

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Modulation of rhizosphere - associated microbiota by insect pests: a
holobiont relationship**

Márcia Leite-Mondin

Thesis presented to obtain the degree of Doctor in
Science. Area: Genetics and Plant Breeding

**Piracicaba
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I dedicate to my aunt Lucia (in memorian), who even without being understood during her lifetime taught us how to keep the mind alive and the heart warm.

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"See, in all our searching, the only thing we've found that makes the emptiness bearable, is each other." – Carl Sagan

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RESUMO

Modulação da microbiota - associada à rizosfera, por pragas de insetos: uma relação holobionte

Atualmente observamos um crescente número de pesquisas que buscam desvendar as causas, os efeitos e as possíveis utilizações biotecnológicas da modulação de comunidades da microbiota de rizosfera nas interações complexas entre plantas e solo. Sabemos também que o ataque de insetos herbívoros é um fator de considerável prejuízo para a agricultura e que tem relações evolutivas bem estabelecidas em sistemas naturais. O presente trabalho procurou testar algumas hipóteses a cerca da relação direta entre a microbiota de rizosfera e o ataque de insetos praga. Partindo do ponto de que plantas possuem mecanismos de defesa contra insetos, bem conhecidos, foi verificado que a microbiota de rizosfera parece contribuir ativamente para esse sistema, e assim estabelecer relações holobiontes. Tivemos um profundo acesso á comunidades do domínio bactéria e fungi, através da tecnologia de sequenciamento de nova geração para amplicons do gene RNAr 16S, região V3 e região intergênica ITS em amostras de solo, semi-solo e intestino de insetos praga (Ordem: *Lepidoptera*) de comportamento generalista. Nossos resultados, resultaram em três artigos aqui apresentados em capítulos. No primeiro capítulo é discutido o efeito modulador da herbívora da praga agrícola *Spodoptera frugiperda* na microbiota de rizosfera de *Arabidopsis thaliana* em diferentes estágios fisiológicos da planta. Como resultados foi possível perceber que o efeito na modulação da estrutura de comunidades de bactérias é diferente do efeito na modulação de comunidades de fungos após o ataque de insetos herbívoros. Os efeitos são diferentes tanto em abundância relativa quando na diversidade para cada um dos domínios de microrganismos estudados. No segundo capítulo destacamos a diferença na modulação da estrutura de comunidades de bactérias para diferentes famílias de plantas. Utilizamos mudas de *A. thaliana*, *Zea mays* Sh2, *Phaseolus vulgaris*, *Solanum lycopersicum* e *Beta vulgaris*, expostas ao ataque de *Trichoplusia ni* durante uma semana. As análises da microbiota de rizosfera de cada um dos grupos de plantas hospedeiras, sugere que a influência da espécie vegetal deve ser considerada na modulação das comunidades de bactérias da rizosfera após a herbívora. Adicionalmente, determinadas espécies de plantas podem ser menos susceptíveis a modulação da rizosfera pela herbívora. Outro destaque foi o efeito da modulação da microbiota de rizosfera, na perda de biomassa de plantas semeadas em semi-solo transplantado. Com base nos dados fenotípicos das diferentes espécies de plantas avaliadas, sugerimos que a modulação da microbiota de rizosfera após a herbívora, pode estar envolvida na inibição da produção de biomassa vegetal na geração seguinte de plântulas. Por fim, no terceiro capítulo exploramos a modulação na microbiota no intestino de larvas de *Trichoplusia ni* através da carga microbiana obtida na alimentação restrita. Larvas *T. ni* de mesma origem foram divididas em três populações. Cada população foi alimentada de forma específica e restrita com folhas de *A. thaliana* ou *S. lycopersicum* ou dieta artificial calórica. Acessamos a microbiota do intestino das larvas, após três gerações de alimentação restrita e verificamos que a microbiota intestinal em lagartas de comportamento generalista, pode ser alterada devido à obtenção de carga microbiana por via alimentar. Essa modulação pode estar relacionada a degradação de metabólitos que podem ser prejudiciais à homeostase dos insetos. A microbiota intestinal de cada população também pode influenciar diretamente as preferências alimentares de gerações sucessivas. Em resumo, todos os nossos resultados, apresentados em cada um dos capítulos a seguir, são chaves no conhecimento e podem ajudar a clarificar as complexas relações entre plantas, insetos e microrganismos. Contribuindo assim para um maior entendimento desse tipo de sistema holobionte.

Palavras-chave: Interação planta/inseto/microrganismo; rRNA 16S; ITS; NGS

ABSTRACT

Modulation of rhizosphere – associated microbiota by insect pest: a holobiont relationship

Currently, we observe a growing number of researches that seek to unravel the causes, effects and possible biotechnological uses of the rhizosphere microbiota communities modulation in the complex interactions between plants and soil. We also know that the attack of herbivorous insects is a factor of considerable damage to agriculture and that has well established evolutionary relationships in natural systems. The present work tried to test some hypotheses about the direct connection between the rhizosphere microbiota and the insect pest attack. Beginning from the point that plants have well-established defense mechanisms against insects, it was verified that the rhizosphere microbiota seems to contribute actively to this system and thus to establish holobionte relationships. We had broad access to communities of the fungi and bacterial domain, through the new generation sequencing for rRNA 16S gene, region V3, and intergenic region ITS amplicons on soil, semi-soil and, insect gut samples from pest insects with general behavior (Order: *Lepidoptera*). Our results from the data analysis to *Illumina Miseq* sequencing outputs and, additional experiments, resulted in three articles presented here in chapters. In the first chapter, we discuss the modulating effect from the pest insect attack (*Spodoptera frugiperda*), on the *Arabidopsis thaliana* microbiota rhizosphere, for different physiological plant's stages. As a result, it was possible to discuss the differences between the modulation in the structure of bacterial communities and the modulation in the structure of the fungal communities after the attack of herbivorous insects. In the second chapter, we highlight the difference in the modulation of the bacterial community structure for different plant families. We used seedlings of *Arabidopsis thaliana*, *Zea mays* Sh2, *Faseolus vulgaris*, *Solano lycopersicum* and, *Beta vulgaris* exposed to the attack of *Trichoplusia ni* for one week. The rhizosphere microbiota analysis for each host plant groups, suggests that the influence of the plant species should be considered on the bacteria rhizosphere communities modulation after the insect attack. Besides, specific plant species may be less susceptible to rhizosphere modulation by insect attack. Another highlight was the microbiota rhizosphere effect in the biomass loss for plants sown on transplanted semi-soil. Based on the phenotypic data, we suggest the rhizosphere microbiota modulation after the herbivore may be involved in the plant biomass inhibition on the next seedlings generation. Finally, in the third chapter, we explore the *Trichoplusia ni* gut microbiota modulation through the microbial load obtained in the restricted feeding. The *T. ni* larvae from the same original population were divided into three populations. Each population was fed individually and restrictively with leaves of *A. thaliana*, *S. lycopersicum* or artificial caloric diet. We accessed the gut microbiota in *T. ni* after three generations of restricted feeding, and we verified that the gut microbiota in caterpillars of general behavior, could be altered due the obtaining of microbial load through alimentary diet. This modulation may be related to the degradation of metabolites that may be harmful to insect homeostasis. The gut microbiota of each population can also directly influence the food preferences of successive generations. In summary, all our results presented in each one of the chapters are important points that can help to clarify the complex relationships between plants/insects/microorganisms and, contributing to a better understanding of this holobiont system.

Keywords: Interaction plant/insect/microorganism; rRNA 16S; ITS; NGS

1 INTRODUCTION

Plants and insects have coexisted in the most diverse types of relationships more than 400 million years ago (Sugio et al., 2014; Franco et al., 2017). Plants are continually evolving their mechanical or chemical defense processes against insects (Willsey et al., 2017; Hahn et al., 2019), while these invertebrates have to develop mechanisms for overcoming, resistance or increased tolerance in relation to strategies of plant defense (Ryam, 1990; Kessler & Baldwin, 2002; Huang et al., 2016). This coexistence can be compared to a continuous arms race (Mello & Silva-Filho, 2002; Sugio et al., 2014).

As an additional element within this context of interaction, plants and insects have relationships with microorganisms that may have resulted directly or indirectly in the interactions between each one (Cardosa et al., 2013; Babikova et al., 2014). The holobionts relationships between microorganisms, insects and plants are currently undergoing in-depth discussion (Hassani et al., 2018). These relationships are of mutual, bi- or tri-directed effect among their components. The holobiont relations have a high capacity for modulation and a direct impact within the evolutionary process and the plasticity of the system. The system as a whole evolves together, and all components have a participation in the action of cause or effect in the face the temporal changes of the system itself (Rosenberg et al., 2006).

Microorganisms can modulate the plant's primary and secondary metabolism (Reichling, 2010; Badri et al., 2013; Mussilova et al., 2016) and plant defense systems (Pieterse et al., 1996; 2012) against insects for the benefit of plants (Kang et al., 2015) or insects' benefit (Antoniou et al., 2017). Microorganisms can also alter insect biology, including behavior (Colman et al., 2012) and may influence plant-insect interactions (Franco et al., 2019). This set of microorganisms (bacteria, fungi, and viruses) that inhabit a particular environment is called microbiota (Schlaeppli & Bulgarelli, 2015). For understand these relationships more profoundly, our work was restricted to access to communities of fungi and bacteria. We focus on two main points which are the influence by the insect pest attack on the microbiota in the region of the soil highly influenced by the root, and the effect of the microbial load of the plants on the herbivorous insects' gut microbiota that act as an agricultural pest.

We maintained our focus based on the holobionts relationships between plants, herbivorous insects and microorganisms by accessing the soil microbiota in contact with the root, and the *Lepidoptera* insect pest gut microbiota. Concerning, the region where the soil and roots of the plants come into contact, that is, the portion of the soil directly influenced by the root system of the plants is called rhizosphere (Vandenkoornhuyse et al., 2015). The community of microorganisms in the rhizosphere is highly differentiated in quantity and community composition found in free soil (Marschner, 1994; Kent & Triplett, 2002). The soil residue

adhered to the root of a plant represents the portion of higher activity of the rhizosphere and is called a rhizoplane (Montiel & Aguilar, 2003).

In different plant species, the rhizosphere microbiota presents different structures, and the microorganisms can be distributed in varying amounts according to the genome and development of the plant (Chaparro et al., 2013). Several studies indicate that these differences in the rhizosphere microbiota for different host plants are directly related to the type and quantity of exudates released by each plant (Haichar et al., 2008, Chaparro et al., 2013; Badri et al., 2013). The root exudates may drive the differentiation of the soil biome in the rhizosphere and have host genotype-dependent factors (Badri & Vivanco, 2009; Sugio et al., 2014). In order to obtain an overview of these relationships, we have studied the rhizosphere microbiota of *A. thaliana* ecotype Col-0, and of different plant species such as Sweet Corn (*Zea mays* Sh2), Bean (*Phaseolus vulgaris*), Tomato (*Solano lycopersicum*), Red Beet (*Beta vulgaris*) after insect attack.

Insects of the order *Lepidoptera* present a variety of species with herbivorous characteristics that can cause enormous economic losses in a considerable number of cultivated plants (Lukhtanov, 2019). Currently, many studies have focused on associations between different insect's species and the bacterial diversity correlations (Colman et al., 2012; Jones et al., 2013), but we still require information from some important insects' herbivorous groups in a general context (e.g., *Lepidoptera*). The general overview emerging from this work, is that herbivorous insects harbor microbial communities of limited diversity, dominated by a few taxa (Colman et al., 2012, Jones et al., 2013).

Several studies analyzing the microbial communities of insects from different orders pointed out that bacterial species diversity is low because that both evolutionary history and insect feeding habits which contributed for its gut microbial diversity are restricted (Colman et al., 2012; Jones et al., 2013). However, it is still unknown for insect pests of the order *Lepidoptera* (e.g., *Spodoptera frugiperda* and *Trichoplusia ni*), whether the microbial load of different plants may influence insect gut microorganism community' structures. In this study, we used insect pests of the order *Lepidoptera* with generalist behavior so that we could test the same herbivorous model in plants of different species.

Current knowledge about some of the interactions between insects/plants/microorganisms, the specific roles of these microbial communities in the rhizosphere of plants and herbivorous insects remain mostly uncertain. Recently, the new generation of sequencing methods and molecular tools for detection of fragments or entire genomes of microorganisms that cannot be cultivated, are advancing our understanding of the influence of the microbiota on plant-insect interactions at the molecular level (Smalla et al., 1996; Kniff, 2014). Our studies about the rhizosphere microbiota of plants and insect gut allowed an advance in our current knowledge on plant/herbivory interactions that are influenced by the associated microorganisms.

The diversity, structure and abundance of bacterial and fungal communities in the host plant (*Arabidopsis thaliana*) rhizosphere and pest insect gut were characterized. In the first chapter, we aim to understand whether there is a significant modulation on rhizosphere microbiota after the attack of the generalist *Spodoptera frugiperda* (*Lepidoptera*) insect pest. In this study sought to verify possible differences in both bacterial and fungal communities after the insect attack. In addition, we seek to understand whether different physiological plant developmental stages (pre-flowering, flowering, and senescence), modulate the rhizosphere-associated microorganism composition by herbivorous insects.

In the second chapter, we compared the rhizosphere microbiota of different plant species after the *Trichoplusia ni* insect attack. It has been accepted that several natural systems can present convergence effects of their components when modulators by some factor inserted in the system. Thus, we tested the hypothesis that the rhizosphere microbiota of different host plants follows a convergence pattern for the communities of bacteria after the insect attack. Second, we checked whether the modulation of the rhizosphere microbiota by the insect attack might reflect some differences in the next plant's generation sown in the same soil that was used in the first experiment.

In the third chapter, we sought to understand the microbial load effect from different plant diets on the modulation of *T. ni* gut microbiota. We have also tested whether there is a difference in the gut microbiota of generalist insects if the population is restricted to one type of diet, for successive generations, and whether this effect is long-lasting. We hypothesized that the gut microbiome modulation could be related to the food preference behavior of the different insects' populations.

This complex network of interactions can be better understood through advances in new generations of sequencing, molecular techniques of genome extraction and amplification for microorganisms that cannot be grown. The increases in proper statistical methodologies also open new interesting perspectives in this field. In the present work, we address some of the open questions of the complex relationships among plants, insects, and microorganisms. The outcomes of this study may provide a scientific basis for the technological advancement of agricultural systems and food security.

2 CONCLUSIONS

First chapter:

- 1) Herbivorous insect attack may be able to modulate the bacterial and fungal community structure in distinct ways in the *A. thaliana* rhizosphere.
- 2) Plant developmental stages contribute to differences in structure and abundance in bacterial and fungal communities in the *A. thaliana* rhizosphere.

Second chapter:

- 1) Structure of the rhizosphere microbiota altered by the insect attack depends on the host plant;
- 2) Specific groups of bacteria are recruited into the rhizosphere microbiota for each type of host plant;
- 3) Modulation of the rhizosphere microbiota by insect attack might negatively influence the biomass of subsequent plant generations.

Third chapter:

- 1) Populations of generalist insects fed with consecutive cycles of different diets show distinct intestinal microbiota.
- 2) Populations of gut microbiota directly influence the food preferences of successive generations.

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