) BO5.10	http://www.broad.mit.edu/annotation/fungi/botrytis_cinerea
T4	http://urgi.versailles.inra.fr/proyects/Botrytis/index.php
Sclerotinia sclerotium (Multi-host rot diseases)	http://www.broad.mit.edu/annotation/fungi/sclerotinia_sclerotium
Saccharomycetes	
Ashbya gossypii (Cotton/citrus fruits disease)	http://agd.vital-it.ch/index.html
Sordariomycetes	
Fusarium graminearum (Wheat/barley head blight)	http://www.broad.mit.edu/annotation/genome/fusarium_group
Fusarium oxysporum (Multi-host wilt disease)	http://www.broad.mit.edu/annotation/genome/fusarium_group
Fusarium verticillioides (Maize seed rot)	http://www.broad.mit.edu/annotation/genome/fusarium_group
Magnaporthe grisea (Rice blast disease)	http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/MultiHome.htlm
Nectria haematococca (Pea wilt)	http://genome.jgi-psf.org/Necha2/ Necha2.home.html
Verticillium dahliae VdLs.17 (Multi-host wilt)	http://www.broad.mit.edu/annotation/genome/verticillium_dahliae/MultiHome.html
Basidiomycota	
Pucciniomycetes	
Puccinia graminis (Cereal rusts)	http://www.broad.mit.edu/annotation/genome/puccinia_graminis
Ustilaginomycetes	
Ustilago maydis (Corn smut disease)	http://www.broad.mit.edu/annotation/fungi/ustilago_maydis

[a]Species are grouped by phylum and class. In parenthesis below the species's name and associated with each species, the most common or most widely recognized diseases are listed.

have been sequenced, 16 of which are phytopathogenic (Table 2), with more than 300 sequencing projects being in progress (Genomes Online database, http://www.genomesonline.org/). Sequence information, while valuable and a necessary starting point, is insufficient alone to answer questions concerning gene function, regulatory networks, and the biochemical pathways activated during pathogenesis. Based on the accumulation of a wealth of fungal genomic sequences, the traditional pursuit of a gene starting with a phenotype (forward genetics) has given way to the opposite situation where the gene sequences are known but not their functions. Thus, the challenge is now to decipher the function of the thousands of genes identified by genome projects, and reverse genetics methodologies are key tools in this endeavour [39].

Table 3: Original proteomics papers and reviews published on plant pathogenic fungi.

Fungus	Proteomic approach (reference)
Aspergillus ssp.	1-DE, MALDI-TOF-MS [40]
Aspergillus flavus	1-DE/2-DE, nanoLC-MS/MS [41]
	1-DE/2-DE, MALDI-TOF-MS [42]
Blumeria graminis f.sp. *hordei*	2-DE, MALDI-TOF/TOF-MS/MS [43]
	nanoLC-MS/MS [44]
	2-DE, MALDI-TOF/TOF-MS/MS, nanoLC-MS/MS, ESI-IT-MS/MS [45]
	2-DE, MALDI-TOF/TOF-MS/MS, ESI-IT-MS/MS [46]
Botrytis cinerea	2-DE, MALDI-TOF/TOF-MS/MS [47]
	1-DE, nanoLC MS/MS [48]
	1-DE, nanoLC MS/MS [49]
Curvularia lunata	2-DE, MALDI-TOF/TOF-MS/MS [50]
	1-DE/2-DE, nanoLC-Q-TOF-MS/MS [51]
Fusarium graminearum	1-DE, CID-LTQ-MS [52]
	2-DE, IT-MS/MS, iTRAQ-MS/MS [53]
	2-DE, ESI-MS/MS [54]
	Interactome [55]
Leptosphaeria maculans	1-DE, liquid-phase IEF, 2-DE [56]
Magnaporthe grisea	Interactome [57]
Phytophthora infestans	2-DE, MALDI-TOF-MS [58]
	2-DE, nanoLC-MS/MS [59]
Phytophthora palmivora	2-DE, MALDI-TOF-MS [58]
Phytophthora ramorum	HPLC-ESI-Q-TOF-nanoLC-MS/MS [60]
Pyrenophora tritici-repentis	2-DE, ESI-Q-TOF-MS/MS [61]
Pleorotus ostreatus	1-DE, MALDI-TOF-MS, ESI-Q-TOF-MS/MS/*de novo* sequencing [62]
Rhizoctonia solani	2-DE, MALDI-TOF-MS [63]
Sclerotinia sclerotiorum	2-DE, ESI-Q-TOF-nanoLC-MS/MS [64]
Stagnospora nodorum	2 -DE, LC-MS/MS [65]
	2-D LC-MALDI-MS/MS [66]
Uromyces appendiculatus	MudPit-MS/MS [67]
Ustilago maydis	2-DE, MALDI-TOF-MS, nanoLC-Q-TOF-MS/MS [68]

The study of gene function in filamentous fungi has made great advances in recent years [69]. Some of the techniques used in high-throughput reverse genetics approaches are targeted gene disruption/replacement (knock-out) [70], gene silencing (knock-down) [71], insertional mutagenesis [72], or targeting induced local lesions in genomes (TILLING) [73] (for review and examples see [39]). Thus, a number of pathogenicity factors have been targeted [74–76], and among them, several signaling pathways such as the cAMP and a mitogen activated protein kinase (MAPK) pathways have been shown to be crucial to virulence in several phytopathogenic fungi [77–82]. Random insertional mutagenesis is an excellent approach for dissecting complex biological traits, such as pathogenicity, because it does not require any prior information or assumptions on gene function. Recently, transposable elements (TEs) have been used for insertional mutagenesis and large-scale transposon mutagenesis has been developed as a tool for the genome-wide identification of virulence determinants in *F. oxysporum* [83].

Otherwise, transcriptomics, the global analysis of gene expression at the mRNA level, is also an attractive method for analyzing the molecular basis of fungal-plant interactions and pathogenesis [84–87]. For understanding the transcriptional activation or repression of genes during the infection process tools such as Differential Display (DD) [88], cDNA-Amplified Fragment-Length Polymorphism (cDNA-AFLP) [89], Suppression Subtractive Hybridization (SSH) [90], Serial Analyse of Gene Expression (SAGE) [91], expressed sequence tags (ESTs) [92], or DNA microarrays [91] have been developed in addition to older techniques such as Northern blotting, and they are reviewed in [84, 93].

3. Proteomics

Until the early 1990s most biological research was focused on the in vitro studies of individual components in which genes and proteins were investigated one at a time. This

strategy shifted in the early and mid 1990s to in vivo and molecular large-scale research, starting with structural genomics and transcriptomics research projects, then moving to proteomics, and recently to metabolomics. All these together constitute the methodological bases of the Modern Systems Biology [37, 94]. Since no single approach can fully clear up the complexity of living organisms, each approach does contribute and must be validated, this being considered as part of a multidisciplinary integrative analysis at different levels, extending from the gene to the phenotype through proteins and metabolites.

Within the "-omics" techniques, Proteomics constitutes, nowadays, priority research for any organism and configures a fundamental discipline in the postgenomic era. It is true that, at 2010, the realities are below the expectations originally generated and that the results gained over the last 15 years have shown that the dynamism, variability, and behaviour of proteins are more complex than had ever been imagined, especially as refers to a number of protein species per gene as a result of alternative splicing, reading frame, and posttranslational modifications, trafficking, and interactions, and considering that protein complexes, rather than individual proteins, are the functional units of the biological machines. However, and differently from other biological systems, mainly yeast [95] and humans [96], the full potential of proteomics is far from being fully exploited in fungal pathogen research, as refers to the low number of fungal pathogen species under investigation at the proteomic level, the low proteome coverage in those species investigated, and the almost unique use of classical, first generation techniques, those based on 2-DE coupled to MS.

The term proteomics was coined by Marc Wilkins, back during the 1994 Siena Meeting, to simply refer to the "PROTein complement of a genOME" [97]. Fifteen years later proteomics has become more than just an appendix of genomics or an experimental approach but a complex scientific discipline dealing with the study of the cell proteome. In the broadest sense, the proteome can be defined as being the total set of protein species present in a biological unit (organule, cell, tissue, organ, individual, species, ecosystem) at any developmental stage and under specific environmental conditions. By using proteomics we aim to know how, where, when, and what for are the several hundred thousands of individual protein species produced in a living organism, how they interact with one another and with other molecules to construct the cellular building, and how they work with each other to fit in with programmed growth and development, and to interact with their biotic and abiotic environment. In the last ten years, excellent reviews and monographs on the fundamentals, concepts, applications, power, and limitations of proteomics have appeared [95, 98–104], some of them dealing with fungal pathogens [37, 105, 106]. It is not the objective of this review to comment or discuss every aspect but instead to show which one has been its contribution to the knowledge of fungal pathogens.

In Proteomics, several areas can be defined: (i) Descriptive Proteomics, including Intracellular and Subcellular Proteomics; (ii) Differential Expression Proteomics, (iii) Post-translational Modifications; (iv) Interactomics; and (v) Proteinomics (targeted or hypothesis-driven Proteomics). In the case of fungi, a new area can also be defined as Secretomics (the secretome is defined as being the combination of native proteins and cell machinery involved in their secretion), since many fungi secrete a vast number of proteins to accommodate their saprotrophic life-style; this would be the case of proteins implicated in the adhesion to the plant surface [107], host-tissue penetration and invasion effectors, [11, 108, 109], and other virulence factors [110].

Proteomics is constantly being renewed to respond to the question of the role of the proteins expressed in a living organism, experiencing, in the last ten years, an explosion of new protocols, and platforms with continuous improvements made at all workflow stages, starting from the laboratory (tissue and cell fractionation, protein extraction, depletion, purification, separation, MS analysis) and ending at the computer (algorithms for protein identification and bioinformatics tools for data analysis, databases, and repositories). These techniques will be briefly introduced and discussed in the next section.

Despite the technological achievements in proteomics, only a tiny fraction of the cell proteome has been characterized so far, and only for a few biological systems (human, fruit fly, *Arabidopsis*, rice). Even for these organisms, the function of quite a number of proteins remains to be investigated [99]. Proteomics techniques have a number of limitations, such as sensitivity, resolution, and speed of data capture. They also face a number of challenges, such as deeper proteome coverage, proteomics of unsequenced "orphan" organisms, top-down Proteomics [100], protein quantification [98], PTMs [105], and Interactomics [55, 57]. Most of these limitations and challenges reflect the difficulty of working with the biological diversity of proteins and their range of physicochemical properties.

The relevance of proteomics in plant fungal pathogens research is very well illustrated by the pioneer work on the *Cladosporium fulvum*-tomato interaction carried out by the Pierre de Wit research group back in 1985 [111] that allowed the characterization of the first avirulence gene product (Avr9) after purification from tomato apoplastic fluids by preparative polyacrylamide gel electrophoresis followed by reverse-phase HPLC and EDMAN N-terminal sequencing [112]. Later on, a number of avirulence gene product effectors have been discovered, mainly by genomic approaches [113]. Curiously, this pioneer work followed the typical proteomics strategy even before MS-based powerful techniques were developed. Another good example is the tomato-*F. oxysporum* pathosystem, in which the first effector of root invading fungi was identified and sequenced, in this case by MS, the Six1, corresponding to a 12 kDa cysteine-rich protein [114]. Other further protein effectors have been characterized in different fungi [115].

Next, we describe the state of the art in the methodology of fungal plant pathogen proteomics and summarize the works published in this field up to December 2009, which so far are 30 (Table 3) out of a total of over 14000 original papers in Proteomics in the last ten years.

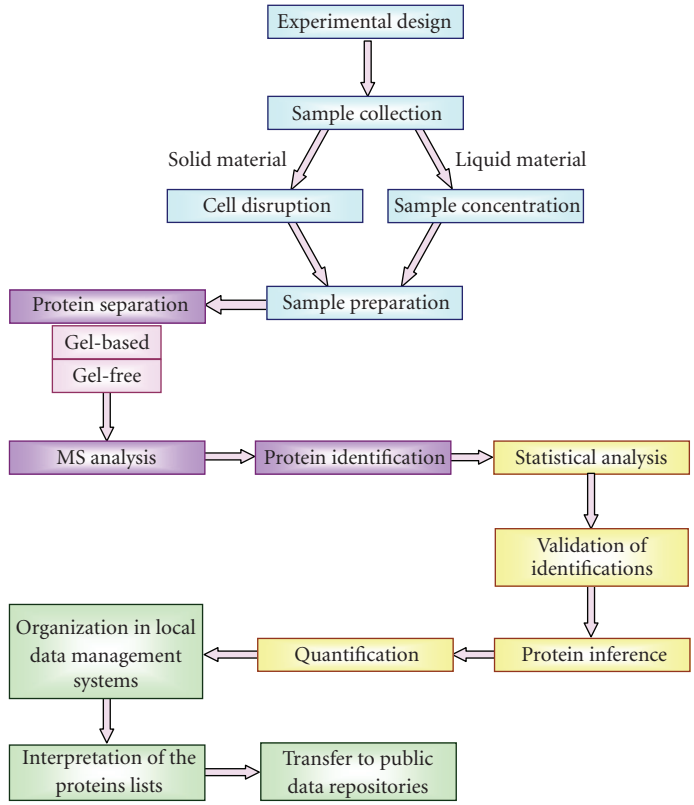

Figure 2: Schematic overview of the work flow in a fungal proteomatic approach (adapted from Deutsch et al., 2008 [116]).

3.1. Methodology. The workflow of a standard MS-based proteomics experiment includes all or most of the following steps: experimental design, sampling, tissue/cell or organelle preparation, protein extraction/fractionation/purification, labeling/modification, separation, MS analysis, protein identification, statistical analysis of data, validation of identification, protein inference, quantification, and data analysis, management, and storage (Figure 2) [102, 116]. The most appropriate protocol to be used depends on and must be optimized for the biological system (i.e., fungal species, plant species, organ, tissue, cells), as well as the objectives of the research (descriptive, comparative, PTMs, interactions, targeted Proteomics) [102].

3.1.1. Experimental Design. A good experimental design is crucial to the success of any proteomics experiment. Eriksson and Fenyö [117] have developed a simulation tool for evaluating the success of current designs and for predicting the performance of future, better-designed proteomics experiments. The simulation gives a holistic view of a general analytical experiment and attempts to identify the factors that affect the success rate. It has been used to predict the success of proteome analyses of Human tissue and body fluids that use various experimental design principles. Several parameters, are required to simulate the steps of a proteome analysis: (i) the distribution of protein amounts in the sample analyzed, (ii) the loss of analyte material and the maximal limit of the amount loaded at each step of sample manipulation (e.g., separation, digestion, and chemical modification), (iii) the dynamic range, the detection limit, and the losses associated with MS analysis. Depending on what experiment is being modelled, the detection limit used in a simulation can represent either protein identification only (lower identification limit) or protein identification with quantification (lower quantification limit) [102].

The establishment of an adequate number of replicates is essential to any differential expression proteomics experiment. This number should be set up while taking into account the dynamic nature of the proteome, and a good number will allow a correct interpretation of the results and the confident assignment of any protein to the group of variable ones [102, 118]. Furthermore, a study of analytical and biological variability should be carried out. Thus, analytical variability examines both the experiment procedures (protein extraction, IEF, SDS-PAGE, gel staining-destaining) and the accuracy of the hardware and software in acquiring and analysing images, and biological variability tests look at several different samples. For example, in the studies to characterize the protein profile of the fungal phytopathogen *B. cinerea* [45], it previously determined the analytical and biological variability. Sixty-four major spots in three 2-DE replicate gels were analyzed and the average CVs were 16.1% for the analytical variance and 37.5% for the biological variance for this fungus. The analytical variance was similar to that reported for bacteria, plants, mice, or human extract and the average biological CV was

higher than that reported for other organisms grown under controlled conditions [45]. While characterizing the *Pinus radiata* needle proteome [119], we found differences in the standard error of mean spot quantity, depending on the number of replicates; the error ranged from 111 and 115 ng for two analytical and biological replicates to 58 and 59 ng for 10 analytical and 12 biological gel replicates.

Using more than six biological replicates did not significantly reduce the standard error; so this figure could be optimal for comparative proteomic experiments. Since normally this is not feasible, most papers in our literature review used only three biological replicates. Given the susceptibility of the data to variation, in 2-DE comparative proteomics it is necessary to be restrictive when deciding whether a spot showed variation. First, all the spots considered had to be consistent, that is, present or absent in all the biological replicates of the particular stage in question; second, when not qualitative (presence vs. absence), differences had to be statistically significant ($P \leq .05$, ANOVA); finally, the variance with respect to a control had to be higher than the average biological coefficient of variance determined for a representative set of at least 150 spots.

3.1.2. Cell Disruption and Sample Preparation. The importance of the extraction protocol in a proteomic experiment can be summarized in the following statement: only if you can extract and solubilize a protein, you have a chance of detecting and identifying it. This sentence sums up the importance of the preparation of protein samples in a proteomic experiment [102]. This is more important in the case of plant tissue or fungal material. In the case of filamentous fungi, the protein extraction is also influenced by the presence of a cell wall which makes up the majority of the cell mass. This cell wall is exceptionally robust [120], and the effective extraction of proteins is a critical step and essential for reproducible results in fungal proteomic studies. Therefore, cell breakdown is an important element in sample preparation for fungal proteomics.

Several early studies were performed to overcome this challenge by providing an effective means of cell lysis for an adequate release of intracellular proteins. For example, mechanical lysis was used to release the cytoplasmic protein via glass beads [59, 121–125], using a cell mill [68], or by sonication [40, 126, 127], these being more efficient than either chemical or enzyme extraction methods [128]. Shimizu and Wariishi [129] utilized an alternative approach to avoid the difficulty of lysing the fungal cell wall by generating protoplasts of *Tyromyces palustris*, since 2-DE patterns from protoplast were better visualized than protein obtained from disrupting the fungal cell wall using SDS extraction. However, the most widely used method for cell disruption is pulverizing the fungus material in liquid nitrogen using a mortar and pestle [45–47, 64, 126, 130, 131].

The extraction protocol most amply utilized implicates the use of protein precipitation media containing organic solvents, such as trichloroacetic acid (TCA)/acetone, followed by solubilization of the precipitate in an appropriate buffer. It allows an increase in the protein concentration and removal of contaminants (salts, lipids, polysaccarides, phenols, and nucleic acids) which can be a problem during IEF [132] and prevents protease activities. This method has often been applied to the preparation of extracts from plants [133–135] and fungi [123, 136]. TCA-treatment makes subsequent protein solubilization for IEF difficult, especially with hydrophobic proteins. These problems have been partly overcome by the use of new zwitterionic detergents [137–142] and thiourea [143], or by a brief treatment with sodium hydroxide [123], which led to an increase in the resolution and capacity of 2-DE gels. Other protein extraction methods have reported an improvement when using acidic extraction solution to reduce streaking of fungal samples caused by their cell wall [144], as well as the use of a phosphate buffer solubilization before the precipitation [45, 46]. In a study to develop an optimized protein extraction protocol for *Rhizoctonia solani*, Lakshman et al. [63] compared TCA/acetone precipitation and phosphate solubilization before TCA/acetone precipitation. Both protocols worked well for *R. solani* protein extraction, although selective enrichment of some proteins was noted with either method. Finally, the combined use of TCA precipitation and phenol extraction gave a better spot definition, because it reduced streaking and led to a higher number of detected spots [47, 145].

In *B. cinerea*, our group has optimized a protein precipitation protocol from mycelium based on a combination of TCA/acetone and phenol/methanol protein extraction methods described by Maldonado et al. [135] with some modifications (Gonzalez-Fernandez et al., unpublished data). Mycelium is lyophilized, pulverized with liquid nitrogen using a mortar and pestle, sonicated in 10% (w/v) TCA/acetone solution with a sonic probe, washed sucessively with methanol and acetone, and finally a protein extraction is released with phenol/methanol precipitation method. We have used a similar protocol from conidia of this fungus by sonicating directly the spores collected in 10% (w/v) TCA/acetone solution (Gonzalez-Fernandez et al., unpublished results). In this sense, specific protocols for protein extraction from spores were optimized in *Aspergillus* ssp. using acidic conditions, step organic gradient, and variable sonication treatment (sonic probe and water bath) [40]. In this study, the use of a sonic probe was the best method to break the robust cell wall of conidia and obtain more proteins.

Special protocols are required in the case of secreted proteins, facing problems such as the very low protein concentration, sometimes below the detection limit of colorimetric methods for determining protein concentration such as Bradford, Lowry, or BCA, and the presence of polysaccharides, mucilaginous material, salts, and secreted metabolites (low-molecular organic acids, fatty acids, phenols, quinines, and other aromatic compounds). Moreover, the presence of these extracellular compounds may impair standard methods for protein quantification and can result in a strong overestimation of total protein [146]. This determination can also be affected by the high concentration of reagents from the solubilisation buffer (such as urea, thiourea or DTT) that may interfere in the spectrophotometric

measurement, producing an overestimation of the total amount of protein. Fragner et al., showed that, depending on the method, the differences varied in the order of two magnitudes, indicating that only the Bradford assay does not lead to an overestimation of the proteins [147].

Francisco and colleagues provided pioneering contributions to this field, establishing a sample preparation protocol for fungal secretome [148], including steps of lyophilization or ultrafiltration plus dialysis, precipitation (TCA/ethanol or chloroform/methanol), deglycosylation, and solubilization for SDS-PAGE or 2-DE.

Comparison of different standard methods for protein precipitation has demonstrated their limited applicability to analyzing the whole fungal secretome [149–152]. Usually, the fungal liquid culture is clarified by filtering and centrifuging, then dialyzed and concentrated up by lyophilizing. Recently, a new optimized protocol has been developed to obtain extracellular proteins from several higher basidiomycetes (*Coprinopsis cinerea*, *Pleorotus ostreatus*, *Phanerochaete chrysosporium*, *Polyporus brumalis,* and *Schizophyllum commune*) [147]. In this work, several precipitation methods, (TCA/acetone, phenol/methanol, other precipitation methods and an optimized protocol by high-speed centrifugation/TCA precipitation/Tris-acetone washes) were compared from liquid cultures of these fungi. The best method was the use of high-speed centrifugation, since it removed a considerable gelatinous material from the liquid culture supernatants. Then fungal proteins were effectively enriched by TCA precipitation and coprecipitated metabolites hampering 2-DE were removed through the application of Tris/acetone washes [147].

Vincent et al. [56], using the plant pathogenic fungus *Leptosphaeria maculans* and symbiont *Laccaria bicolor* grown in culture, have established a proteomic protocol for the analysis of the secretome. They evaluate different protocols, including ultrafiltration followed by TCA/acetone precipitation or phenol/ammonium acetate-phase partition, successive TCA/acetone precipitations without ultrafiltration, phenol/ether extraction without ultrafiltration, lyophilization, and prefractionation of secretome samples using liquid-phase IEF. Liquid-phase IEF followed by dialysis and lyophilization as a prefractionation prior to IPG-IEF produced the best results, with up to 2000 spots well resolved on 2-DE. This protocol can be applicable to a reduced number of samples and be very useful for descriptive proteomics but not for comparative proteomics experiments because of the excessive manipulation of the sample. Thus, in our work with *B. cinerea,* we aim to compare the proteome of wild-type and a high number of mutant strains (close to 100) affected in pathogenicity, and therefore we have optimized a protocol including the following steps: liquid media filtering, dialyzing, lyophilizing, and TCA/acetone-phenol/methanol protein precipitation. It is true that the number of resolved spots is much lower, but still enough for our purposes (Gonzalez-Fernandez et al., unpublished data).

A new field of study has been opened up with the analysis of infection structures such as appressorium and haustoria. In this case, specific protocols for the isolation of such structures are required [85] and the main problem is the large amount of material required for proteomic analysis if compared to transcriptomics. Godfrey et al. have developed a procedure for isolating haustoria from the barley powdery mildew fungus based on filtration and the use of differential and gradient centrifugation [44].

Up to now we have made reference to experiments with in vitro grown fungi; studies *in planta* are much more complicated due to the presence of both proteomes than of the plant and the pathogen. Bindschedler et al. [153] undertook a systematic shotgun proteomics analysis of the obligate biotroph *B. graminis* f. sp. *hordei* at different stages of development in the host: ungerminated spores, epiphytic sporulating hyphae, and haustoria, this being, as far as we know, the only large-scale comparative study of proteomes of phytopathogenic fungi during *in planta* colonization in addition to those analyses of whole extracts from host infected tissue [154, 155] or intercellular washing fluids [156].

In short, since no single protein extraction protocol can capture the full proteome, the chosen protocol should be optimized for the research objective. The ideal method should be highly reproducible and should extract the greatest number of protein species, while at the same time reduce the level of contaminants and minimize artifactual protein degradation and modifications [148, 157, 158]. Fortunately, each protocol takes us to a specific fraction of the proteome, thus being complementary [135]. Another issue to consider is the extreme complexity of the proteome and the wide dynamic range in protein abundance, which exceeds the capability of all currently available analytical platforms. Sample prefractionation is a good approach to reducing the complexity of the proteome sample and decreasing the dynamic range [159], with EQUALIZER being the last developed technology [160].

3.1.3. Protein Separation, Mass Spectrometry and Protein Identification. In fungal plant pathogen research electrophoresis, including denaturing 1-DE SDS-PAGE and 2-DE, with IEF as first dimension, and SDS-PAGE as the second, is almost the only protein separation technique employed, with both crude total protein extracts and protein fractions obtained from various prefractionation procedures [161]. Despite its simplicity, 1-DE can still be quite a valid technique providing relevant information, especially in the case of comparative proteomics with large numbers of samples to be compared. Thus, using this technique, it is possible to distinguish between genotypes of different wild-type strains of *B. cinerea* and identify proteins involved in the pathogenicity mechanisms (Figure 3) (Gonzalez-Fernandez et al., unpublished results). With appropriate software, 1-DE is a simple and reliable technique for finger-printing crude extracts and it is especially useful in the case of hydrophobic and low-molecular-weight proteins [162]. The combination of 1-DE, band cutting, trypsin digestion, and LC separation of the resulting peptides remains the proteomic technique capable of providing the greatest protein coverage [163, 164]. Therefore, the 1-DE is a good approach to obtaining preliminary results before the study by 2-DE

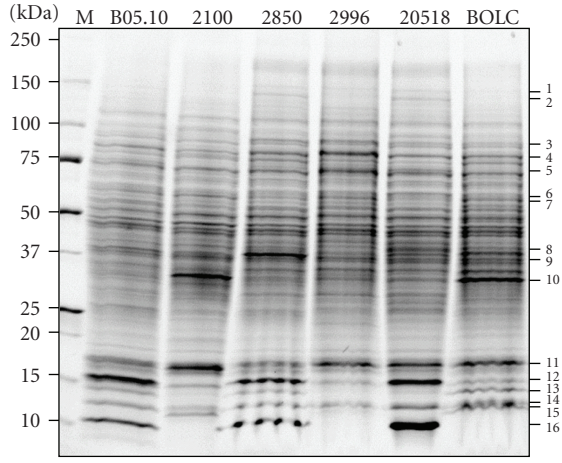

Figure 3: One-DE of 15 μg of mycelium protein extract of six different strains of *B. cinerea* (B05.10, CECT 2100, CECT 2850, CECT 2996, CECT 20518, BOLC (isolated from infected lentil plants)). This approach allowed us to observe differences in the protein band patterns among strains. The bands were cut out and the protein identification was made using MALDI-TOF/TOF MS/MS, and PMF search and a combined search (+MS/MS) were performed in nrNCBI database of proteins using MASCOT. Some of these proteins identified have been reported to be involved in pathogenicity in *B. cinerea* or in other phytopathogenic fungi, such as malate dehydrogenase (10), woronin body major protein (11), peptidyl-prolyl cis-trans isomerase (14) and PIC5 protein (15), or implicated in fungal growth and differentiation, such as nucleoside diphosphate kinase (12). The abundance of these proteins was different among isolated (Gonzalez-Fernandez et al., unpublished results).

[41, 42, 50, 165]. For example, the use of 1-DE in combination with MS/MS analysis has allowed the detection of both known [166] or new proteins [62] of interest in fungal pathogenicity.

In fungal proteomics, 2-DE separation techniques [132, 167, 168] are widely used (Table 3), these having the advantage of separating the proteins at the protein species level with a high resolution of up to 10,000 spots [169]. Briefly, the 2-DE consists of a tandem pair of electrophoretic separations: in the first dimension, proteins are resolved according to their isoelectric points (pIs) normally using IEF, and in the second dimension, proteins are separated according to their approximate molecular weight using SDS-PAGE. These proteins can then be detected by a variety of staining techniques: (i) organic dyes, such as colloidal Coomassie blue staining, (ii) zinc-imidazole staining, (iii) silver staining, and (iv) fluorescence-based detection. Excellent reviews describe and discuss the features and protocols of electrophoretic separations in proteomics strategies [132, 170]. The main advantages of 2-DE are high protein separation capacity and the possibility of making large-scale protein-profiling experiments. However, the reproducibility and resolution of this technique are still remaining challenges. Moreover, this method was reported to under-represent proteins with extreme physicochemical properties (size, isoelectric point, transmembrane domains) and those of a low abundance [171]. These limitations to analytical protein profiling have led to the more recent development of techniques based on LC separation of proteins or peptides, including two-dimensional liquid-phase chromatography (based on a high-perfomance chromatofocusing in the first dimension followed by high-resolution reversed-phase chromatography in the second one) [172], and one-dimensional 1-DE-nanoscale capillary LC-MS/MS, namely, GeLC-MS/MS (this technique combines a size-based protein separation with an in-gel digestion of the resulting fractions). Recorbert et al. [173] explored the efficiency of GeLC-MS/MS to identify proteins from the mycelium of *Glomus intraradices* developed on root organ cultures, reporting on the identification of 92 different proteins. Overall, this GeLC-MS/MS strategy paves the way towards analysing on a large-scale fungal response environmental cues on the basis of quantitative shotgun protein profiling experiments.

Despite the existence of quite a number of different methods developed for protein extraction and separation, it is clear that, all in all, it is not enough to allow for the analysis of entire proteomes (organelle, cell, tissue, or organ). Some methodologies have proven to be more powerful and decisive than others, with regard to the number of proteins identified. This is the case of Multidimensional Protein Identification Technology (MudPIT), an LC-based strategy, which allows the detection of a much larger number of proteins compared to gel-based methods, its drawback being the lack of quantitative data [174]. MudPIT was first applied to the fungal proteome of the *S. cerevisiae* and yielded the largest proteome analysis to date, in which a total of 1484 proteins were detected and identified [175]. The categorization of these hits demonstrated the ability of this technology to detect and identify proteins rarely seen in proteome analysis, including low abundance proteins like transcription factors and protein kinases [175]. It has been reported that a set of proteins can only be detected by a specific technology [176, 177], which is in agreement with the idea that a combination of different methodologies is still needed to characterize entire proteomes [131]. MudPIT has been used to compare the proteome from germinating and ungerminated asexual uredospores of the biotrophic fungal pathogen *Uromyces appendiculatus,* which is the causal agent of rust disease in beans [67].

Mass spectrometry is the basic technique for global proteomic analysis due to its accuracy, resolution, and sensitivity, small amounts of sample (femtomole to attomole concentrations), and having the capacity for a high throughput. It allows not only to profile a proteome from a qualitative and quantitative point of view but also, and more important, to identify protein species and characterize postranslational modifications. Proteins are identified from mass spectra of intact proteins (top-down proteomics) or peptide fragments obtained after enzymatic (mostly tryptic peptides) or chemical treatment (bottom-up proteomics). Protein species are identified by comparison of the experimental spectra and the theoretical one obtained in silico from protein, genomic, ESTs sequence, or MS spectra databases. For that purpose, different instrumentation, algorithms, databases, and repositories are available [178, 179].

Different strategies and algorithms can be used for protein identification (i) including peptide mass fingerprinting (PMF, only valid if the protein sequence is present in the database of interest, generally used if the genome of the organism of study is fully sequenced); (ii) tandem mass spectrometry (where peptide sequences are identified by correlating acquired fragment ion spectra with theoretical spectra predicted for each peptide contained in a protein sequence database, or by correlating acquired fragment ion spectra with libraries of experimental MS/MS spectra identified in previous experiments); (iii) *de novo* sequencing, where peptide sequences are explicitly read out directly from fragment ion spectra; and (iv) hybrid approaches, such as those based on the extraction of short sequence tags of 3–5 residues in length, followed by "error-tolerant" database searching [179]. In a genomic-based proteomics strategy the percentage of proteins identified from MS data is dependent on the availability of genomic DNA or EST sequences. The construction of protein repositories with signature peptides and derived MS spectra will open up new possibilities for protein identification. The availability of ESTs from unsequenced "orphan" organisms as is the case of most plant fugal pathogens of interest will increase the percentage of identified proteins [93]. There are relatively few and a slow accumulation of EST data derived from a number of plant fungal pathogens and related species in public databases (e.g., dbEST at the National Centre for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/dbEST/; and COGEME, Phytopathogenic Fungi and Oomycete EST Database [180, 181] at Exeter University, UK, http://cogeme.ex.ac.uk/). In Table 4, the number of EST entries for a number of fungi at the NCBI and Dana Faber databases is listed.

3.1.4. Second-Generation Techniques for Quantitative Proteomics. By using proteomics it is not only aimed to identify protein species (main objective of descriptive proteomics) but also quantify them, at least in relative terms, by comparing two biological units (genotypes, cells, organules) under different spatiotemporal parameters and environmental conditions. Absolute, rather than relative, protein quantitation remains one of the main challenges in proteomics [102]. There are different methods to dissect the proteome in a quantitative manner: (i) methods based on 2-DE with poststaining, such as colloidal Comassie blue staining [182] and fluorescence staining [183], or prelabeling such as two-dimensional fluorescence difference gel electrophoresis (2-DIGE) [184, 185]; and (ii) gel-free methods based on in vitro or in vivo protein targetting with a stable isotope, such as isotope-coded affinity tags (ICATs) [186, 187], ^{18}O labeling [188], or stable isotope labelling in cell culture (SILAC) [189], or isobaric tags, such as isobaric tag for relative and absolute quantitation (iTRAQ) [190]. Novel, label-free approaches, such as spectral counting, are being developed [191, 192]. The use of second generation proteomic techniques based on protein labelling and those label-free ones are far from being fully exploited in fungal pathogen research.

Table 4: Number of ESTs entries for some fungi of interest up to December 2009.

Fungus	Dana Faber/NBCI[a]
Aspergillus flavus	20372/22452
Aspergillus niger	No entries/47082
Blumeria graminis hordei	No entries/17142
Botrytis cinerea	No entries/28531
Claviceps purpurea	No entries/8789
Curvularia lunata	No entries/1488
Fusarium graminearum	No entries/58011
Fusarium oxysporum	No entries/17478
Fusarium verticillioides	86908/87134
Leptosphaeria maculans	No entries/20034
Magnaporthe grisea	87403/110613
Puccinia graminis	No entries/209
Phytophthora infestans	90287/164143
Phytophthora palmivora	No entries/14824
Pyrenophora tritici-repentis	No entries/1
Nectria haematococca mpVI	No entries/33122
Sclerotinia sclerotiorum	No entries/2578
Stagonospora nodorum	No entries/16447
Ustilago maydis	No entries/39717

[a]Data taken from The Gene Index Proyect at the Dana Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi/plant.html) and NBCI (http://www.ncbi.nlm.nih.gov/).

In 2-DIGE, proteins in two samples are labeled in vitro through cysteine or lysine residues using two different fluorescent cyanine dyes differing in their excitation and emission wavelengths, but with an identical relative mass. Labeled samples are then mixed and subjected to 2-DE on the same gel. After consecutive excitation with both wavelengths, the images are overlaid and normalized, whereby only differences between the two samples are visualized [193]. 2-DIGE enables to perform high-throughput, differential protein expression analysis to compare directly, on a single gel, the differences in protein expression levels between different complex protein samples. The main advantage of 2-DIGE on 2-DE is its unrivalled performance, attributable to a unique experiment, in which each protein spot on the gel is represented by its own internal standard [105].

The classical proteomic quantification electrophoretic methods utilizing dyes, fluorophores, or radioactivity have provided very good sensitivity, linearity, and dynamic ranges, but they suffer from two important shortcomings: first, they require high-resolution protein separation typically provided by 2-DE gels, which limits their applicability to abundant and soluble proteins; and second, they do not reveal the identity of the underlying protein [194]. Both of these problems are overcome by modern LC-MS/MS techniques [95, 195–197]. However, the MS-based techniques are not inherently quantitative because proteolytic peptides exhibit a wide range of physicochemical properties (size, charge, hydrophobicity) that lead to large differences in mass spectrometric response. Therefore, in MS-based gel-free quantification it is necessary

to use isotopic labeling. Observed peak ratios for isotopic analogs are highly accurate, because there are no chemical differences between the species, and they are analyzed in the same experiment. Mass spectrometry can recognize the mass difference between the labeled and unlabeled forms of a peptide and the quantification is achieved by comparing their respective signal intensities [194].

A number of isotopic labeling techniques have recently been proposed that share the requirement of the chemical modification of the peptides or proteins. One of these strategies is the ICAT method for relative quantitation of protein abundance [186]. In this approach, an isotopically labeled affinity reagent is attached to particular amino acids in all proteins in the population. After digestion of the protein to peptides, as a necessary step in all mainstream proteomic protocols, the labeled peptides are affinity-purified using the newly incorporated affinity tag, thereby achieving a simplification of the peptide mixture at the same time as incorporating the isotopic label. This method addresses many of the above limitations and leads to a larger number of identifications of cysteine-containing peptides. However, the method is performed by cross-linking peptides to beads via their cysteine groups and photo-releasing them afterwards, which may compromise low-level analysis. The iTRAQ is used to identify and quantify proteins from different sources in one experiment. The method is based on the covalent labeling of the N-terminus and side-chain amines of peptides from protein digestions with tags of varying mass. The fragmentation of the attached tag generates a low molecular mass reporter ion that can be used to relatively quantify the peptides and the proteins from which they originated [198]. In SILAC (stable isotope labeling by amino acids in cell culture), labeled, essential amino acids are added to amino acid-deficient cell culture media and are therefore incorporated into all proteins as they are synthesized, "encoded into the proteome" [189]. No chemical labeling or affinity purification steps are performed, and the method is compatible with virtually all cell culture conditions. Finally, label-free protein quantification methods are promising alternatives. It is based on precursor signal intensity, which is, in most cases, applied to data acquired on high mass precision spectrometers. The mass spectral peak intensities of the peptide ion correlate well with protein abundances in complex samples [199–201]. Another label-free method is spectral counting, which simply counts the number of MS/MS spectra identified for a given peptide and then integrates the results for all measured peptides of proteins that are quantified [202]. An advantage of this technique is that relative abundances of different proteins can in principle be measured. These new quantification techniques have become powerful tools to overcome the inherent problems of the 2-DE including identification of proteins of a low abundance, high hydrophobicity, extreme pl or high MW.

These second-generation MS technologies for Quantitative Proteomics have not begun to be applied to Fungal Proteomics yet. The only one nongel-based quantitative proteomics example is the use of iTRAQ to study the profile protein expression differences on *F. graminearum* which allowed the identification of numerous candidate pathogenicity proteins [53].

Although this review is focused on MS-based proteomics, we want to make a brief mention of protein microarrays because they are powerful tools for individual studies as well as systematic characterization of proteins and their biochemical activities and regulation [203]. The arrays can be used to screen nearly the entire proteome in an unbiased fashion and have an enormous utility for a variety of applications. These include protein-protein interactions, identification of novel lipid- and nucleic acid-binding proteins, and finding targets of small molecules, protein kinases, and other modification enzymes.

In short, all these technologies have a great potential in protein separation and remain a challenge for future research works in Fungal Proteomics.

3.1.5. Data Analysis and Statistical Validation. Proteomics tools generate an important amount of data, because a single proteomics experiment reveals the expression information for hundreds or thousands of proteins. Therefore, data analysis and bioinformatics are essential for this type of research and in many cases take more time than the actual experiment and require special skills and tools (for review see [116, 118]). All 2-DE software permits a fast and reliable gel comparison, and multiple gel analyses, including filtering of 2-DE images, automatic spot detection, normalization of the volume of each protein spot, and differential and statistical analyses [204–206]. A great resource for finding software tools for proteomics can be found in the website https://proteomecommons.org/.

Before the protein identification, the remaining challenge is to determine whether the putative identification is, in fact, correct. Statistical tools help us to validate information. Although postsearch statistical validation still does not enjoy universal application, its importance has been recognized by most researchers and codified in the editorial policies of some leading journal [207]. In this decade, an important number of commercial software involving even more powerful algorithms and statistical tools than those of the previous generations have been designed to help researchers deal with the sheer quantity of data produced [194, 195, 208].

Statistical data analyses can be classified as univariated or multivariated [209]. The univariated methods, such as the Student's *t*-Test, are used to detect significant changes in the expression of individual proteins. They are the simplest to interpret conceptually and the most common ones used. The multivariated methods, such as principle component analysis (PCA), look for patterns in expression changes and utilize all the data simultaneously. Early expression studies compared one sample with another, generally by the calculation of a ratio, and the analyses were restricted to looking for changes above a threshold determined by the system experimental noise. This method of analysis limits the sensitivity of the system, as biologically relevant changes smaller than the threshold cannot be detected. Using a threshold is a rather simplistic approach and does not take into account the variability of each protein, running the risk of selecting

Table 5: Useful online resources and Fungal Genome and Proteome Databases.

Name/description	URL
Genome Databases	
National Center for Biotechnology Information (NCBI).	http://www.ncbi.nlm.nih.gov/
NIH genetic sequences database.	http://www.ncbi.nlm.nih.gov/Genbank/
Fungal Genomes Central, information and resources pertaining to fungi and fungal sequencing projects.	http://www.ncbi.nlm.nih.gov/projects/genome/guide/fungi/
The Gene Index proyect (GI). The Computational Biology and Functional Genomics Laboratory, and the Dana-Faber Institute and Public School of Public Health.	http://compbio.dfci.harvard.edu/tgi/fungi.html
Fungal Genome Initiative of The Broad Institute (FGI).	http://www.broadinstitute.org/science/projects/fungal-genome-initiative/fungal-genome-initiative
Genoscope, Sequencing National Centre.	http://www.genoscope.cns.fr/spip/Fungi-sequenced-at-Genoscope.html
Joint Genome Institute (JGI).	http://www.jgi.doe.gov/
The Genome Center at Washington University (WU-GSC).	http://genome.wustl.edu/genomes/list/plant_fungi
The Sanger Institute fungal sequencing.	http://www.sanger.ac.uk/Projects/Fungi/
Genome projects.	http://genomesonline.org/
The MIPS *F. Graminearum* Genome Database.	http://mips.gsf.de/projects/fungi/Fgraminearum/
The MIPS *U. Maydis* Database.	http://mips.gsf.de/genre/proj/ustilago
The MIPS *Neurospora crassa* Genome Database.	http://mips.helmholtz-muenchen.de/genre/proj/ncrassa/
COGEME, Phytopathogenic Fungi and Oomycete EST Database (v1.6), constructed and maintained by Darren Soanes (University of Exeter, UK).	http://cogeme.ex.ac.uk/
SGD, *Saccaromyces* Genome Database, scientific database of the molecular biology and genetics of the yeast *Saccharomyces cerevisiae*.	http://www.yeastgenome.org/
e-Fungi, warehouse which integrates sequence data (genomic data, EST data, Gene Ontology annotation, KEGG pathways and results of the following analyses performed on the genomic data) from multiple fungal sequences in a way that facilitates the systematic comparative study of those genomes (School of Computer Science and the Faculty of Life Sciences at the University of Manchester and the Departments of Computer Science and Biological Sciences at the University of Exeter).	http://beaconw.cs.manchester.ac.uk/efungi/execute/welcome
CADRE, Central Aspergillus Database Repository, resource for viewing assemblies and annotated genes arising from various Aspergillus sequencing and annotation projects.	http://www.cadre-genomes.org.uk/
FungalGenome, website with several links and references for the currently available fungal genomes sequences or proposed fungal genomes.	http//:fungalgenomes.org/wiki/Fungal_Genomes_Links
Proteome Databases	
The Expasy (Expert Protein Analysis System) proteomics server of Swiss Institute of Bioinformatics (SIB). Analysis of protein sequences, structures and 2-D-PAGE.	http://ca.expaxy.org/
MIPS, Munich Information Center for Protein Sequences.	http://mips.gsf.de/
The PRIDE, Proteinomics IDEntifications Database. EMBL-EBI (European Bioinformatic Institute).	http://www.ebi.ac.uk/pride/

Table 5: Continued.

Name/description	URL
Integr8, Integrated information about deciphered genomes and their corresponding proteomes. EMBL-EBI.	http://www.ebi.ac.uk/integr8/EBI-Integr8-HomePage.do
SNAPPVIEW (Structure, iNterfaces and Aligments for Protein-Protein Interactions).	http://www.compbio.dundee.ac.uk/SNAPPI/download.jsp
Phospho3. Database of three-dimensional structures of phosphorylation sites.	http://cbm.bio.uniroma2.it/phospho3d/
Proteome Analyst PA-GOSUB 2.5. Sequences, predicted GO molecular functions and subcellular localisations.	http://www.cs.ualberta.ca/~bioinfo/PA/GOSUB/
RCSB, The Research Collaboratory for Structural Bioinformatics. Protein Database (PDB).	http://www.rcbs.org/pdb/home/home.do
PDB-Site. Comprehensive structural and functional information on PTMs, catalytic active sites, ligand binding (protein-protein, protein-DNA, protein-RNA interacions) in the Protein Data Bank (PDB).	http://wwwmgs.bionet.nsc.ru/mgs/gnw/pbbsite/
WoLF PSORT, Protein Subcellular Localization Prediction.	http://wolfpsort.org/
NMPdb, Nuclear Matrix Associated Proteins.	http://cubic.bioc.columbia.edu/db/NMPdb/
TargetP, predicts the subcellular location of eukaryotic proteins, based on the predicted presence of the N-terminal presequences.	http://www.cbs.dtu.dk/services/TargetP/
MitoP2, Mitochondrial Database. This database provides a comprehensive list of mitochondrial proteins of yeast, mouse, *Arabidopsis thaliana*, neurospora and human.	http://www.mitop.de:8080/mitop2/
The SecretomeP, Prediction of protein secretion and information on various PTMs and localisational aspect of the protein.	http://www.cbs.dtu.dk/services/SecretomeP/
MASCOT, a powerful search engine that uses MS data to identify proteins from primary sequence databases.	http://www.matrixscience.com/
VEMS, Virtual Expert Mass Spectrometrist. Program for integrated proteome analysis.	http://www.yass.sdu.dk/
The NetPhos server produces neural network predictions for serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins.	http://www.cbs.dtu.dk/services/NetPhos/
ProPrInt, Protein-Protein Interaction Predictor. Compilation of web-resources in the field of Protein-Protein Interaction (PPI).	http://www.imtech.res.in/raghava/proprint/resources.htm
ProteomeCommons, public proteomics database for annotations and other information linked to the Tranche data repository and to other resources. It provides public access to free, open-source proteomics tools and data.	https://proteomecommons.org
Fungal Proteome Specialized Databases	
MPID, Protein-protein interaction Database of *M. grisea.*	http://bioinformatics.cau.edu.cn/cgi-bin/zzd-cgi/ppi/mpid.pl
FPPI, Protein-protein interaction database of *F. graminearum.*	http://csb.shu.edu.cn/fppi

variable proteins due to sample selection. The use of a fold change has a potential role in preliminary experiments but the limitation of this method must always be considered when interpreting the data [209].

Hypothesis tests, for example, Student's *t*-Test, assess whether the differences between groups are an effect of chance arising from a sampling effect or reflect a real statistical significant difference between groups [209]. Hypothesis tests are usually stated in terms of both a condition that is in doubt (the null hypothesis) and a condition that is believed to exist (the alternative hypothesis). The tests calculate a *p*-score, which is the probability of obtaining these results assuming that the null hypothesis is correct. Hypothesis tests can be divided into two groups: parametric and nonparametric. Parametric tests assume that the distribution of the variables being assessed belong to known probability distributions. For example, the Student's *t*-Test assumes that the variable comes from a normal distribution. Nonparametric tests, also called distribution-free methods, do not rely on estimation of parameters such as the mean. An example of nonparametric tests is the Mann-Whitney *U*-Test, which ranks all values from low to high and then compares the mean ranking the two groups. Otherwise, Student's *t*-Test or Mann-Whitney *U*-Test compares two groups and ANOVA or Krustal-Wallis compares more than two groups. These tests analyze individual spots instead of the complete set, omitting information about correlated variables. Biron et al. recommend assessing the normality for each protein species and then selecting either a parametric or nonparametric test [208].

In expression studies, many thousands of statistical tests are conducted, one for each protein species. A substantial number of false positives may accumulate which is termed the multiple testing problem and is a general property of a confidence-based statistical test when applied many times [209]. One approach to addressing the multiple testing problem is to control the family wise error rate (FWER), which control the probability of one or more false rejections among all the tests conducted. The simplest and most conservative approach is the Bonferroni correction, which adjusts the threshold of significance by dividing the *per* comparison error rate (PCER) by the number of comparisons being completed [210]. This has led to the application of methodologies to control the false discovery rate (FDR), where the focus is on achieving an acceptable ratio of true- and false-positives. The FDR is a proportion of changes identified as significant that are false [211]. An extension to the FDR calculates a *Q*-value for each tested feature and is the expected proportion of false positives incurred when making a call that this feature has a significant change in the expression [212]. For each *P*-value, a *Q* -value will be reported on an overall estimation for the proportion of species changing in the study.

Multiple testing correction methods, such as the Bonferroni correction and testing for the false discovery rate (FDR) [213], fit the Student *t*-test or ANOVA values for each protein spot to keep the overall error rate as low as possible. Multivariate data analysis methods, such as PCA, are now used to pinpoint spots that differ between samples. These multivariate methods focus not only on differences in individual spots but also on the covariance structure between proteins [214]. However, the results of these methods are sensitive to data scaling, and they may fail to produce valid multivariate models due to the large number of spots in the gels that do not contribute to the discrimination process [215]. One of the limitations of PCA analysis is that it does not allow to miss values, a problem that can be avoided by imputing them when possible (if enough replicates are available) [216].

3.1.6. Databases and Repositories. The huge amount of data generated are being deposited and organized in several databases available to the scientific community: the UniProt knowledgebase reported by Schneider et al. [217] and other Proteome Databases mentioned in Table 5. After 20 years of Proteomics research, it is possible to look back at previous research and publications, identifying errors from the experimental design, the analysis, and the interpretation of the data [179]. In addition, data validation is done in a purely descriptive or speculative manner, as well as it is common to find low-confidence protein identification in the literature, especially in the case of unsequenced organisms and inappropriate statistical analyses of results have often been performed. It is interesting to see how many manuscripts contain the term "proteome" when probably only a tiny fraction of the total proteome has been analysed. About this problem, HUPO's Proteomic Standard Initiative has developed guidance modules [218] that have been translated into Minimal Information about a Proteomic Experiment (MIAPE) documents. The MIAPE documents recommend proteomic techniques that should be considered and followed when conducting a proteomic experiment. Proteomics journals should be, and in fact are, extremely strict when recommending that investigators follow the MIAPE standards for publishing a proteomic experiment. On the other hand, many journals recommend or require the original data generated in a proteomic experiment to be submitted to public repositories [207, 219]. A shift in the protein identification paradigm is currently underway, moving from sequencing and database searching to spectrum searching in spectral libraries. This underscores the importance of repositories for Proteomics [220–222]. The main public peptide and protein identification repositories are GPMDB (Global Proteome Machine database) [223], PeptideAtlas [224], and Proteomics IDEntifications database (PRIDE) [225]. Other emerging and smaller systems include Genome Annotating Proteomic Pipeline database (GAPP database) [226], Tranche (Falkner, J. A., Andrews, P. C., HUPO Conference 2006, Long Beach, USA, Poster presentation), PepSeeker [227], Max-Planck Unified database (MAPU) [228], the Open Proteomic Database (OPD) [229], and the Yeast Resource Center Public Data Repository (YRC PDR) [230].

3.2. Proteomics of Plant Pathogenic Fungi. Several proteomic studies have been carried out in order to understand fungal pathogenicity or plant-fungus interactions

Table 6: Original proteomics papers published in plant-pathogenic fungi interactions.

Pathogen-Host	Description of study (References)
Alternaria bassicicola-Arabidopsis	Study of change in the *Arabidopsis* secretome in response to salicylic acid and identifying of several proteins involved in pathogen response such as GDSL LIPASE1 (GLIP1) [231].
Aphanomyces eutiches-Medicago truncatula	Identification of several proteins which play a major role during root adaptation to various stress conditions [232], and study of parasitic plant-pathogen interactions formed between legumes and this oomycete [233, 234].
Black point disease-Barley	Identification of a novel late embryogenesis abundant (LEA) protein and a barley grain peroxidase 1 (BP1) that were specifically more abundant in healthy grain and black pointed grain, respectively [235].
B. graminis hordei-Barley	Systematic shotgun proteomics analysis at different stages of development of powdery mildew in the host to gain further understanding of the biology during infection of this fungus [153].
Cladosporium fulvum-Tomato	Identified 3 novel fungal secretory proteins [236].
Cronartium ribicola-Pinus strobus	Study of molecular basis of white pine blister rust resistance [237].
Diploidia scrobiculata-Pinus nigra Sphaeropsis sapinea-Pinus nigra	Study about defense protein responses in phloem of Austrian pine inoculated with *D. scrobiculata* and *S. sapinea* [238].
Erysiphe pisi-Pea	Identification of proteins implicated in powdery mildew resistance [239].
F. graminearum-Barley	Identification of proteins associated with resistance to *Fusarium* head blight in barley [240].
F. graminearum-Wheat	Identification of proteins associated with resistance to *Fusarium* head blight in wheat [241] and which have a role in interaction between *F. graminearum* and *T. aestivum* [155].
F. graminearum-Wheat	Identification of proteins associated with resistance to scab in wheat spikes [238].
F. moniliforme-Arabidopsis	Study of changes in the extracellular matrix of *A. thaliana* cell suspension cultures with fungal pathogen elicitors of *F. moniliforme* [242].
F. oxysporum-Sugar beet	Study of resistance to *F. oxysporum* disease [243].
F. oxysporum-Tomato	Identification of 21 tomato and 7 fungal proteins in the xylem sap of tomato plants infected by *F. oxysporum* [156].
F. verticillioides-Maize	Identification of protein change patterns in germinating maize embryos in response to infection with *F. verticillioides* [244].
Fusarium-Arabidopsis	Identification of differentially expressed proteins in response to treatments with pathogen-derived elicitors to identify pivotal genes' role in pathogen defence systems [245].
Fusarium-Maize	Study of the role of the extracellular matrix in signal modulation during pathogen-induced defence responses [246].
Gossypium hirsutum-Cotton	Identified pathogen-induced cotton proteins implicated in post-invasion defence reponses (PR-proteins related to oxidative burst), nitrogen metabolism, amino acid synthesis and isoprenoid synthesis [247].
Hyaloperonospora parasitica-Arabidopsis B. cinerea-Arabidopsis	Study of pathogenic resistance of Arabidopsis wild-type and CaHIR1-overexpressing transgenic plants inoculated with these fungi, among other pathogens [248].

Table 6: Continued.

Pathogen-Host	Description of study (References)
Leptosphaeria maculans-Brassica	Identification of *Brassica* proteins involved in resistance to this fungus [249].
L. maculans-Brassica carinata L. maculans-Brassica napus	Study of changes in the leaf protein profiles of *Brassica napus* (highly susceptible) and *Brassica carinata* (highly resistant) in order to understand the biochemical basis for the observed resistance to *L. maculans* [250].
M. grisea-Rice	Change protein analysis during blast fungus infection of rice leaves with different levels of nitrogen nutrient [251]. Analysis of differentially expressed proteins induced by blast fungus in suspension-cultured cells [252] in leaves [253] and during appressorium formation [254]. Proteomic approach of differentially expressed proteins in rice plant leaves at 12 h and 24 h after treatment with the glycoprotein elicitor CSB I, purified from ZC(13), a race of the rice blast fungus *M. grisea* [255].
Marsonina brunnea f. sp. *Multigermtub-Populus euramericana*	Identification of proteins related to black spot disease resistance in poplar leaves [256].
Moliniophtora perniciosa-Cocoa	Optimization of protein extraction for cocoa leaves and meristemes infected by this fungus that causes witches' broom disease [257].
Nectria haematococca-Pea	Study of extracellular proteins in pea roots inoculated with *N. haematococca* [258].
Penincillium exposum-Piccia membranefaciens-Peach fruit	Peach fruit inoculated with *P. exposum* and treated with SA and *P. membranefaciens* [259].
Phellinus sulphurasces-Pseudotsuga menzeii	Comparative proteomic study to explore the molecular mechanisms that underlie the defense response of Douglas-fir to laminated root rot disease caused by *P. sulphurascens* [260].
Peronospora viciae-Pea	Catalogued host (pea) leaf proteins, which showed alternation in their abundance levels during a compatible interaction with *P. viciae* [261].
Plasmodiophora brassicae-Brassica napus	Study of changes in the root protein profile of canola with clubroot disease [262].
Puccinia triticina-Wheat	Change analysis in the proteomes of both host and pathogen during development of wheat leaf rust disease [154].
Rhizoctonia solani-Rice	Identification of proteins and DNA markers in rice associated with response to infection by *R. solani* [263].
Rust-Phaseolus vulgaris	Study of basal and R-gene-mediated plant defense in bean leaves against this pathogen [264].
S. sclerotiorum-Brassica napus	Study of changes in the leaf proteome of *B. napus* accompanying infection by *S. sclerotiorum* [265].

(for reviews see [266–268]), although the plant-fungus association has been the one most studied by Proteomics approaches (Table 6), which is outside the scope of this review. On the other hand, some fungal species have attracted an increasing interest in the biotechnological industry, in food science, or in agronomy as biocontrol agents (Table 7), which is also beyond the objectives of this work. At this point, this review describes studies published up to December 2009 in plant pathogenic fungi in descriptive proteomics (intracellular proteomics, subproteomics, and secretomics), differential expression proteomics, as well as some basic knowledge about the Interactomics in fungi (Table 3).

3.2.1. Descriptive, Subcellular, and Differential Expression Proteomics. Within this section, papers devoted to establishing reference proteome maps of fungal cells and structures and subcellular fractions, and to study changes in the protein profile between species, races, populations, mutants, growth and developmental stages, as well as growth conditions, are discussed, paying special attention to proteins related to pathogenicity and virulence.

Most of the reported work mainly uses mycelia from in vitro grown fungi, and 2-DE coupled to MS as proteomic strategy. Thus, a partial proteome map has been reported for the ascomycete *B. cinerea* Pers. Fr. (teleomorf *Botryotinia fuckeliana* (de Bary) Whetzel), a phytopathogenic

Table 7: Original proteomics papers published on fungi for biotechnological or agricultural applications.

Fungus	Interest (reference)
Boletus edilus	Study of salinity stress of this ectomycorrhizal fungus for its importance in reforestation in saline areas [269].
Coprinopsis cinerea *Pleorotus ostreatus* *Phanerochaete chrysosporium* *Polyporus brumalis* *Schizophyllum commune*	Optimization of a protocol for 2-DE of extracellular proteins from these wood-degrading fungi [147].
Glomus intraradices	Study of arbuscular mycorrhiza symbiosis [173].
Metarhizium anisopliae	Study of bioinsecticidal activity of this fungus to develop novel compounds or produce genetically modified plants resistant to insect pests [270].
Monascus pilosus	Study of the influence of nitrogen limitation for industrial production of many poliketide secondary metabolites [271].
Phanerochaete chrysosporium	Several studies of ligninolytic processes for wood biodelignification in cellulose pulp industries [130, 131, 149, 150, 272].
Pleurotus sapidus	Study of secretome for wood biodelignification for peanuts industry applications [152].
Trichoderma atroviride *T. harzianum*	Several studies in these fungus for their biocontrol properties [126, 273–275]
T. reesei	Study of cell-wall-degrading enzymes in *A. bisporigera* (an ectomycorrhizal basidiomycetous fungus) comparing with a MS/MS-based shotgun proteomics of the secretome of *T. reesei* [276].
Amanita bisporigera	

necrotroph pathogen causing significant yield losses in a number of crops, by Fernández-Acero et al. They have reported the detection of 400 spots in Coomassie-stained 2-DE gels, covering the 5.4–7.7 pH and 14–85 kDa ranges. Out of 60 spots subjected to MS analysis, twenty-two proteins were identified by MALDI-TOF or ESI IT MS/MS, with some of them corresponding to forms of malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and a cyclophilin, proteins that have been related to virulence [45]. In a second study, comparative proteomic analysis of two *B. cinerea* strains differing in virulence and toxin production revealed the existence of qualitative and quatitative differences in the 2-DE protein profile. Some of them were the same proteins mentioned above and they appeared overexpressed or exclusively in the most virulent strain [46]. A third and more exhaustive work tried to establish a proteomic map of *B. cinerea* during cellulose degradation [47]. Using 2-DE and MALDI-TOF/TOF MS/MS, 306 proteins were identified, mostly representing unannoted proteins. The authors conclude that since cellulose is one of the major components of the plant cell wall, many of the identified proteins may have a crucial role in the pathogenicity process, be involved in the infection cycle, and be potential antifungal targets.

A close relative to *B. cinerea* is the soil-borne *Sclerotinia sclerotiorum*. Yajima and Kav [64] performed the first comprehensive proteome-level study in this important phytopathogenic fungus, in order to gain a better understanding of its life cycle and its ability to infect susceptible plants. For the high-throughput identification of secreted as well as mycelial proteins, they employed 2-DE and MS/MS. Eighteen secreted and 95 mycelial proteins were identified. Many of the annotated secreted proteins were cell wall-degrading enzymes that had been previously identified as pathogenicity or virulence factors of *S. Sclerotiorum*. Furthermore, this study has allowed the annotation of a number of proteins that were unnamed, predicted, or hypothetical proteins with undetermined functions in the available databases.

Xu et al. [50] analyzed the proteome profile of six different isolates of *Curvularia lunata*, a maize phytopatogenic fungus, by means of 1-DE and 2-DE, in an attempt to correlate the band or spot pattern with virulence. According to the 1-DE band pattern, isolates were clustered into three groups consisting of different virulent types. By 2-DE 423 spots were resolved with 29 of them being isolate-specific, and 39 showed quantitative differences. Twenty proteins were identified by MALDI-TOF-TOF, most of them associated with virulence differentiation, metabolisms, stress response, and signal transduction. One of them was identified as Brn1 protein which has been reported to be related to melanin biosynthesis and the virulence differentiation in fungi.

The fungal pathogen *F. graminearum* (teleomorph *Gibberella zeae*) is the causal agent of *Fusarium* head blight in wheat, barley, and oats and *Gibberella* ear rot in maize in temperate climates worldwide. It synthesizes trichothecene mycotoxins during plant host attack to facilitate spread within the host. In order to study proteins and pathways that are important for successful host invasion, Taylor et al. [53] conducted experiments in which *F. graminearum* cells were grown in aseptic liquid culture conditions conducive to trichothecene and butenolide production in the absence of host plant tissue. Protein samples were extracted from three biological replicates of a time course study and subjected to iTRAQ (isobaric tags for relative and absolute quantification) analysis. Statistical analysis of a filtered dataset of 435 proteins revealed 130 *F. graminearum* proteins that

exhibited significant changes in expression, 72 of which were upaccumulated relative to their level at the initial phase of the time course. There was good agreement between upaccumulated proteins identified by 2-DE-MS/MS and iTRAQ. RT-PCR and northern hybridization confirmed that genes encoding proteins that were upregulated based on iTRAQ were also transcriptionally active under mycotoxin-producing conditions. Numerous candidate pathogenicity proteins were identified using this technique, including many predicted secreted proteins. Curiously, enzymes catalyzing reactions in the mevalonate pathway leading to trichothecene precursors were either not identified or only identified in one replicate, indicating that proteomics approaches cannot always probe biological characteristics. Two-DE with MS has been used to compare the proteome of virus-free and virus-(FgV-DK21-) infected *F. graminearum* cultures [54]. The virus perturbs fungal developmental processes such as sporulation, morphology, and pigmentation and attenuates its virulence. A total of 148 spots showing differences in abundance were identified. Among these spots, 33 spots were subjected to ESI-MS/MS, with 23 identified. Seven proteins including sporulation-specific gene SPS2, triose phosphate isomerase, nucleoside diphosphate kinase, and woronin body major protein precursor were upaccumulated while 16, including enolase, saccharopine dehydrogenase, flavohemoglobin, mannitol dehydrogenase, and malate dehydrogenase, were downaccumulated. Variations in protein abundance were investigated at the mRNA level by real-time RT-PCR analysis, which confirmed the proteomic data for 9 out of the representative 11 selected proteins.

There are a few proteomics studies on fungal spores published. The results in the characterization of *Penicillium* spores by MALDI-TOF MS with different matrices demonstrated its ability for the classification of fungal spores [277]. Recently, Sulc et al. [40] have reported protein profiling of intact *Aspergillus* ssp. spores, including some plant pathogenic species, by MALDI-TOF MS, and they built up a mass spectral database with twenty-four *Aspergillus* strains. Thus, these mass finger-printing generated by MS can be used for typing and characterizing different fungal strains and finding new biomarkers in host-pathogen interactions. Another study on *B. graminis* f.sp. *hordei* (*Bgh*) spores concluded with the first proteome of *Bgh*, using a combination of 2-DE and MS analyses and matched to NCBInr EST on *Bgh*-translated genome databases [43]. The identity of 123 distinct fungal gene products was determined, most of them with a predicted function in carbohydrate, lipid, or protein metabolism indicating that the conidiospore is geared for the breakdown of storage compounds and protein metabolites during germination correlating with previously reported transcriptomic data [278, 279]. These results allowed a functionally annotated reference proteome for *Bgh* conidia.

Holzmuller et al. [118] have reported a technique to isolate the fungal haustorium (specialised structures, existing in intimate contact with the host cell, are required by the pathogen to acquire nutrients from the host cell) from infected plants, using the barley powdery mildew as an experimental system. The technique is of relevance in the study of the molecular bases of biotrophy considering that biotrophic fungi, including downy mildews (Oomycota), powdery mildews (Ascomycota), and rust fungi (Basidiomycota), are some of the most destructive pathogens on many plants. Extracted proteins were separated and analyzed by LC-MS/MS. The searches were made against a custom *Bgh* EST sequence database and the NCBInr fungal protein database, using the MS/MS data, and 204 haustorium proteins were identified. The majority of the proteins appeared to have roles in protein metabolic pathways and biological energy production. Surprisingly, pyruvate decarboxylase (PDC), involved in alcoholic fermentation and commonly abundant in fungi and plants, was absent in both their *Bgh* proteome data set and in their EST sequence database. Significantly, BLAST searches of the recently available *Bgh* genome sequence data also failed to identify a sequence encoding this enzyme, strongly indicating that *Bgh* does not have a gene for PDC [44].

In order to overcome the low proteome coverage of most of the proteomic platforms available, this being related to the physicochemical and biological complexity and high dynamism range of proteins, different strategies directed at subfractionating the whole proteome have been developed, most of them involving cell fractionation. The analysis of the subcellular proteomes [280] not only allows a deeper proteome coverage but also provides relevant information on the biology of the different organules, protein location, and trafficking. The number of intracellular subproteomic studies carried out with fungal plant pathogens is minimum. Next we introduce a couple of papers appearing in the literature. The number of them devoted to the cell wall and extracellular fraction is much higher, and because of that a specific section is devoted to them. Hernández-Macedo et al. [131] have reported differences in the patterns of cellular and membrane proteins obtained from iron-sufficient and iron-deficient mycelia from *P. chrysosporium* and *L. edodes* by using SDS-PAGE and 2-DE. Mitochondria have also received attention. Grinyer et al. [121] were the first to publish a mitochondrial subproteome, describing a successful sample preparation protocol and mitochondrial proteome map for *T. harzianum*. Based on protein databases of *N. crassa*, *A. nidulans*, *A. oryzae*, *S. cerevisiae*, and *Schizosaccharomyces pombe*, they identified 25 unique mitochondrial proteins involved in the tricarboxylic acid cycle, chaperones, binding-proteins and transport proteins, as well as mitochondrial integral membrane proteins. More recently, the same researchers separated and identified 13 of the 14 subunits of the *T. reesei* 20S proteasome [281], providing the first filamentous fungal proteasome proteomics and paving the way for future differential display studies addressing intracellular degradation of endogenous and foreign proteins in filamentous fungi.

Relevant information on biological systems and processes comes from comparative studies in which genotypes, including mutants, developmental stages, or environmental conditions supply the knowledge inferred from the observed differences. Fungal pathogenicity requires the coordinated regulation of multiple genes (and their protein products) involved in host recognition, spore germination, hyphal

penetration, appressorium formation, toxin production, and secretion. To study the infection cycle and to identify virulence factors, proteomics provides us with a powerful tool for analyzing changes in protein expression between races and stages. However, most of these studies are made *in planta* after the plant inoculation, which is outside the scope of this review. In the case of plant fungal pathogens at least four papers have reported changes in the proteome at different developmental stages or strains. The dimorphic phytopathogenic fungus *Ustilago maydis* has been established as a valuable model system to study fungal dimorphism and pathogenicity. In its haploid stage, the fungus is unicellular and multiplies vegetatively by budding and undergoing a dimorphic transition infective filamentous growth. This process is coordinately regulated by the bW/bE transcription factor. Böhmer et al. [68] reported the first proteome reference map of *U. maydis* cells, in which proteins were identified combining 2-DE with MALDI-TOF MS and ESI-MS/MS analyses. The authors observed 13 proteins spots accumulated in greater abundance in the bW/bE-induced filamentous form than in the budding state. The majority of the identified proteins might have putative roles in energy and general metabolism. Comparison of Rac1- and -b-regulated protein sets supports the hypothesis that filament formation during pathogenic development occurs *via* stimulation of a Rac1-containing signalling module. The proteins identified in this study might prove to be potential targets for antibiotic substances specifically targeted at dimorphic fungal pathogens. Detailed information on the proteins can be found in an interactive map accessible at the MIPS Ustilago Maydis Database (MUMDB; http://mips.gsf.de/genre/proj/ustilago/Maps/2D/). The reference map generated from *U. maydis* had a coverage of 4% of all annotated genes, indicating the low proteome coverage encompassed by standard proteomic techniques.

By using 2-DE and MALDI-TOF MS, specific proteins of asexual life stages from *Phytophthora palmivora*, a pathogen of cocoa and other economically important tropical crops, were analyzed [58]. From 400 (cyst and germinated cyst) to 800 (sporangial) could be resolved. Approximately 1% of proteins appeared to be specific for each of the mycelial, sporangial, zoospore, cyst, and germinated cyst stages of the life cycle. Moreover, they made the protein profiles of parallel samples of *P. palmivora* and *P. infestans* and demonstrated that precisely 30% of proteins comigrated suggesting that proteomics could be used to proteptype *Phytophthora* spp. In this work, only three identified proteins were reported, corresponding to actin isoforms. More recently, Ebstrup et al. [59] performed a proteomic study of proteins from cysts, germinated cysts, and appressoria on *P. infestans* grown in vitro, identifying significant changes in the amount of several proteins. These identified proteins were most likely important for disease establishment and some of the proteins could therefore be putative targets for disease control. For example, downregulation of the crinkling- and necrosis-inducing (CRN2) protein in appressoria compared to germinated cysts and the discovery of upregulation of a putative elongation factor (EF-3) are of great interest. On the one hand, CRN2 protein might have an important function in the interaction with the host-plant before and after penetration into the leaf, this being a putative target for disease control. Since plants presumably do not contain EF-3, it could represent a putative antioomycete as well as a putative antifungal target. Furthermore, several representatives of housekeeping systems were upaccumulated, and these changes are most likely involved in the runup to the establishment of the infection of the host plant. The biotrophic fungal pathogen *U. appendiculatus* is the causal agent of rust disease of beans. Cooper et al. [67] surveyed the proteome from germinating and ungerminated asexual uredospores of this pathogen, using MudPIT MS/MS. The proteins identified revealed that uredospores require high energy and structural proteins during germination, indicating a metabolic transition from dormancy to germination.

The role of signal transduction in the pathogenecity of *Stagonospora nodorum* is well established and the inactivation of heterotrimeric G protein signaling caused developmental defects and reduced pathogenicity [282]. In a follow-up study, the *S. nodorum* wild-type and Galpha-defective mutant (gna1) proteomes were compared via 2-DE coupled to LC-MS/MS. By matching the protein mass spectra to the translate *S. nodorum* genome, the study identified several Gna1-regulated proteins, including a positively regulated short-chain dehydrogenase (Sch1) [65].

Cao et al. [61] released an evaluation of pathogenic ability of *Pyrenophora tritici-repentis* and the possible adaptation to a saprophytic habit of an avirulent race. This fungus causes tan spot, an important foliar disease of wheat, and produces multiple host-specific toxins, including Ptr ToxB, which is also found in avirulent isolates of the fungus. In order to improve the understanding of the role of this homolog and evaluate the general pathogenic ability of *P. tritici-repentis*, the authors compared both full mycelial and secreted proteomes of avirulent and virulent isolates of the pathogen, by 2-DE and ESI-q-TOF MS/MS. The proteomic analysis revealed a number of the proteins found to be upregulated in a virulent race, which has been implicated in microbial virulence in other pathosystems, such as the secreted enzymes a-mannosidase and exo-b-1,3-glucanase, heat-shock and bip proteins, and various metabolic enzymes, which suggests a reduced general pathogenic ability in avirulent race of *P. tritici-repentis*, irrespective of toxin production.

3.2.2. Extracellular and Cell Wall Proteins: The Secretome. Most eukaryotic plant pathogens initially invade the space between host cell walls (the apoplastic space), and much of the initial host defence and pathogen counter defence happens in the apoplast and commonly involves secreted pathogen and host-derived proteins and metabolites [283]. While some pathogens remain exclusively in the apoplast, such as *Cladosporium fulvum*, others, including mildews, rusts smuts, *Phytophthora*, and *Magnaporthe* species, breach host cell walls but remain external to and separated from the host cytoplasm by host and pathogen cell membranes. Some host wall-breaching pathogens, like rusts, mildews, and oomycetes, form specialised expanded hyphal protuberances

called haustoria whereas others, like maize smut and the rice blast fungi, use unexpanded but probably specialised intrahost cell wall hyphae [284]. The role of these structures was initially thought to be primarily nutrient acquisition, but recently their additional role in secretion of effectors, some of which are translocated to the host cytoplasm, has become more apparent. These issues have been recently reviewed by Ellis et al. [113].

The secretome has been defined as being the combination of native secreted proteins and the cell machinery involved in their secretion [285]. A defining characteristic of plant pathogenic fungi is the secretion of a large number of degradative enzymes and other proteins, which have diverse functions in nutrient acquisition, substrate colonization, and ecological interactions [286–288]. Several extracellular fungal enzymes, such as polygalacturonase, pectate lyase, xylanase, and lipase, have been shown or postulated to be required for virulence in at least one host-pathogen interaction [289–295]. Proteomics is the right approach to study the interaction between plants and microbes mediated by excreted molecules, the role of the cell wall and the interface, and to identify fungal protein effectors facilitating either infection (virulence factors, enzymes of the toxin biosynthesis pathways) or trigger defence responses (avirulence factors). In the light of this, it has been said that, unlike animals, "fungi digest their food and [then] eat it" [296], illustrating the large number of extracellular hydrolytic enzymes necessary to digest a plethora of potential substrates. Therefore, many of these proteins are of special interest in the study of plant pathogens [46, 64]. This might also be owing to the fact that secretome sample preparation is much faster and simpler than extraction and preparation of intracellular proteins. Next, a number of papers covering this topic are presented, including those dealing with the secretome of *Trichoderma* spp, a study directed at identifying proteins related to its biofungicidal activity.

Pioneering work on this field comes before the arrival of proteomics during the 1990s, with typical studies focused on the identification, purification, and characterization of single secreted proteins, under the influence of the biotechnology industry for the production of enzymes for commercial and industrial use [297]. The first complete proteomic study of secreted proteins was released on the filamentous fungus *A. flavus* [41, 42]. The interest of this study was the ability of both *A. flavus* and *A. parasiticus* to degrade the flavonoids that plants produce as typical secondary metabolites against invading microorganisms. The secreted proteins were analyzed by 2-DE and MALDI-TOF mass spectrometry, with 15 rutin-induced proteins and 7 noninduced proteins identified, among them enzymes of routine catabolism pathway and glycosidases.

In *F. graminearum*, a devastating pathogen of wheat, maize, and other cereals, Phalip et al. [51] investigated the exoproteome of this fungus grown on glucose and on plant cell wall (*Humulus lupulus*, L.). The culture medium was found to contain a larger amount of proteins and these were more diverse when the fungus grew on the cell wall. Using both 1-DE and 2-DE coupled to LC-MS/MS analysis and protein identification based on similarity searches, 84 unique proteins were identified in the cell wall-grown fungal exoproteome and 45% were implicated in plant cell wall degradation. These cell wall-degradating enzymes were predominantly matches to putative carbohydrate active enzymes implicated in cellulose, hemicelluloses, and pectin, catabolism. As expected, *F. graminearum* grown on glucose produced relatively few cell wall-degrading enzymes. These results indicated that fungal metabolism becomes oriented towards the synthesis and secretion of a whole arsenal of enzymes able to digest almost the complete plant cell wall.

The secretome has also been analyzed in *S. sclerotium* as commented above [64]. In this study, 52 secreted proteins were identified and many of the annotated secreted proteins were cell wall-degrading enzymes that had been identified previously as pathogenic or virulence factors of *S. sclerotium*. However, one of them, α-L-arabinofuranosidase, which is involved in the initiation or progression of plant diseases, was not detected by previous EST studies, clearly demonstrating the merit of performing proteomic research.

Two studies have been published reporting the *B. cinerea* (B05.10) secreted proteins analysis [48, 49]. First, secretions were collected from fungus grown on a solid substrate of cellophane membrane while mock infecting media supplemented with the extract of full red tomato, ripened strawberry, or *Arabidopsis* leaf extract. Overall, 89 *B. cinerea* proteins were identified by high-throughput LC-MS/MS from all growth conditions. Sixty of these proteins were predicted to contain a SignalP motif indicating the extracellular location of the proteins. The proteins identified were transport proteins, proteins well-characterized for carbohydrate metabolism, peptidases, oxidation/reduction, and pathogenicity factors that could provide important insights into how *B. cinerea* might use secreted proteins for plant infection and colonization [48]. In the second work, the impact of degree of esterification of pectin on secreted enzyme of *B. cinerea* was studied, because changes during the ripening process of fruits appear to play an important role in the activation of the dormant infection. All the major components of the fruit cell wall (pectin, cellulose, hemicellulose) undergo these changes. By 1-DE and LC-MS/MS, 126 proteins were identified and 87 proteins were predicted secreted by SignalP, some of them being pectinases. The results showed that the growth of *B. cinerea* and the secretion of proteins were similar in cultures containing differently esterified pectins, and therefore it is likely that the activation of this fungi from dormant state is not solely dependent on changes in the degree of esterification of the pectin component of the plant cell wall [49]. Therefore, future studies of the *B. cinerea* secretome in infections of ripe and unripe fruits will provide important information for describing the mechanisms that the fungus employs to access nutrients and decompose tissues.

Using the plant pathogenic fungus *L. maculans* and symbiont *Laccaria bicolor* grown in culture, Vincent et al. [56] established a proteomic protocol for extraction, concentration, and resolution of the fungal secretome. These authors used both broad and narrow acidic and basic pH range in IEF. The quality of protein extracts was assessed by both 1-DE and 2-DE and MS identification. Compared

with the previously published protocols for which only dozens of 2-DE spots were recovered from fungal secretome samples, in this study, up to approximately 2000 2-DE spots were resolved. This high resolution was confirmed with the identification of proteins along several pH gradients as well as the presence of major secretome markers such as endopolygalacturonases, beta-glucanosyltransferases, pectate lyases, and endoglucanases. Thus, shotgun proteomic experiments evidenced the enrichment of secreted protein within the liquid medium.

One of the earliest works was released on *Trichoderma reesei* mycelium cell wall, one of the most powerful producers of extracellular proteins, this study being justified in order to find out the protein secretory pathways and the effect of the fungal genus, strain, and media condition on the excretion through the cell wall [298]. A total of 220 cell envelope-associated proteins were successfully extracted and separated by 2-DE from *Trichoderma reesei* mycelia actively secreting proteins and from mycelia in which the secretion of proteins is low. Out of the 52 2-DE spots subjected to ESI-TOF MS, 20 were identified, with HEX1, the major protein in Woronin body, a structure unique to filamentous fungi, being the most abundant one. Suárez et al. [299] studied the secretome of *T. harzianum* grown using either chitin (a key cell wall component) or cell wall of other fungi (*R. solani*, *B. cinerea*, or *Pythium ultimum*) as a nutrient source. For each different substrate, they found significant differences in 2-DE maps of extracellular proteins. However, despite these differences, the most abundant protein under all conditions was a novel aspartic protease (P6281), which showed a strong homology with polyporopepsin from *Irpex lacteus*. This led to speculation that this protein plays a fundamental role in the parasitic activity of *Trichoderma* spp. Marra et al. [273] have studied interactions between *T. atroviride*, two different fungal phytopathogens (*B. cinerea* and *R. solani*), and plants (bean). Two-DE was used to analyze separately collected proteomes from each single, two- or three-partner interaction. Then, differential proteins were subjected to MALDI-TOF MS and in silico analysis to search homologies with known proteins. Thus, a large number of protein factors associated with the multiplayer interactions examined were identified, including protein kinases, cyclophilines, chitine synthase, and ABC transporters. Recently, another similar study was released between *T. harzianum* and *R. solani* by analysing the secretome to identify the target proteins that are directly related to biocontrol mechanism [274]. Seven cell-wall degrading enzymes, chitinase, cellulase, xylanase, β-1,3-glucanase, β-1,6-glucanase, mannanase, and protease, were revealed by activity assay, in-gel activity stain, 2-DE, and LC-MS/MS analysis, these being increased in response to *R. solani*.

A cell wall proteome has been proposed for the oomycete *Phytophthora ramorum*, the causal agent of sudden oak death, in order to study its pathogenic factors [60]. This study showed an inventory of cell wall-associated proteins based on MS sequence analysis. Seventeen secreted proteins were identified by homology searches. The functional classification revealed several cell wall-associated proteins, thus suggesting that cell wall proteins may also be important for fungal pathogenicity.

The filamentous fungus *Neurospora crassa* is a model laboratory organism but in nature is commonly found growing on dead plant material, particularly grasses. Using functional genomics resources available for *N. crassa*, which include a near-full genome deletion strain set and whole genome microarrays, Tian et al. [300] undertook a system-wide analysis of plant cell wall and cellulose degradation, identifying approximately 770 genes that showed expression differences when *N. crassa* was cultured on ground *Miscanthus* stems as a sole carbon source. An overlap set of 114 genes was identified from expression analysis of *N. crassa* grown on pure cellulose. Functional annotation of upregulated genes showed enrichment for proteins predicted to be involved in plant cell wall degradation, but also many genes encoding proteins of an unknown function. As a complement to expression data, the secretome associated with *N. crassa* growth on Miscanthus and cellulose was determined using a shotgun MudPIT proteomic strategy. Over 50 proteins were identified, including 10 of the 23 predicted *N. crassa* cellulases. Strains containing deletions in genes encoding 16 proteins detected in both the microarray and mass spectrometry experiments were analyzed for phenotypic changes during growth on crystalline cellulose and for cellulase activity. While growth of some of the deletion strains on cellulose was severely diminished, other deletion strains produced higher levels of extracellular proteins that showed increased cellulase activity. These results show that proteomics in combination with other powerful tools available in model systems such as *N. crassa* allow for a comprehensive system level understanding of fungal biology.

3.2.3. Interactomics. The biological organization in living cells can be regarded as being part of a complex network [301–303]. Traditional approaches studied a single gene or unique protein and therefore did not provide a complete knowledge of the biological processes. Proteins release their functional roles through their interactions with one another in vivo. Thus, developing a protein-protein interaction (PPI) network can lead to a more comprehensive understanding of the cell processes [304]. Interactomics is a discipline at the intersection of bioinformatics and biology that deals with studying both the interactions and the consequences of those interactions between and among proteins and other molecules within a cell [305]. The network of all such interactions is called the interactome. Interactomics thus aims to compare these interaction networks (i.e., interactomes) between and within species in order to find how the traits of such networks are either preserved or varied. Interactomics is an example of top-down systems biology, which takes an overhead, as well as overall, view of a biosystem or organism.

In recent years, high-throughput methods have been implemented to identify PPIs [306–310] and these have recently been reviewed in [311, 312]. Using these experimental methods, such as yeast two-hybrid screens, PPI networks for a series of model organisms were determined and allowed

us to better understand the function of proteins at the level of system biology. Two-hybrid screening (also known as yeast two hybrid system or Y2H) is a powerful tool for identifying PPI. The premise behind the test is the activation of dowstream reporter gene(s) by the binding of a transcription factor onto an upstream activating sequence (UAS). For the purposes of two-hybrid screening, the transcription factor is split into two separate fragments, called the binding domain (BD) and activating domain (AD). The BD is the domain responsible for binding to the UAS and the AD is the domain responsible for activation of transcription [313].

In parallel with the large-scale experimental determination of PPI, many PPI prediction methods were also developed. These methods are based on diverse attributes, concepts, or data types, such as interolog [314], gene expression profiles [315], gene ontology (GO) annotations [316], domain interactions [317], coevolution [318], and structural information [319]. Some machine learning methods, such as support vector machines (SVMs) have also been used to predict PPIs [320, 321]. Among the above-mentioned computational methods the interolog approach has been widely implemented [322] and has proved to be reliable for predicting PPI from model organisms [323]. The core idea of the interolog approach is that many PPIs are conserved in different organisms [324]. Accumulated PPI data from model organisms as well as advances in detecting orthologous proteins in different organisms [280] have continuously made the interolog method an increasingly powerful tool for constructing PPI maps for entire proteomes.

Using the interolog method, He et al. [57] constructed the first PPI network for *M. grisea*. Thus, 11674 PPIs among 3017 *M. grisea* proteins were deduced from the experimental PPI data in different organisms, although the predicted PPI network covered approximately only one-fourth of the fungal proteome and may still contain many false-positives. Moreover, they built two subnets called pathogenicity and secreted proteins networks, which may be helpful in constructing an interactome between the rice blast fungus and rice (MPID website, http://bioinformatics.cau.edu.cn/zzd_lab/MPID.html).

A *F. graminearum* protein-protein interaction database providing comprehensive information on protein-protein interactions based on both interologs from several protein-protein interaction of seven species and domain-domain interactions experimentally determined based on protein is available at http://csb.shu.edu.cn/fppi [55]. It contains 223 166 interactions among 7406 proteins for *F. graminearum*, covering 52% of the whole *F. graminearum* proteome.

4. Concluding Remarks

In the current scientific scenario, Proteomics should be understood to be part of a multidisciplinary approach. A combination of high-throughput "Omics" (Genomics, Transcriptomics, Proteomics, and Metabolomics) and classical biochemistry and cell biology techniques should be used for data validation and to deepen the knowledge of living organisms. Proteomic techniques are used to characterize a specific protein or a structural or functional group of proteins. This is what we can call "Hypothesis-driven Proteomics", "Targeted Proteomics", or "Proteinomics". This type of study will provide relevant information on protein structure and function, isoforms, organs, cells, and subcellular location and trafficking, processing, signal peptides, PTMs, expression kinetics, and correlation with RNA and metabolites. At the same time, it is a method for validating data obtained using one specific approach [102].

Despite the continuous development and improvement of powerful proteomic techniques, protocols, equipments, and bioinformatic tools, just a minimal fraction of the cell proteome, and for only a few organisms, has been characterized so far. This is mainly due to the enormous diversity and complexity of proteomes, and to technical limitations in quantification, sensitivity, resolution, speed of data capture, and analysis.

In the field of Fungal MS-based Proteomics, great progress has been made in past years. This is because of the increasing number of fungal genomes available and the developments in sample preparation, high-resolution protein separation techniques, MS, MS software for effective protein identification and characterization, and bioinformatics technology. The tremendous diversity and genome flexibility in fungi, however, will make this task a difficult one. Thus, a key step in sequence analysis is the annotation. The existing programs for automated gene prediction are not perfect and need to be improved or trained better. Follow-up manual annotation is also necessary to improve the accuracy of automated annotation, but this is time-consuming and labor-intensive. Ultimately, a comprehensive genome database similar to YPD (http://www.yeastgenome.org/) will be desirable for fungal pathogens.

To date, most proteomic studies in plant pathogenic fungi have been limited to 1- and 2-DE analysis. However, various powerful proteomic methods have been developed for genomewide analysis of protein expression, protein localization, and protein-protein interaction in fungi. Whole-genome protein arrays and systematic yeast two-hybrid assays have been used to characterize the yeast proteome and interactome. Integration of large-scale genomics and proteomics data enables the elucidation of global networks and system biology studies in yeast. It is necessary for similar advanced proteomic resources to be soon available for some fungal plant pathogens. Moreover, second-generation MS technologies for Quantitative Proteomics such as 2-DIGE, stable isotope labeling, (ICAT, iTRAQ and SILAC), or label-free methods (peak integration, spectral counting) have not yet begun or are beginning to be applied to Fungal Proteomics research.

Otherwise, the major challenge is the analysis and significance of PTMs because proteins have properties arising from their folded structure and so generic methods are difficult to design and apply.

In conclusion, since plant pathogenic fungi cause important losses in a number of crops, it is necessary to make high-throughput studies on these organisms to identify pathogenicity factors. Although genomics-based investigation of host-pathogen interactions can provide valuable

information on the changes in gene expression, the investigation into changes in protein abundance is also important, in order to identify those proteins that are essential during such interactions. This is because there is often a poor correlation between transcript and protein abundance [325]. Proteomics analysis is an excellent tool that can give us a great deal of information about fungal pathogenicity by high-throughput studies. This approach has allowed the identification of new fungal virulence factors, characterizing signal transduction or biochemical pathways, studying the fungal life cycle and their life-style. We can use this information to provide new targets for disease crop diagnosis focused on fungicide design. Otherwise, the secretome analysis is especially important because fungi secrete an arsenal of extracellular enzymes to break down the plant cell wall for pathogen penetration and nutrient consumption. In this sense, Proteomics allows us to identify numerous differential proteins involved in multiple-player cross-talk normally occurring in nature between plants and pathogens, the so-called "interaction proteomes". Finally, MS-based Proteomics can help us to characterize fungal strains and find new biomarkers in host-pathogen interactions.

In short, Fungal Proteomics is in the first step. Therefore, we still have a long way to go in the "Omics" of Plant Pathogenic Fungi compared to studies made in Humans, Bacteria, Yeast, or Plants. The important investment made by both the public and private sector in recent years augurs good prospects in fungal proteomics research in the future.

Abbreviations

1-DE:	One-dimentional electrophoresis
2-DE:	Two-dimentional electrophoresis
ESI:	Electrospray ionization
HPLC:	High performance liquid chromatograohy
IEF:	Isoelectrofocusing
LC:	Liquid chromatography
MALDI:	Matrix-assisted laser desorption/ionization
MS:	Mass spectrometry
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TOF:	Time of flight.

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