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An Overview of Proteomics Tools for Understanding Plant Defense Against Pathogens

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Abstract
Plant diseases are responsible for important losses in crops and cause serious impacts in agricultural production. In the last years, proteomics has been used to examine plant defense responses against pathogens. Such studies may be pioneer in the generation of crops with enhanced resistance. In this review, we focus on proteomics advances in the understanding of host and non-host resistance against pathogens.

Introduction
The term "Proteomics" was first introduced by Marc Wilkins in 1994, as a data-rich discipline that uses Mass Spectrometry (MS) and bioinformatics to characterize the proteome of a cell (Rossignol, 2001; Wilkins et al., 2008; Wilkins, 2009). Unlike genomic tools, proteomics are useful to know the identity, abundance, turnover rate, Post-Translational Modifications (PTMs) and interactions of different proteins in a mixture (Hirano et al., 2004). Protein identification has become possible by coupling gel-based techniques to MS in which samples are converted into gaseous ions and detected by a mass spectrometer according to their mass to charge ratio (m/z) (Quirino et al., 2010; Lodha et al., 2013). With a second mass spectrometer (MS/MS) the amino acid sequence can be inferred (Quirino et al., 2010). Also, gel-free approaches have been recently developed such as Liquid Chromatography (LC) (Quirino et al., 2010).

Nowadays, proteomics assays can be solved by two different approaches referred to as 'bottom-up' and 'top-down'. In bottom-up proteins are digested with a protease prior to MS identification (Tipton et al., 2011). On the other hand, top-down proteomics is applied to intact proteins (Catherman et al., 2014; Kiss et al., 2014; Tran et al., 2011).

Worldwide, plants are affected by pathogens and insects attacks, which are serious threats to agricultural industries and biodiversity conservation. To protect themselves, plants have developed innate immune and resistance systems. Once the first barrier (cell wall) is overcome by the pathogen, the plant detects conserved pathogen structures named Pathogen or Microbial Associated Molecular Patterns (PAMPs or MAMPs, respectively) through their recognition with specific plant receptors named Pattern Recognition Receptors (PRRs). These interactions trigger PAMP-Triggered Immunity (PTI) that constitutes the first step of active plant defense. PTI response involves signaling through the second messenger cGMP, Mitogen-Activated Protein Kinases (MAPKs), Ca^{2+} and H^{+} influx, early accumulation of Reactive Oxygen Species (ROS), cell wall thickening, and enhanced expression of Pathogenesis-Related (PR) genes (Lannoo and Van Damme, 2014). Successful pathogens suppress and overcome PTI responses by the secretion of effectors resulting in Effector-Triggered Susceptibility (ETS). This interaction is named compatible and causes plant disease. However, some plants have evolved Resistance (R) proteins which are typically intracellular NB-LRR (Nucleotide-Binding Sites-Leucine-Rich Repeat) type receptors that recognize the pathogen effectors activating the so-called Effector-Triggered Immunity (ETI). The ability to trigger ETI is strain or race specific and is associated with programmed cell death, which is referred to as the Hypersensitive Response (HR) and Systemic Acquired Resistance (SAR). This interaction is named incompatible and includes non-host interactions (Jones and Dangl, 2006; Chisholm et al., 2006; Afroz et al., 2013; Zimaro et al., 2011).

In this review, we will specifically discuss current proteomics techniques that constitute valuable approaches to characterize the defense that plants mount during their battle against pathogens.

Protein identification by Mass Spectrometry
Mass spectrometry is crucial for protein identification and specifically Peptide Mass Fingerprinting (PMF) employs different algorithms to compare analyzed proteins spectra with known standards (Li and Assmann, 2000; Quirino et al., 2010; Lodha et al., 2013). Moreover, tandem mass spectrometry (MS/MS) allows 'de novo' sequencing (Hirano, et al., 2004; Quirino et al., 2010). MS includes: an ion source, a mass analyzer and a detector (Hirano et al., 2004). Although there are several ionization techniques, Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption/Ionization (MALDI) are the most commonly employed (Lodha, et al., 2013; Hirano et al., 2004; Quirino, et al., 2010).

Besides, major technical breakthroughs such as the generation of more complex analyzers (Time-Of-Flight or TOF, and Quadrupole or Q mass analyzer) and the advent of high-resolution mass spectrometers, like Orbitrap, have

turned MS in a key player to study the proteome (Yang, et al., 2015). However, it is necessary to select the accurate MS instrument for a particular analysis. In general, Q-MS and Ion Trap-MS (IT-MS) are combined with ESI while TOF-MS is associated with MALDI. TOF has been coupled to quadrupole (Q-TOF), and combinations such as MALDI-TOF/TOF and MALDI-Q-TOF were also developed (Hirano et al., 2004). The evolution of MS has turned it into an essential technology for many proteomics applications in the study of plant resistance. We next describe some of the most used techniques based on gel electrophoresis and also gel-free technologies in plant resistance characterization. In Figure 1, a schematic representation of them is depicted (Figure 1).

Two Dimensional Polyacrylamide Gel Electrophoresis

Two Dimensional PolyAcrylamide Gel Electrophoresis (2D-PAGE) is used to separate proteins from a mixture providing information such as: molecular weight, isoelectric point, presence or absence of proteins in a sample and PTMs (Görg et al., 2004; Quirino et al., 2010; Lodha, et al., 2013).

2D-PAGE methods combined with PMF or MS/MS have been used to study the interaction of rice with *Magnaporthe oryzae* that causes the most devastating disease in cultivated area. Differentially expressed protein spots were observed in rice apoplast after *M. oryzae* infection and a

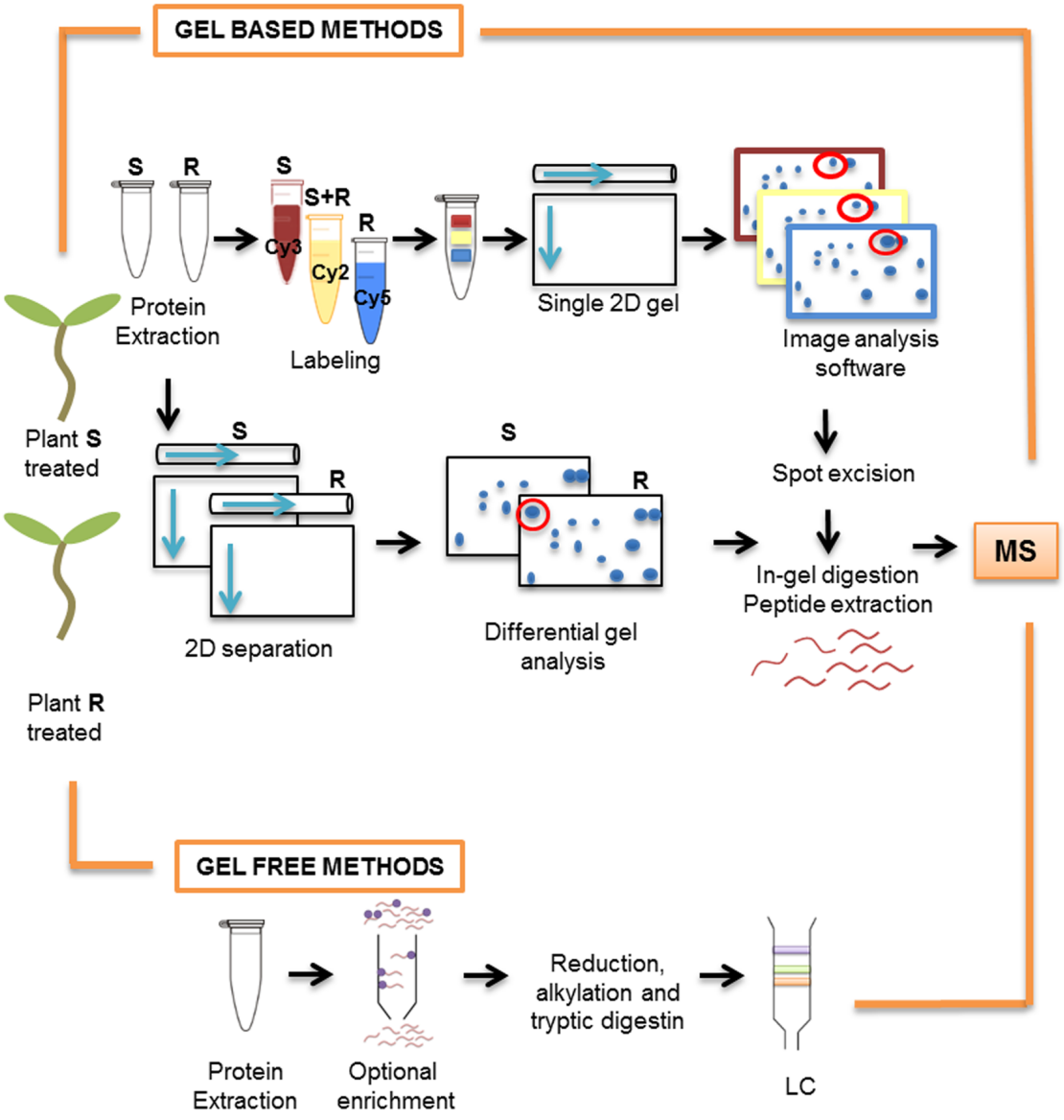

Figure 1. Schematic overview of protocols employed for some of the gel-based and gel-free proteomics techniques described in this review. In this example, protein extracts are obtained from two plants samples: susceptible and resistant denoted S and R, respectively. Differential spots are red highlighted. MS: Mass Spectrometry, LC: Liquid Chromatography.

number of proteins were identified by MALDI-TOF-MS. A number of proteins including: Glycosyl Hydrolase (GH) proteins, PR and antioxidant proteins, subtilisin-like proteases and OsDUF26 were strongly expressed, suggesting that secreted proteolytic enzymes modulate plant resistance in this plant-pathogen interaction (Wu et al., 2014).

MS/MS was used to study corn resistance to the fungus *Curvularia lunata*. Protein markers related to host resistance were identified using four maize inbred lines with different resistance levels. The number of spots presenting quantitative differences was higher in resistant inbred lines than in susceptible ones. Also, a larger amount of up-regulated protein was detected in resistant maize. These proteins were identified as GLPs (Germin-Like Proteins), eIF-5A (Translation Initiation Factor 5A), OEE 1 (Oxygen-Evolving Enhancer Protein 1), OEE 2 (Oxygen-Evolving Enhancer Protein 2), and OEC (Oxygen-Evolving Complex), and they have been associated with host defense and resistance to pathogen infection (Huang et al., 2009).

Non-host resistance has also been studied by 2D-PAGE followed by MS/MS analyzing the non-host response of rice to the rust fungi *Puccina striiformis* f. sp. *tritici*. The proteins related to this non-host resistance were mainly involved in H_2O_2 production and glycerol-3-phosphate signaling (Zhao et al., 2014).

Two-Dimensional Differential in-Gel Electrophoresis

Two Dimensional Differential in-Gel Electrophoresis (2D-DIGE), a 2D-PAGE modification, is a powerful technique that detects reproducibly differences between two protein samples simultaneously on the same gel (Unlü et al., 1997). When performing 2D-DIGE, different samples are covalently labeled with distinct spectrally resolvable fluorophores, combined and then fractionated together on a single 2D gel. Frequently, a third sample known as internal standard, composed of a mixture of equal amounts of experimental samples, is applied in the analysis. This enables to relativize each protein to its corresponding spot in the internal standard in the same gel. Then, changes between proteins abundances are visualized using fluorescence imaging (Marouga et al., 2005). 2D-DIGE and MALDI-TOF-MS/MS were used to detect differential proteins spots in resistant and susceptible maize ecotypes during sugarcane mosaic virus infection. Identified proteins were subdivided into functional categories: stress and defense response, energy and metabolism, photosynthesis, protein synthesis and folding, carbon fixation, structure protein and signal transduction/transcription. A high proportion of proteins identified as stress and defense related, were up-accumulated in the resistant maize genotype. Furthermore, 17 novel proteins that have not been previously described as virus-responsive were identified, providing novel data into the molecular mechanism of plant-virus interactions (Wu et al., 2013).

Another example in which 2D-DIGE was used led to the identification of rice defense related proteins in the incompatible interaction with the fungus *M. oryzae* when compared to proteins detected in the compatible interaction. Noticeably, similar proteins were identified in both incompatible and compatible interactions. However, the incompatible interaction caused an earlier and higher expression of these proteins in resistant plants in comparison with susceptible ones, which might determine resistance to the pathogen (Kim et al., 2013).

Gel-Free Technologies

Although gel based techniques allow rapid, simple and reproducible separation of proteins, they present several limitations, such as separation of high molecular weight and basic proteins (Rossignol, 2001; Agrawal, 2013; Quirino et al., 2010). These restrictions have been overcome with the application of the "shotgun" method and gel-free approaches like LC-MS/MS (Hirano et al., 2004). Hence, prior to MS analysis, LC separation is performed which is based on Strong Cation eXchange (SCX) and Reversed Phase (RP) chromatography (Hirano et al., 2004; Agrawal et al., 2013). LC-MS/MS was used to study the compatible *Pseudomonas syringae-Arabidopsis thaliana* interaction (Hurley et al., 2014). Virulence of *P. syringae* relies on the Type III Secretion System (T3SS) to inject effector proteins into host cells (Afroz et al., 2013; Hurley et al., 2014). These effectors are able to suppress the initial PTI during compatible plant-pathogen interaction by targeting PRR complexes and downstream signaling components. Recognition of the AvrRpt2 effector by the *A. thaliana* the RPS2 (Resistance to *P. syringae* 2) immune receptor activates ETI. Nevertheless, HopF2 effector suppresses this ETI-response. Using LC-MS/MS, the proteins that co-purified with HopF2 in *A. thaliana* were identified, and this analysis was relevant to determine protein functionality. A high molecular weight complex co-purified with HopF2, and members corresponded to membrane associated proteins, that included AHA2 and ERD4 (involved in the closing of stomata in response to *P. syringae)*, and others proteins (AtHIR2 and AtREM1.3) that play a role in pathogen perception (Hurley et al., 2014).

Proteomics methods to analyze post-translational modifications

PTMs of amino acids extend the range of functions of the protein. Enzymes can cleave amino acids or append different functional groups such as acetate, phosphate, various lipids and carbohydrates, changing the activity state, localization, turnover, and interactions with other proteins (Mann and Jensen, 2003). Plants proteins undergo PTMs in response to pathogens and other stimuli. The most intensely studied PTM is phosphorylation (Jones et al., 2013) that is crucial in plant resistance events by fine-tuning regulation of protein function (Yang et al., 2013). Phosphoproteins are usually present in a low copy number, so their detection is challenging. Generally an enrichment step is performed to obtain protein mixtures in which phosphoproteins are abundant.

We next summarize proteomics methods involving the study of PTMs in plant defense. For instance in tomato, *Cladosporium fulvum* resistance genes (Cf) recognize fungal effectors resulting in HR and resistance, and

phosphorylation mediates Cf cascades. Leaves extracts from infected and no infected tissue were subjected to phosphopeptide identification and label-free quantification by LC-MS/MS (Matthiesen et al., 2005). Titanium dioxide (TiO_2) enrichment followed by LC-ESI-QTOF allowed the identification of phosphoproteins. TiO_2 possess high affinity for phosphopeptides, which are then eluted using ammonia solution (Yu and Veenstra, 2013). Results showed that photosynthesis is quickly suppressed upon HR and differentially phosphorylated Hsp90 (Heat Shock Protein 90) chaperon isoforms are expressed, suggesting a role in plant defense. Likewise, infected tissue underwent a switch to anaerobic respiration (Stulemeijer et al., 2009). Plant defense related proteins were indentified in wheat resistance to the fungus *Septoria tritici* by comparing resistant and susceptible infected plants (Yang et al., 2013). Protein extracts were subjected to TiO_2 enrichment. Hydrophilic Interaction LIquid Chromatography (HILIC) fractionation and LC-MS/MS analysis were conducted. Differential phosphorylation was observed for GTP-binding proteins, 14-3-3 proteins, and calcium-binding proteins. HILIC allows separation of small polar compounds because they present good solubility in the mobile phase of HILIC, overcoming the poor solubility encountered in LC (Kahsay et al., 2014).

In addition, rice phosphoproteome was studied during the interaction with *M. oryzae*. Changes of phosphoprotein profiles in rice leaves were analyzed during early compatible and incompatible interactions using PEG (Polyethylene Glycol) prefractionation to deplete RuBisCO, and by enrichment of low abundant phosphoproteins through $Al(OH)_3$ Metal-Oxide Affinity Chromatography ($Al(OH)_3$ MOAC), 2D-PAGE, Pro-Q diamond phospho-protein stain (Pro-Q DPS) and MS. Spots were subjected to MALDI-TOF/TOF-MS and many *M. oryzae*-regulated phosphoproteins involved in plant resistance were found. Among them, signaling-related, microtubule-associated, amino acid synthesis-related proteins and energy-related enzymes were successfully identified (Li et al., 2015).

N-Glycosylation and Lys-Acetylation were analyzed in grapevine proteins in response to *Lobesia botrana* (Melo-Braga et al., 2012). Enrichment in glycopeptides was achieved by Zwitterionic-Hydrophilic Interaction Liquid Chromatography (ZIC-HILIC). This method involves Trifluoroacetic Acid (TFA) and Ion Pairing (IP) to increase the hydrophilicity difference between glycopeptides and non-glycosylated peptides. Also, Lys-Acetylation was studied using an anti-acetyllysine antibody to immunoprecipitate acetylated peptides. At last, about 30% grapevine proteins differed in abundance in infected tissue related to an uninfected control (Melo-Braga et al., 2012). Another type of PTM observed in resistance proteins is the S-acylation (palmitoylation) that consists in the addition of fatty acids to cysteine residues (Kim et al., 2005). To study this PTM, sample preparation must include blocking the free cysteines with N-ethylmaleimide followed by removal of S-acyl groups by hydroxylamine treatment. Exposed cysteines are tagged with biotin and then purified and analyzed by MS. For example, FLS2, the plant receptor for bacterial flagellin, has been found to be S-acylated. Apart from FLS2 and RPS2 previously observed to be palmitoylated (Kim et al., 2005), several other S-acylated proteins have been identified, such as RPS2-containing complexes and the calcium channels ACA8 and ACA10. This information confers an important role for S-acylation in pathogen recognition and plant resistance (Hemsley et al., 2013).

Surveying plant cellular compartments and tissues through proteomics

Subcellular proteomics consist in the study of specific plant fractions such as the apoplast, organelles or plasma membrane. By this approach, the complexity of protein extracts can be reduced to have a major representation in a defined compartment of the plant cell (Delaunois et al., 2013; Quirino et al., 2010).

Membrane fractions of *A. thaliana* following activation of RPS2 have been analyzed by a large scale proteomics approach. Plasma Membrane (PM) proteins enrichment was achieved using the aqueous two-phase partitioning method and they were analyzed by LC-MS/MS. In total 2303 *Arabidopsis* proteins were detected and 22% of the up-regulated proteins were previously characterized in plant immunity or cell death regulation (Elmore et al., 2012). These results suggest that the PM proteome is dynamic during plant immune signaling and the activation of RPS2 initiates ETI signaling and elicits HR.

Many important events take place in the apoplast where pathogen and host interact, thus this compartment has a crucial role in plant defense mechanisms. Apoplastic fluid is usually isolated using Vacuum Infiltration Centrifugation (VIC) method. The tissue is infiltrated with extraction buffer, then the surface is dried and leaves are rolled and inserted into tubes. Finally, centrifugation allows the recovery of apoplastic proteins (Delaunois et al., 2014). In a recent report, grapevine leaves apoplastic fluid showed defense proteins such as osmotin and thaumatin-like proteins, suggesting the presence of preformed defenses. Chitinases and glucanases, that degrade fungal cell walls, were also represented. Furthermore, peroxidases and superoxide dismutase were found actively involved in the plant defense response (Delaunois et al., 2014).

MALDI Imaging Mass Spectrometry

MALDI Imaging Mass Spectrometry (MALDI-IMS) is the combination of molecular mass analysis and spatial information. This most recent technology can be used to detect, identify and localize specific molecules such as drugs, lipids and proteins directly from intact tissue sections. Essentially, MALDI-IMS allows direct measurement of mass spectra from tissue, and the distribution of the detected compounds can be visualized as an image, representing 2D distribution maps of selected components (Amstalden van Hove et al., 2010; Burnum et al., 2008; Goodwin et al., 2008; Rohner et al., 2005). Until today, MALDI-IMS has not been used to identify proteins involved in plant-pathogen interactions, even though recent reports showed the identification and localization of other molecules involved in plant resistance. For instance, the differential accumulation of the flavonoid hesperidin in

Citrus sinensis grafted on *Citrus limonia* cv Pêra using MALDI-IMS was studied with the aim to analyze if the accumulation of this compound and tolerance to citrus variegated chlorosis bacterium were correlated (Soares et al., 2015). Transversal sections of leaves and petioles were analyzed to obtain different ion intensities of hesperidin from infected and healthy tissue, as well as to determine its anatomical distribution in both tissues. By MALDI-IMS, the highest ion intensities of hesperidin were observed from infected plants when compared with healthy tissues in all analyses. In petioles of infected plants, hesperidin was detected in whole tissues, while in leaf transversal sections it was observed mainly in xylem, collenchyma and mesophyll cell types. In healthy plants hesperidin ions were low-abundant and after infection, relocalization occurred (Soares et al., 2015).

MALDI-IMS has also been used to investigate the *in-situ* response to the pathogen *Plasmopara viticola.* This plant produces high amounts of toxic compounds against fungi such as resveratrol*,* pterostilbene and viniferins. Grapevine infected leaves showed differential presence of compounds in susceptible and resistant varieties. In a susceptible variety, less toxic compounds for fungi were detected on the infection sites, and the viniferins localized too far away from infection sites to play a real antifungal role. Using this technology, locations from a range of compounds in both susceptible to resistant grapevine species is now possible to understand their role in defense (Becker et al., 2014). Even if this technique has not been used to analyze proteins yet, it is challenging to apply this technology to the *in situ* identification of plant defense proteins during both PTI and ETI.

Concluding remarks

A major challenge for scientists is to direct efforts to control plant diseases, and improve resistance to pathogens. Phytopathogens threaten agriculture, and despite efforts to manage crop diseases, pathogens account for losses of 15% of worldwide food production. The understanding of the host molecular events leading to the setting up of resistance mechanisms and its functional characterization is essential to adopt new technologies for plant breeding and crop improvement. In this means, the current omics technologies provide a range of methods which offer new opportunities to develop strategies that are likely to provide durable disease resistance.

Data from genomes states that human beings are 98% chimpanzees, and chimpanzees are 98% human beings as well. According to this, only 2% of the genomic information differentiates both species, but this is not enough to explain the apparent differences. A genome containing 30,000 genes can usually generate 100,000 mRNAs. Moreover, those mRNAs can generate 400,000 proteins that together constitute the proteome in a certain condition or stage. Thus, the genome provides insights about events that could potentially occur in a cell or in an organism and transcriptomic data report transcripts levels that are not always translated into a functional protein. The proteome tells us exactly what is occurring in a specific state of a cell, a certain tissue or an organism and, the interactome, defined by physical interactions between proteins and molecules, allow us to predict what might occur in the living cell or tissue analyzed.

To elucidate plant-pathogen interactions, numerous studies were carried out from plant and pathogen perspectives, revealing that these interactions involve a complex and specific communication. Here, we present evidence of the efficacy and convenience to apply these techniques to the study of plant-pathogen interactions and the consequent leading molecular events at the protein level that ultimately confer resistance to the host plant.

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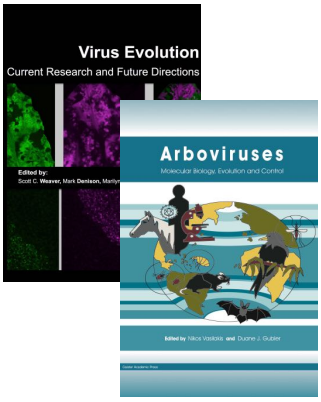

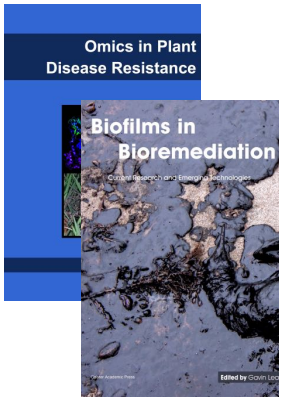

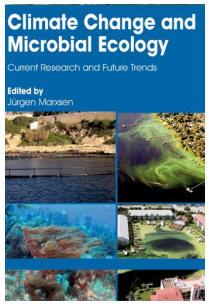

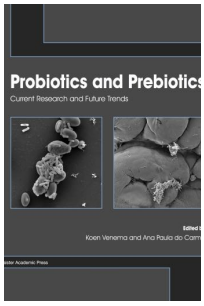

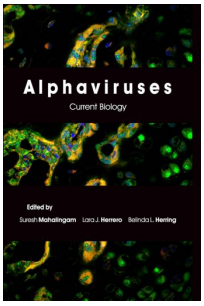

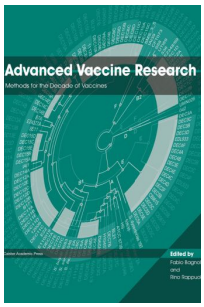

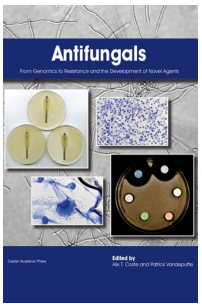

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