Root-Microbe Interactions: the Importance of Protein Secretion

Clelia De-la-Peña*1 and Jorge M. Vivanco2

1Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, 97200 Mérida, Yucatán, México
2Department of Horticulture and Landscape Architecture and Center for Rhizosphere Biology, Colorado State University, Fort Collins, CO 80523, USA

Abstract: The interactions between plants and microbes have been widely studied using gene expression studies and small molecule exchange between organisms. For the most part, these studies have focused on aboveground interactions and fewer studies have examined these types of processes belowground. The purpose of this review is to summarize the current literature looking at the interactions between roots and soil microbes, with an emphasis on the exchange of proteins between the organisms. Roots can establish close contact with different microorganisms in the rhizosphere, from pathogens to beneficial bacteria such as nitrogen fixers; recent data indicate that protein exchange is an integral part of these associations. These interactions include the release by roots of defense proteins, proteins involved in bacterial chemotaxis and proteins found inside root border cells, and release of proteins from bacteria that could activate innate immunity in plants. The overall goal of this review is to convey recent proteomic information related to root-microbe exchange to identify potential areas of development to improve agriculture.

Keywords: Proteomics, root exudates, nodulation, pathogenesis, symbiosis, defense, interaction, microbes.

INTRODUCTION

The advance on “omics” approaches in plant-microbe interaction has increased rapidly. The sequencing of the genomes of both plants and pathogens have provided new tools to probe disease resistance [1-5]. Proteomics approaches are complementary to genomics and provide a link between transcript expression and protein level examinations [6-9]. In plant-microbe interactions, proteomics have become a necessary tool for recognizing macromolecules involved in the mechanism of response to biotic stress by plants [10-13]. Recently, the concept of “rooteomics” was coined [14] to emphasize the importance of proteomics in roots and in root-microbe interactions. When the proteome of Arabidopsis roots and leaves was compared [9], it was observed that proteins involved in defense represented 13% of the root proteome and only 7% in leaves, suggesting that roots are more engaged in defense against potential threats than the aerial plant parts.

Roots, compared to leaves, are in close contact with a high diversity of microbes, which may explain why a greater proportion of proteins involved in defense are found in roots vs. leaves. Furthermore, the population of microorganisms is between 2.5 and 1260 times higher in the rhizosphere than in bulk soil [15], and in leaves is even lower than in bulk soil [16]. Studies of proteins involved in root-microbe recognition [17, 18], root defense [19-21], biofilm formation [22], quorum sensing [23], response to wounding [24], fungal infection [25-27] and fungal elicitors [27, 28] have provided strong evidence about their role in root-microbe interactions through root exudation [29, 30]. Some root proteins are produced constitutively regardless of the microbe’s identity, while others are specifically induced [31], and a similar situation is observed in the proteins found in the root exudates [30]. In roots and root exudates, proteins related to metabolism are constitutively accumulated or secreted, while proteins related to defense, signaling and protein destination are specifically induced according to the identity of the microbe that interacts with the root. Some pathogenesis-related (PR) proteins have been found to be constitutively secreted by the roots of healthy plants, but in other plants these PR proteins are induced by specific external cues [30].

It is important to mention that there is a small difference between proteins secreted by roots and proteins secreted by root border cells. Although in both cases PR proteins have been found, the secretion dynamics are different. When a plant is attacked by a pathogen, the plant produces a range of PR proteins that are synthesized in the rough endoplasmic reticulum (ER) and then secreted from the cell. Genes encoding ER resident chaperones, such as the lumenal binding protein (BiP) [32], are also induced under infection by pathogens [33]. It has been proposed that BiP is required as an early response to enhance PR protein synthesis in the rough ER that induce the trigger for a rapid defense response [33]. In the case of border cells, which are cells that disperse into suspension when root tips are placed into water [21], pathogen attack causes the number of border cells released to be higher [21, 34], contributing to the production and secretion of proteins related to defense.

Proteomic approaches are likely to provide new clues about root-microbe interactions that could lead to the development of novel agricultural strategies to improve crop
yield. Initial cataloguing of proteins secreted into the rhizosphere will help to build models to understand the basic principles of the root response to intruders vs. the response to beneficial microbes. Here, we review the proteomics information available for root-pathogen, root-fungus and root-rhizobia interactions, and discuss the importance of secreted proteins in the rhizosphere (Fig. 1). Also, we designate one section to discuss and compare four different proteomic approaches in root exudates.

**PROTEIN COMMUNICATION BETWEEN ROOTS AND RHIZOBIUM**

Symbiotic associations are not restricted to those between plants and fungi, but can also happen with bacteria. There is the well-known association of rhizobacteria such as *Azorhizobium*, *Bradyrhizobium* and *Sinorhizobium* with legumes that leads to symbiosis, which starts with chemical exchange and culminates in nodule formation in the roots. Infection of legume roots is the first event leading to nodulation. The nodule is a very sophisticated structure, in which bacteria provide nitrogen to the plant in exchange for nutrients and shelter [35]. The way rhizobia invade roots is through the root hairs [36] and wounds of emerging lateral roots [37, 38]. The function of proteins in the symbiosis process has been widely studied [39-42]. For the most part, the studies have focused either on nodule proteins [13, 43, 44] or on the proteins from the isolated bacteria [45-49], but proteomic studies of root exudates during this interaction are scarce.

Bacterial-secreted proteins have important roles in the successful nodulation in certain rhizobia-legume interactions [50, 51]. Proteins such as NodO, glycanases and cellulases are important for nodulation, symbiotic nitrogen fixation and exopolysaccharides’ modification [51]. Using proteomics approaches, De-la-Peña *et al.* [30] analyzed extracellular proteins in rhizobia by two-dimensional gel electrophoresis (2-DE), followed by Q-TOF MS/MS at 6, 12 and 24 hours of bacterial growth. It was found that *S. meliloti* strain Rm1021 secretes more proteins at six hours than at 24 h and many of these proteins are related to signaling (such as flagellin), cell processes and metabolism. Flagellin is a bacterial-secreted protein [50] that functions as key signal for the initiation of defense responses by plants [52]. De-la-Peña *et al.* [30] not only analyzed the proteins secreted by *S. meliloti* alone, but also when it was in contact with legume and non-legume plants. The *M. sativa-S. meliloti* interaction caused an increase in the secretion of four bacterial proteins (a superoxide dismutase (SOD), a hypothetical protein SMc02156, a putative glycine betaine-binding ABC transporter protein, and a putative outer membrane lipoprotein), in comparison with the secretion of proteins from *S. meliloti* in the absence of *M. sativa*. However, the profile of secreted bacterial proteins when *S. meliloti* was in contact with a non-legume plant such as *A. thaliana* was very different. For instance, a
hypothetical protein SMC02156, a probable leu/ile/val binding protein, a putative phosphate-binding periplasmic protein and a carbamate kinase were downregulated in this interaction, in comparison with the *M. sativa-S. meliloti* interaction. Because the protein exchange is reciprocal between bacteria and roots, it was hypothesized that roots would also change their secretion according to the microbe’s identity. Thus, *M. sativa* was inoculated with *P. syringae* DC3000 or *S. meliloti*; the proteomic profile included high levels of several PR proteins soon after initial contact with *P. syringae*, but this effect was not observed in the interaction with *S. meliloti* [30].

Most of the proteomics studies published on legumes have been reported on the model plant *Medicago truncatula* [10, 11, 53-60]. Bestel-Corre and coworkers [11] analyzed the proteome of *M. truncatula* roots inoculated with the nitrogen fixing bacteria *S. meliloti* and with the mycorrhizal fungus *Glomus mosseae*. Interestingly, in response to nodulation, four proteins were upregulated, one was downregulated and 46 were newly induced; these proteins were related to nitrogen fixation and carbon metabolism, and some elongation factors were observed as well. On the other hand, when *M. truncatula* was in contact with *G. mosseae*, most of the upregulated and newly induced proteins were related to defense responses, root physiology and respiratory pathways. The most intriguing result was that the proteins identified from the interaction fungus-plant were only from plant origin, while proteins present in bacteria-plant interactions belong to both partners; no common proteins were found to be induced in both mycorrhizal and rhizobial symbiosis. It is also important to mention that proteins upregulated in roots during nodulation, such as elongation factor Tu (EF-Tu), enolase and superoxide dismutase, were also found to be secreted in *M. sativa-S. meliloti* during the first six hours of contact [30]. In studies of soybean root hairs infected with *B. japonicum*, the following proteins accumulated in the root hairs: lipoxygenase, lectin, phénylaniline-ammonia lyase, actin, ascorbate peroxidase, phosphoglucomutase and phospholipase D [61] which have not been found in the rhizosphere. Plant-secreted/produced lectins serve as important signal proteins for: symbiosis [62, 63], endophytic association [63], biofilm formation and bacterial attachment to roots [64, 65], and also for pathogenesis [66]. In addition, phospholipase D found in the root hairs of soybean infected with *B. japonicum* [61] could be participating in lipid signaling pathways for the formation of new membranes around the nodule which could induce the secretion of proteins into the rhizosphere.

Root-secreted proteins have been involved in chemotaxis between *S. meliloti* and alfalfa or *Lotus corniculatus* [67-69]. Currier and Strobel [69] found an ~60 kDa root glycoprotein named trefoil chemotactin that was able to attract six different strains of rhizobia. Furthermore, secreted proteins not only have been found to participate in chemotaxis, but they are also able to modify Nod factors from *Rhizobia* [40]. Nod factors are the perfect substrate for chitinases because of their chitin-like structure [70-73] that could be hydrolyzed by specific plant chitinases [70]. Studies in root hairs of white clover and pea have documented the increase in peroxidase activity when roots were inoculated with heterologous *R. leguminosarum*, a non-symbiont strain, which has been interpreted as a plant defense response [74]. In summary, additional studies on the secretome of root-symbiont interactions are needed to identify possible proteins related to recognition.

**PROTEOMICS OF INTERACTIONS BETWEEN ROOTS AND PATHOGENIC BACTERIA**

Roots secrete a vast diversity of proteins into the soil, from defense proteins such as glycosidases to proteins involved in signaling and regulatory processes like peroxidases [30]. Inside root cells, peroxidases catalyze many oxidation-reduction reactions in the presence of *H2O2* as an oxidizing agent, which is a reactive oxygen species (ROS). Another enzyme involved in the detoxification of free radicals is SOD. SOD has been found in root border cells, which contain proteins that play a vital role in the innate immunity of plants [29]. Among the proteins found to be secreted in the rhizosphere are peroxidases [30, 75], phosphatases [76, 77], esterases [78], proteases [30, 79-81], chininases [30, 82], glucanaganes [30, 83], thaumatin-like proteins [30] and ribosome-inactivating proteins [84]. Some of these enzymes are induced by wounding [24] and pathogen attack [85-87]. There are several examples of root-secreted proteins that are used by the plant for defense responses against soil pathogens [19, 29, 30, 84, 88-91]. Also, the biological properties attributed to glucanaganes and chininases have been enormous. Glucanaganes and chininases have been recognized for their antimicrobial properties. Most plant chininases possess lysozyme activity; i.e., they can hydrolyze bacterial peptidoglycan and fungal chitin [92]. Thaumatin-like protein is also part of the large PR protein family. This protein is included in the group 5 of the PR proteins, PR-5. These extracellular proteins inhibit the growth of fungi [93]. PR proteins, such as chininases, osmotin and thaumatin-like proteins, have been found in root exudates secreted constitutively, as well as under pathogen contact [30, 68, 94]. Root-secreted proteins are not only important for defense, but also for attracting microbes to the roots, a process known as chemotaxis [69]. Chemotaxis is one of the earliest essential events in the interaction between plants and microbes [95, 96].

Although bacteria rely on diverse secretion systems (type I to V) to overcome plant defenses and colonize the host [97], the most important system involved in secretion of extracellular enzymes, toxins and virulence factor is the type II secretion system. Proteomic and genomic studies of the Gram-positive bacteria *Bacillus subtilis* secretome started 10 years ago [98, 99], revealing nearly 300 possible proteins secreted into the soil. However, so far only 113 proteins have been identified [100]. Some questions that remain to be answered are why bacteria need to secrete so many proteins and what the best way to identify these proteins is. The secretome analysis of bacterial pathogenicity has revealed new and important virulence proteins [101-105]. For instance, Kazemi-Pour and coworkers analyzed the secretome of *Erwinia chrysanthemi*, a well-known plant pathogenic bacteria, using pH 3-10 linear and pH 6-11 non-linear 7 cm IPG strips [101]. About 50 proteins were visualized on a two-dimensional gel electrophoresis (2-DE) stained with silver nitrate at a protein load of 20-30μg. The following proteins were identified: Avr-like protein, elongation factor EF-Tu,
flagellin, pectate lyases and metalloproteases, which are related to virulence, disease symptoms and pathogenicity. Furthermore, _E. chrysanthemi_ and _E. carotovora_ secrete proteins that degrade plant cell walls, such as pectin lyase, cellulase, protease and polygalacturonase [106, 107]. Polygalacturonases are considered to be key enzymes involved in pathogenesis [108]. Using a similar approach, Watt and coworkers investigated the _Xanthomonas campestris_ secretome. The bacteria was cultured in minimal medium and the cell-free culture supernatant was separated by 2-DE. The proteome map revealed 97 proteins, which were identified using a matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF). Some of these proteins are involved in element acquisition, protein maintenance and folding, compound degradation, and proteins with unknown functions [102]. The interesting thing is that both bacteria, _E. chrysanthemi_ and _X. campestris_, share secreted proteins that could be linked with pathogenicity. For instance, the work of Kazemi-Pour [101] found that _E. chrysanthemi_ secreted an elongation factor, a chaperonin GroEL, flagellin and celluloses that also were found in the work of Watt with _X. campestris_ [102] and the secretion of these proteins have found to increase in the presence of plants and plant compounds [30, 101].

Secretion of proteins is a two-way street, with microbes releasing extracellular enzymes that degrade cellulose, chitin, lignin, etc., and as a counter-attack by plants in the form of root exudates [29, 30]. Because roots are surrounded by a large number and variety of beneficial and pathogenic microbes, they have achieved a sophisticated level of recognition that allows them to defend against pathogens and to establish symbiotic contact with beneficial microbes. In order to colonize the roots, bacteria usually congregate together using of quorum-sensing signals. The most common type of quorum-sensing (QS) signal are the N-acyl-homoserine lactones (AHL) [109], which affect the expression of more than 600 genes in bacteria [110]. Under a pathogenic attack, QS regulates and control genes involved in production of extracellular polysaccharides, degradative enzymes, antibiotics, Hrp protein secretion, Ti plasmid transfer, biofilm formation and others [109]. Recent studies indicate that plants are also able to sense and respond to bacterial AHL. Proteomics studies have revealed that _Medicago truncatula_ roots are able to detect low concentrations of bacterial quorum-sensing signals from the pathogenic bacterium _Pseudomonas aeruginosa_ by change and accumulation of 154 proteins, from which 21 are related to defense and stress responses [31]. Because the recognition between the pathogen and the host is mediated by proteins produced by bacteria, it has been suggested that some posttranslational modifications, such as glycosylation, of these bacterial proteins are important for infection. Furthermore, it has been observed that protein phosphorylation in Arabidopsis is essential for early signaling and defense responses [111].

Because of the lack of correlation between the transcriptome and the proteome of an organism [112], functional analysis of secreted proteins would represent the precise biological function of how bacteria react to the presence of different plants. This was the case of a bacterial mutant impaired in polygalacturonase secretion, which exhibited a reduced virulence [105].

**IMPORTANCE OF FUNGAL AND OOMYCETES INTERACTION WITH ROOTS**

Today, more than 158 fungal secretomes have been annotated, including those corresponding to fungal pathogens of humans and plants, as well as oomycetes [113]. These proteomic tools have created ample information about gene products involved in biochemical pathways, gene regulation, cell division and host-parasite interactions [93, 114-118]. There are pathogenic fungi that cause serious damage and loss to agriculture. In contrast, symbiotic mycorrhizal fungi provide predominantly phosphorous to the host plant in exchange for carbon. Proteomics have been applied to complement the genomics analysis of the fungal secretome [119]. A combination of MALDI-TOF and LC-MS/MS was used to identify a novel aspartic protease found in the mycoparasit _Trichoderma harzianum_ secretome [114]. By using both 1D and 2D electrophoresis followed by mass spectrometry analysis, it was possible to identify only 84 proteins (from 196 total spots) of low abundance from a large amount of protein (600 µg) of the devastating plant pathogenic fungus, _Fusarium graminearum_ [119]. This low number of identified proteins suggests that probably the sample was not in solution in the 2-DE buffer because of the lack of thiourea, which normally helps to resuspend more proteins than urea alone [120]. This fungus secretes cellulases, endoglucanases, proteases and chitinases; 45% of the proteins are actually involved in cell wall degradation. Pathogenic fungi also secrete proteins named hydrophobins that are implicated in growth and development [121]. Other fungal secretomes studied are those of _Phytophthora ramorum_ [122], the causal agent of sudden oak death, and _Sclerotinia sclerotiorum_, a phytotrophic fungus [123]. Yamada and Kaw [123] identified 18 secreted proteins from the liquid culture of this fungus that were separated by 2-DE using ESI-q-TOF MS/MS. A proteomic study of the secretome of _S. sclerotiorum_ identified alfa-l-arabinofuranosidase, which is involved in the virulence process of this fungus and was not detected by expressed sequence tag (EST) studies. Proteome research not only in the secretome of the pathogen alone, but also in the presence of the host should be persuaded in order to know how the interaction between pathogen and host is established. The proteomic analysis of the interaction could lead to the identification of effector proteins produced by fungi and defense proteins produced by plants that are specific to the given fungus. These potential proteins include AVR proteins, small cysteine-rich proteins, translamatinas, cellulose binding elicitor lectin and ethylene inducing-like protein [124].

Usually when a fungus senses a plant host its genetic and biochemical systems generate a series of signals to start the invasion, which sometimes can trigger the host defense response [125]. However, some fungi are able to penetrate the host cuticle and invade the roots by secreting an enzyme able to degrade cutin called cutinase [126] (Fig. 2). Because cutinase plays an important role in virulence, proteomic and molecular approaches have been done to understand more about this enzyme [127, 128]. On the other hand, other fungal secreted proteins, such as elicinitins, glycoproteins and proteins of low molecular weight, are effective elicitors of plant defense response [125] and roots combat the invader with enzymes such as glucosyl hydrolase [129]. Chitinases
and glucanases have been found in root exudates, which are effective in repressing the growth of root pathogenic fungi [68]. By using 2-DE following MALDI-TOF-MS, the root protein profile of *M. truncatula* infected with the pathogen *Aphanomyces euteiches* was analyzed [10]. The first difference between infected and non-infected roots was detected as quickly as 6 h post infection; the majority of the induced proteins belonged to the family of class 10 pathogenesis-related proteins (PR10).

An interesting discovery about antifungal plant secreted proteins was that they are specific for pathogenic vs non-pathogenic fungi. For instance, the antimicrobial Dm-AMP1 protein found in the root exudates of *Dahlia merckii* acts by reducing the growth of the pathogenic fungus *Verticillium albo-atrum*. However, this protein is innocuous against the arbuscular mycorrhizal fungus *Glomus mosseae* and does not interfere with the symbiosis recognition and establishment between the plant and the mycorrhizal [130]. This is the case of osmotin, a protein found in the root exudates [30], which participates in plasma membrane permeabilization, which is associated with fungal spore lysis [131]. Although mycorrhiza are beneficial fungi for plants, the induction of plant-defense-related genes still takes place at early stages of the interaction [132-134]. During the earlier stages of the development of the vesicular-arbuscular mycorrhizal symbiosis association between *Allium porrum* L. and *Glomus versiforme*, the root chitinase activity was almost twice as high as in uninfected roots [26]. However, once the symbiosis was fully established, the chitinase activity in mycorrhizal roots was even lower than in the control roots. The possible explanation for this observation is that at the earliest stages of the interaction with the fungus, roots respond with a defense response. However, once the symbiotic interaction is established, the fungus is able to suppress the plant stress reaction and grow inside the roots.

**PROTEOMIC APPROACHES FOR ROOT EXUDATES**

It is clear that some differences exist between natural secretion in soil vs. a hydroponic culture setting in the laboratory. However, proteomic approaches in the laboratory can help to understand what could be happening in the soil.
It is well known that greater amount of carbon derived from roots is lost in soil compared to hydronic systems. In general, 5-10% of the net fixed carbon can be recovered in soil [135] while in hydronic systems only 0.5-3% is lost as rhizodeposition [135, 136]. This is because soil particles affect localized root exudation due to the large concentration gradient that exists between the root cytoplasm and the soil [135]. In this section we describe the principal proteomic approaches used to analyze root exudates. One of the major technical problems to analyze this secretome is the presence of pigments and phenolics in the root exudates that interfere with protein extraction. To overcome the problem of phenolic interference with protein analysis, some strategies have been developed [137, 138]. Another concern is whether the proteins are indeed secreted from roots or from damaged root cells. There are some steps that the researchers working with secreted proteomics must take into account related to this issue. Bioinformatics is one the tools used to determine protein translocation from the cytoplasm into the extracellular environment. Other tools involve specific methodologies to minimize cellular damage [139]. Furthermore, bioinformatics has been used by De-la-Peña et al. [30] to differentiate between the proteins that are secreted by bacteria from the proteins that are secreted by plants.

Among the different methods to extract and separate root-secreted proteins are those published by Charmont et al. [137] in A. thaliana, Basu et al. [94] in Brassica napus and A. thaliana, Wen et al. [29] in pea and maize, and De-la-Peña et al. [30] in A. thaliana and M. sativa, all of which used bioinformatics’ tools to be sure that the identified proteins found in their experiments were indeed secreted by plants.

Although the four methods relate to root exudates’ proteomics, many differences are observed among the methods (Fig. 2) that could account for such different results in resolution as well as number of resolved proteins in the gel. For instance, Basu et al. [94], Wen et al. [29] and De-la-Peña et al. [30] used LC MS/MS for protein identification and reported 52, 184 and 148 proteins, respectively. Charmont et al. [137], on the other hand, identified 112 proteins using MALDI-TOF-MS. Although Charmont et al. [137] included polyvinyl-polypyrrolidone (PVPP), which binds to phenolics, in their protocol of purification, it is still difficult to explain why only 52 proteins were identified in comparison with the more than 100 proteins identified by other methods that did not use PVPP. It is worth noting that Charmont et al. [137], Basu et al. [94] and De-la-Peña et al. [30] worked with A. thaliana as a model. However, differences in the number of resolved proteins were found that could be due to the amount of loaded protein. The methodology differences and similarities between protocols are summarized in Fig. (2). The four groups concluded in their reports that proteomics is the best approach to identify new sets of apoplastic proteins [137], to facilitate the identification of low abundance extracellular proteins in the rhizosphere [94], to identify proteins from detached border cells [29], and to identify proteins in plant-microbe interactions [30].

CONCLUSION AND FUTURE PERSPECTIVES

Considerable progress has been made toward understanding the proteomics of root-symbiont associations and more recently the interactions involving compatible and non compatible root-microbe interactions. A relatively minor subset of these studies is addressing the proteomics of the exudates involved in these interactions with the assumption that these are early signals that could be potentially manipulated to enhance crop productivity. However, most of these studies have been conducted under controlled conditions and the true presence and importance of proteome signals in soils and in the rhizosphere remains to be determined. Although much progress has been achieved in relating the processes of symbiosis with those of pathogenesis, it is necessary to expand our knowledge not only about the proteins that are accumulated in roots in response to nodulation or defense, but also the proteins that are secreted by both plants and bacteria and could be potential candidates for recognition signals. Also, it would be of great interest to investigate the functional characterization of secreted proteins, such as by studying enolase or superoxide dismutase impaired-mutants, to determine if they have any effect on nodulation. Future studies should address the proteomics of these interactions under rhizosphere conditions.

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