

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

**Interactions of maize bushy stunt phytoplasma with the leafhopper vector,  
*Dalbulus maidis* (Delong and Wolcott) (Hemiptera: Cicadellidae) and  
associated microbiota**

**Javier García González**

Thesis presented to obtain the degree of Doctor in  
Science. Area: Entomology

**Piracicaba  
2016**

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*Dalbulus maidis* (Delong and Wolcott) (Hemiptera: Cicadellidae) and associated  
microbiota**

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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## **Dedication**

Perhaps you do not perceive what the effort I am doing just to support your dreams and issues since the distance. But I am sure life will allow me to be more proud and gratified than today for the person I contributed to realize with his dreams. TO NICOLAS GARCÍA GONZÁLEZ.

It is not just the ideal to share our minds and souls; it is also the happiness you have given me sharing the creation and education of our miracle many times spoken and finally born, LAURA MARIANA. TO MARISOL GIRALDO JARAMILLO.

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## EPIGRAPH

Every natural action is performed by the very nature of the way and in the shortest time possible. No action may be abbreviated nature, because nature is generated as soon as possible.

Practice should always be based on a good knowledge of the theory

Leonardo Da Vinci

I have gathered what I observed to be useful. And brought it together as a single body

Vitruvius



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## RESUMO

### **Interações de ‘maize bushy stunt phytoplasma’ com a cigarrinha vetora *Dalbulus maidis* (Delong and Wolcott) (Hemiptera: Cicadellidae) e microbiota associada**

Fitoplasmas são bactérias transmitidas de forma persistente propagativa por insetos vetores, havendo interações diretas e indiretas envolvendo tais organismos. Para entender algumas dessas interações no caso do enfezamento vermelho do milho, associado ao ‘maize bushy stunt phytoplasma’ (MBSP) e à cigarrinha vetora *Dalbulus maidis* (Hemiptera: Cicadellidae), foram desenvolvidas duas linhas de trabalho. Na primeira determinou-se o efeito indireto de MBSP no comportamento e biologia do vetor, enquanto que na segunda estudaram-se as interações diretas do fitoplasma com o vetor durante sua movimentação através de órgãos internos e associação com a microbiota do inseto. O efeito indireto no comportamento foi demonstrado em testes de escolha nos quais se variou o período de incubação de MBSP na planta (plantas com sintomas iniciais ou avançados da doença, e plantas infectadas assintomáticas) sobre a preferência para pouso e oviposição pelo vetor. O efeito indireto na biologia do *D. maidis* foi estabelecido em dois experimentos de tabela de vida, alimentando o inseto durante seu ciclo de vida em plantas de milho saudáveis ou plantas infectadas com o fitoplasma em dois tempos de incubação (plantas com sintomas avançados da doença e plantas infectadas assintomáticas). Os testes de escolha mostraram que a preferência de *D. maidis* para pouso e oviposição em plantas infectadas por MBSP em relação a plantas saudáveis depende do período de incubação do patógeno na planta. A cigarrinha preferiu plantas infectadas por MBSP na fase assintomática da doença, mas rejeitou plantas com sintomas avançados. Ocorreu aquisição do fitoplasma pelo vetor em plantas assintomáticas a partir de 3 dias após a inoculação, mas a eficiência de transmissão aumentou após 14 dias de incubação do patógeno na planta-fonte, e diminuiu o tempo para expressão de sintomas nas plantas-teste inoculadas. Os resultados sugerem que MBSP modula a preferência do vetor para plantas infectadas no estágio inicial da cultura de modo a permitir sua rápida disseminação. O efeito da infecção de plantas por MBSP na biologia de *D. maidis* mostrou ser neutro para a maioria dos parâmetros biológicos estimados; houve menor taxa líquida de reprodução ( $R_0$ ), que foi compensada por maior razão sexual, para insetos criados em plantas com sintomas avançados. Verificou-se, por PCR, aquisição do fitoplasma por todos os estádios ninfais do vetor e sua presença em órgãos reprodutivos dos adultos. Por microscopia eletrônica de transmissão, observaram-se células do tipo fitoplasma no lúmen, microvilosidades e células epiteliais do mesêntero de *D. maidis*, sugerindo que o MBSP penetra no epitélio através das microvilosidades. No epitélio intestinal, foram observadas massas de células do tipo fitoplasma próximas a mitocôndrias e células bacterianas, possíveis endossimbiontes. Na hemocele, também foram observadas células do tipo fitoplasma agrupadas numa matriz, em associação com bactérias similares às observadas no intestino. Associações semelhantes foram observadas na glândula salivar. A técnica de FISH revelou uma variação na riqueza e abundância das espécies na da microbiota no mesêntero e glândula salivar de *D. maidis* em função do tempo após a aquisição de MBSP. A abundância de *Sulcia* sp., *Cardinium* sp. e eubactéria aumentou, enquanto que a de *Rickettsia* sp. decresceu. A associação frequente dessas bactérias com fitoplasma em alguns tecidos de *D. maidis* sugere que endossimbiontes possam ter um papel nas interações fitoplasma-vetor.

Palavras-chave: Cigarrinha-do-milho; Enfezamento vermelho; Preferência hospedeira; Ultraestrutura; Endossimbiontes



## ABSTRACT

### **Interactions of maize bushy stunt phytoplasma with the leafhopper vector, *Dalbulus maidis* (Delong and Wolcott) (Hemiptera: Cicadellidae) and associated microbiota**

Phytoplasmas are bacteria with a persistent propagative transmission by insect vectors that generates direct and indirect interactions among them. In order to understand these interactions for maize bushy stunt phytoplasma (MBSP) and the leafhopper vector *Dalbulus maidis* (Hemiptera: Cicadellidae), two research lines were addressed. The first one aimed to determine the indirect effects of maize infection by MBSP on some biological and behavioral parameters of the vector, whereas a second line investigated direct interactions of the phytoplasma with *D. maidis* during its movement through the vector body following acquisition from plants, and associated microbiota. Indirect effects were investigated in choice experiments in which alighting and oviposition preferences by *D. maidis* were compared on healthy vs. MBSP-infected plants with variable incubation time (diseased plants with early and advanced symptoms, or still asymptomatic). Likewise, indirect effect of MBSP on the *D. maidis* biology was determined in two life table experiments in which the vector was reared on healthy vs. MBSP-infected plants expressing advanced disease symptoms or still asymptomatic. Choice experiments showed that alighting and oviposition preferences of *D. maidis* on MBSP-infected plants compared to healthy plants depend on the pathogen incubation period in the plant. The leafhopper preferred MBSP-infected plants over healthy ones during the asymptomatic phase of the disease, but rejected infected plants with advanced symptoms. The vector was able to acquire MBSP from asymptomatic infected plants shortly (3 days) after inoculation, but transmission efficiency increased when acquisition occurred at later stages of the pathogen incubation period ( $\geq 14$  days) in the source plants and the test plants showed disease symptoms faster. These results suggest that MBSP modulates *D. maidis* preference for asymptomatic infected plants in the early stages of the crop, allowing rapid spread of this pathogen. Maize infection by the phytoplasma had a neutral effect on most life table parameters of *D. maidis*; a lower net reproductive rate ( $R_0$ ) was observed in the cohort reared on MBSP-infected plants with advanced symptoms, which was compensated to some extent by a higher sexual ratio. MBSP acquisition by all vector nymphal stadia was confirmed by PCR, and the pathogen as detected in both male and female reproductive organs. Concerning direct MBSP-vector interactions, transmission electron microscopy analyses showed phytoplasma-like cells in the midgut lumen, microvilli and epithelial cells, suggesting that MBSP enters the epithelium midgut through the microvilli wall. Within the epithelial cells, mitochondria and bacteria-like cells (possibly endosymbionts) were observed together with masses of phytoplasma-like cells. In the hemocoel, phytoplasma-like cells grouped into a matrix were also observed in association with bacteria-like cells similar to those observed in the midgut epithelium. Similar associations were found in the salivary gland. Interestingly, in-situ hybridization (FISH) technique revealed a variation in diversity and abundance of the microbiota in intestine and salivary glands of *D. maidis* adults over time after MBSP acquisition from plants. *Sulcia* sp., *Cardinium* sp. and eubacteria increased their abundance over time, whereas *Rickettsia* sp. decreased. The frequent association of the vector microbiota with the phytoplasma in some tissues of *D. maidis* suggests that endosymbiotic bacteria may play some role in MBSP-vector interactions.

**Keywords:** Corn leafhopper; Maize bushy stunt phytoplasma; Host preference; Ultrastructure; Endosymbionts



**ACRONYM LIST**

AAP – Acquisition Access Period

IAP – Inoculation Access Period

IP – Incubation period

LP – Latent Period

MBSP- Maize Bushy Stunt Phytoplasma

TEM – Transmission Electronic Microscopy

SEM – Scanning Electronic Microscopy

*Ca* - Candidatus



## 1 INTRODUCTION

Leafhoppers and planthoppers are important vector insects of phytopathogenic organisms thanks to its feeding behavior on the plant vascular system, and for both virus and bacteria phytopathogen transmission including spiroplasmas and phytoplasmas (AMMAR et al., 2011).

The leafhopper *Dalbulus maidis* (DeLong and Wolcott) (Hemiptera: Cicadellidae) is a monophagous insect colonizing maize crops in the Americas, being identified as vector of three maize phytopathogens: ‘Corn Stunt Spiroplasma’ (CSS; *Spiroplasma kunkelii*), ‘Maize bushy stunt phytoplasma’ (MBSP, or Maize phytoplasma) and *Maize rayado fino virus* (MRFV) (AMMAR et al., 2011; ÖZBECK et al., 2003; WAQUIL et al., 1999). These pathogens produce economically important diseases in Brazil (FERNANDES, 1998) and several countries of Latin America as the pale stunt, reddish stunt and corn streak virus, respectively (NAULT, 1980).

In Brazil CSS and MBSP are the focus of research because of their difficulty to control them and the absence of plant resistant varieties. In order to obtain maize tolerant plants, it is fundamental to deal studies on the interaction pathogen – plant – vector insect, looking the comprehension for the involved mechanisms in the pathogenicity and dissemination of MBSP and CSS. Actually, some advances have been obtained for some Phytoplasmas (SUGIO et al., 2011a; SUGIO; HOGENHOUT, 2012), that could be extended to the MBSP – *D. maidis* interactions.

Phytoplasmas are phytopathogenic bacteria belonging to the Mollicute class inhabiting the sieved plate of the phloem plant, they cannot be cultivated in artificial media, they live in both organisms insect and plant affected, and for its acquisition and inoculation they depend exclusively of its vector insect. Literature mentions different phytoplasma species associated with more than 200 plant diseases, almost of them transmitted by leafhoppers of both Cicadellidae and Delphacidae families. Phytoplasma acquisition from sieve plaques occurs by the alimentary canal of the insect stylet with passing through the digestive tube and entrance to the body cavity (hemocele) and hemolymph. To inoculate new plants, the phytoplasma must colonize the insect salivary glands just to be released at the moment of the feeding on healthy plants (CHRISTENSEN et al., 2005; BAI et al., 2006; HOGENHOUT et al., 2008).

Symptoms produced by MBSP on maize plants are generally observed 30 days after inoculation, with initially signs are chlorosis of margins of whorl leaves, followed by

reddening of the tips of the older leaves (NAULT, 1980). With progressive infection times, infected plants can exhibit axillary buds, size plant reduction, as well as both spikes and grain deformation. Plants showing the mentioned symptoms reduce its production between a 35 to 93% depending on the infectious insect population (TOFFANELLI; BEDENDO, 2002).

Phytoplasma – vector insect studies indicate mutualistic interactions, where both organisms gains benefits. As a co-evolution force, phytophagous insects uses their own armament battery or that offered by the carrying microorganisms inside its body, with the aim to break the plant resistance for both feeding and reproduction, for colonizing new host plants, for increasing its fertility and fecundity or just to fly longer distances (KINGDOM; HOGENHOUT, 2007).

Aphid vectors, for example, during its stylet penetration on plant tissues produces both gel and sheath saliva secreting with them proteins into the cells attacked that induce or suppress defense biological response in favor of the insect. Other Hemiptera also salivate into the host plant, inducing phytophathogen transmission or introducing salivary compounds affecting the plant (HOGENHOUT; BOS, 2011).

Most of the leafhopper species are important pest because of their feeding behavior on the sieve elements moving by the plant vascular tissue, and because of the virus and bacterial transmission such as the case of spiroplasm, phytoplasma, and *Xylella fastidiosa* (AMMAR et al., 2011; AMMAR, 1986).

In some cases, phytopathogen infection can encourage host plant modifications in order to attract more insect vectors to feed or reproduce (HOGENHOUT et al., 2008). Research on the spiroplasma CSS and the phytoplasma MBSP in *D. maidis* populations showed a 37% increase in survival of insect infective cohorts when compared with health individuals (EBBERT; NAULT, 2001).

Recent researches revealed that the vector leafhopper *Macrostelus quadrilineatus* Forbes (Hemiptera: Cicadellidae) survive and reproduce more on Arabidopsis plants infected with ‘Aster yellows phytoplasma’, Witches’ broom (AY-WB) race, than on healthy plants (SUGIO et al., 2011a, 2011b; KINGDOM; HOGENHOUT, 2007). Sugio et al. (2011a, 2011b) demonstrated that AY-WB produce effector proteins (e.g. SAP11) causing reduction to the Arabidopsis plant response against the *M. quadrilineatus* attack, favoring its survival and reproduction. The research revealed that *M. quadrilineatus* increased in 60% their progeny when the insect grew on Arabidopsis plants infected with AY-WB in relation with health plants.

Perhaps, plant modification by the phytopathogen microorganism is the most common interaction studied; indeed these microorganisms do interact with their vectors when they pass within their bodies during their latent period before being feasibly transmitted to new host plants.

Insects and microorganisms present into their body exhibit several cooperation means, being the most common to find insects in symbiosis with bacteria (endosymbionts) serving to complement the nutritional needs of the insects when they feed on host plants with poor nutritional compounds (DOUGLAS, 2009; SANCHEZ-CONTRERAS; VLISIDOU, 2008).

Latest studies determined that insect endosymbionts also could help phytoplasmas in both movement and passage of the microorganisms through the physiological barriers of the insect as well as in the protection to the insect immune system. Similarly, it has been theorized that endosymbiont and phytoplasma sharing the same insect location, could facilitate the phytopathogen transmission process. In the aphid hemocel, for example, the Primary endosymbiont (P-endosymbiont) *Buchnera* is present releasing the protein Symbionin mediating in the phytopathogen movement through the hemocel.

In the vector insect *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) and their biotypes a dissimilar bacterial endosymbiont community has been demonstrated. P-endosymbiont *Portiera* and Secondary endosymbionts (S-endosymbionts) *Hamiltonella* e *Arsenophonus* produce a chaperonin protein GroEL with 63 kDa linking to the geminivirus particles and acting as layer protection against the enzyme degradation in the intestine and hemolymph of the insect (CZOSNEK et al., 2001). Likewise in the *Cotton leaf curl virus* (CLCuV) (Geminiviridae: Begomovirus) transmission, a GroEL protein of the P-endosymbiont *Arsenophonus* was found interacting with the protein capsid of the virus CLCuV (RANA et al., 2012).

Interactions between endosymbionts and phytoplasma transmission were recently demonstrated for the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae). The P-endosymbiont *Cardinium* present in *S. titanus* is assumed to interact with the phytoplasma responsible of the grapevine yellowish, helping on its transmission (GONELLA et al., 2011).

The discovery was done by the localization, multiplication and higher concentrations of the microorganisms into the insects possessing both phytoplasma and endosymbiont (ALMA et al., 2010).

Considering the conceivable interactions between arthropods –plants –microorganisms and their implications in the development of novel pest control techniques, new research lines

can emerge looking for the understanding of factors and metabolic routes involved in both plant and arthropod regulation by these microorganism (HOGENHOUT, BOS, 2011).

The thesis proposed had two research lines developed in cooperation with a European research team. The first research line consisted in the effect of the mayze bushy stunt phytoplasma MBSP on the biology and behavior of the insect vector *D. maidis*. The second research line was related with the recent demonstrations that both endosymbiont and phytoplasma can interact in the same insect vector structures in order to facilitate the phytopathogen transmission. To do so a method employed by Prof. Dr. Daniele Daffonchio at the Universita degli Studi di Milano (Italy), allowed the identification and localization of both microorganisms in the pathosystem MBSP – *D. maidis* – Maize plants.

The two hypothesis proposed were: 1-) The MBSP infection of maize plants and/or *D. maidis* affects the biology and behavior of the insect vector 2-) During the latent period of the phytoplasma MBSP within the vector, insect microbiota is present and cooperates with the phytopathogenic microorganism on its passage, movement and posterior transmission to new host plants.

## Objectives

- a. To investigate the effect of Maize bushy stunt phytoplasma MBSP on *D. maidis* preference to maize plants.
- b. Evaluate the effect of the phytoplasma MBSP on the *D. maidis* survival, reproduction and acquisition in maize plants.
- c. Identify and localize the microbiota present in *D. maidis* joining the phytoplasma passage and movement for the insect physiological barriers.

## 1.1 Methodology

The research was developed at the insect vector laboratory, Entomology and Acarology department Universidade de São Paulo – Escola Superior de Agricultura Luiz de Queiroz (ESALQ) in collaboration with Professor Dr. Daniele Daffonchio at the Milan University (Italy) who helped in the identification and localization of both phytoplasma and microbiota present in the insect physiological structures.

### **1.1.1 Obtention of healthy maize plants**

Hybrid maize seeds codified as 2B710PW and 2B688PW (Dow Agrosience ) susceptible to *D. maidis* were grown under greenhouse conditions in amount of 3 to 4 seeds per pot every four days during whole the research.

### **1.1.2. Obtention of a health vector colony.**

A *D. maidis* colony was initiated from a vector population maintained on greenhouse conditions. Young adults were confined during 72h in rearing cages containing 15d healthy maize plants as oviposition substrate. Plants with postures were isolated during 5d in order to dissect postures from plants. Dissected postures were placed on new health maize plants for ecloding nymph feeding. Every 5d maize plants were renewed until adult emergence. New emerged adults were freely reared on health maize plants for maintaining the health vector population.

### **1.1.3 Obtention and maintenance of an MBSP isolate**

Maize plants with typical symptoms of the phytoplasma were collected in an experimental field at the genetic department of the University of São Paulo ESALQ, and carried out to the laboratory for phytoplasma isolation. In laboratory, infected plants were covered with a cloth cage and infested with 150-200 *D. maidis* adults for a 96h Acquisition Access Period (AAP) ( $27 \pm 8$  °C,  $70 \pm 9$  % R.H; 14:10 h). After the AAP, insects were separated from the plants, released in amount of 20 insects per rearing cages containing healthy plants and maintained for a 23d Latent Period (LP) in controlled conditions ( $25 \pm 3$  °C y  $55 \pm 10$  %HR e 14:10 h). Once completed the LP, 10 infectious adults were released in a new rearing cage containing 15d maize plants during a 96h IAP and maintained under the same controlled conditions mentioned. Plants reaching the IAP were isolated from the insects and kept separated into cloth cages for symptom expression. The process was replicated during all the research time.

In order to confirm the phytoplasma infection in planta, the nested PCR was applied following the protocol followed by Rapussi et al (2012). A 4,0 cm<sup>2</sup> piece of leaf was soaked in 800 ml of CTAB 2x buffer solution. The product was disposed in an eppendorf tube and incubated at 65 °C in heat water during 40 minutes. Posteriorly 600 ml of CIA solution was added to the samples, mixed by inversion, vortexed and centrifugated for 10 minutes (1832,73

g.). Supernatant was separated in a new tube, adding 540 µl of cooled isopropanol (-20 °C), mixing gently to separate nucleic acids and cooling them overnight at 4°C.

At the following day samples were centrifuged for 10 minutes (1832,73 g.) and the liquid phase was separated from the resulting pellet. Pellet was washed two times in 500 µl ethanol 80% with a posterior washing in NaCl 1M (500 µl), and a posterior incubation in the NaCl solution during 40 minutes (4°C). Samples were again centrifuged for 10 minutes (1832,73 g.), eliminating the liquid solution from the pellet. Tubes containing the pellets were dried at room temperature. The pellet was finally re-suspended in a 100 µl buffer TAE.

Two amplifications were made to the samples, using for the first one the general primers R16mF2 (CATGCAAGTCGAACGGA) and R16mR1 (CTTAACCCCAATCATCATCGAC) for mollicute detection. The result of the first amplification was diluted in milli-Q water and re-amplified using the primers R16 (DF1) (TAAAAGACCTAGCAATAGG) - R16(DR1) (CAATCCGAACTGAGACTGT) for detection of group I phytoplasma. Conditions for both amplification were: 94°C ,2' one time; 94 °C, 1'; 50 °C, 2'; 27°C 3' 35 times; 72°C, 5' one time; 4°C indefinitely. Band signal was observed on agarose gel 1% placed into a dark chamber with UV light and the visualization on a computer with the software eagle®.

#### **1.1.4 Obtention of infected maize plants with the phytoplasma MBSP**

One maize seed was planted in a 15cm diameter pot, planting 40 plants per time. The material was preserved in a specific greenhouse until the formation of two truly leaves. Once the plants reached the desired stage, plants were exposed individually to five infectious *D. maidis* adults for a 72h Inoculation Access Period (IAP). After the IAP, insects were took off and plants were relocated in the greenhouse until the stage required for the experiments.

#### **1.1.5 Rearing of healthy *D. maidis***

A *D. maidis* population collected from Sete lagoas (Minas Gerais, Brazil) was maintained in greenhouse following protocols developed by Oliveira and Lopez (2004). The Insects were fed with maize plants of 40-45 days after sowing contained in cages of 40x40x60 cm. Every four days plant material was awarding to maintained quality food to the insect.

In order to maintain the insect colony, four maize plants of 25-30 days after sowing were weekly offered to healthy insects of known age during a 96 h oviposition period. After the mentioned time, plants with ovipositions were isolated in a new cage for the development of the immature stages. Ecloded nymphs were fed with healthy maize plants until the adult emergence.

### 1.1.6 Rearing of infectious *D. maidis*

To obtain infectious vector individuals, groups of healthy nymph IV, nymph V and adults were fed during a 72h AAP adults with infected plants of 45-55 days after inoculation in cages of 30x30x90 cm size. After the AAP the infectious individuals were isolated and relocated in rearing cages containing healthy maize plants during 23-25 latent period (LP) in controlled conditions. After the LP adults were able to inoculate the material required to the experiments, providing them healthy maize plants permanently for the maintenance of the infectious colony.

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**2 EARLY STAGES OF INFECTION BY MAIZE BUSHY STUNT PHYTOPLASMA ENHANCE HOST PLANT ATTRACTIVENESS FOR ITS LEAFHOPPER VECTOR, *Dalbulus maidis* (DELONG & WOLCOTT) (HEMIPTERA: CICADELLIDAE)**

**Abstract**

Vector-borne plant pathogenic bacteria can induce changes in infected host plants that may favor their own spread by altering the insect vector behavior and biology. This study aimed to investigate the effects of different stages of maize infection by the maize bushy stunt phytoplasma (MBSP) on host plant selection by the corn leafhopper, *Dalbulus maidis*. In a series of choice tests, the alighting and oviposition preferences of *D. maidis* adults were evaluated on healthy vs. infected MBSP-infected plants at 25-30, 30-35 and 40-45 days post-inoculation, when the maize plants showed early, intermediate and advanced disease symptoms, respectively. In separate experiment, we were able to detect MBSP infection by nested PCR in asymptomatic maize plants as early as 2, 5, 10, 15 and 20 days post-inoculation; thus, we also evaluated *D. maidis* preference for healthy vs. MBSP-infected (but still asymptomatic) plants, with 7, 9 and 13 days of disease incubation period. Finally, transmission efficiency by *D. maidis* was measured after acquisition on MBSP-infected, but still asymptomatic maize plants, with 3, 5, 10, 14, 20 and 27 days post-inoculation. *D. maidis* adults preferred to settle and oviposit on healthy than on symptomatic diseased plants, but preferred MBSP-infected plants in the asymptomatic phase of the disease. The phytoplasma was detected in maize leaves by nested-PCR since 2 days after inoculation, but with higher frequency at 20 days. Acquisition and transmission efficiency of MBSP by *D. maidis* was positively correlated with the disease incubation period in the source plants of the pathogen, with the higher rates of transmission detected when acquisition occurred on source plants with 14 days (27/30) and 27 days (28/30) post inoculation. These results suggest that MBSP modulates *D. maidis* preference for asymptomatic infected maize plants in the early stages of the crop, allowing rapid spread of this pathogen.

Keywords: Host preference; Incubation time; Leafhopper; Phytoplasma; Transmission efficiency

**2.1 Introduction**

Choice host by phytophagous insects comply a series of steps with a natural hierarquic structure, where each consecutive phase will occur when the previous one has been succeeded with the sufficient stimulus (JONES, 1991; VAN LOON, 1996; KÜHNLE; MÜLLER, 2012).

Commonly, insects are attracted at the distance from potential plant host by both visual and olfactive cues, but plant contact and probe by tactile, gustative and sensorial organs of the insect determines plant acceptance or rejection (VAN LOON, 1996; SISTERTSON, 2008; HILKER; MEINER, 2010, 2011).

As soon as an insect herbivore starts to feed on a plant, several defense signals are induced, leading to different defense responses. Plants can evaluate the quality and quantity of

leaf tissue damaged or can recognize compounds in insect oral secretions, as the case of sucking insects, which elicit more intense volatile responses than mechanical damage alone, as observed in *Zea mays* (L.) with the volicitin production when wounded (FÜRSTENBERG-HÄGG; ZAGROBELNY; BAK., 2013).

Vector-borne pathogens and other parasites often alter the traits of their host in such a way that influence the frequency and nature of interactions between hosts and vectors by inducing changes in host phenotype with important implications in the pathogen transmission (MAUCK et al., 2012; INGWELL, EIGENBRODE; BOSQUE-PEREZ, 2012).

In this relation the mode of transmission should be the major factor shaping the effects on plant viruses and other vector-borne parasites on aspects of the host phenotype that influence interactions with the insect vector (MAUCK; DE MORAES; MESCHER, 2010; MAUCK et al., 2012). Phytoplasma as a persistent propagative pathogen requires from the insect vector sustained food periods for hours or days to acquire the pathogen (BAI et al., 2006; HOGENHOUT et al., 2008) needing to maintain the ideal conditions of the plant for the insect feeding and reproduction.

There is evidence that a coevolution between a parasite and its host, often leads to lessened virulence of the host-parasite interaction, although this does not imply that continually decreasing virulence or avirulence response is the inevitable outcome (PURCELL, 1982; PURCELL; NAULT, 1997). For example, in the leafhopper *Macrostelus quadrilineatus* (Hemiptera: Cicadellidae) vector of AY- phytoplasma, the insect survival and egg oviposition increased when the insect fed on infected plants when compared with healthy individuals non exposed to the phytoplasma (BEANLAND et al., 2000; SUGIO et al., 2011; SUGIO; HOGENHOUT, 2012).

Contrary to the observed for *M. quadrilineatus*, in the leafhopper *Scaphoideus titanus* (Homoptera: Cicadellidae) vector of the phytoplasma Flavescence Doree (FDP), the average number of eggs carried by FDP-infected females was about half that of uninfected females and the average number of hatched nymphs per FDP-infected female was about one-third that of healthy females, suggesting that fecundity was limited by FDP infection (BRESSAN; GIROLAMI; PADIEU, 2005).

*Dalbulus maidis* (DeLong and Wolcott) (Hemiptera: Cicadellidae) is a monophagous species completing its development on the genus *Zea* (maize and teosintes) and living in a wide range of altitudes on its host from sea level to 3200m a.s.l. in the Peruvian Andes (NAULT, 1990).

*D. maidis* is identified as vector of three maize phytopathogens: ‘Corn Stunt Spiroplasma’ (CSS; *Spiroplasma kunkelii*), ‘Maize bushy stunt phytoplasma’ (MBSP, or Maize phytoplasma) and *Maize rayado fino virus* (MRFV) (AMMAR et al., 2011; ÖZBECK et al., 2003; WAQUIL et al., 1999).

Symptoms produced by MBSP on maize plants are generally observed 30 days after inoculation, with initially signs as chlorosis of margins of whorl leaves, followed by reddening of the tips of the older leaves (NAULT, 1980). With progressive infection times, infected plants can exhibit axillary buds, size plant reduction, as well as both spikes and grain deformation. Plants showing the mentioned symptoms reduce its production between a 35 to 93% depending on the infectious insect population (TOFFANELLI; BEDENDO, 2002).

In the present research we want to determine the effect of maize bushy stunt phytoplasma plant infection on the *D. maidis* preference measured as settled adults and laid eggs per plant.

## 2.2 Materials and Methods

Maize infected plants with MBSP (MBSP+) were evaluated in separated experiments for determining their preference to both healthy and infectious *D. maidis* adults. Three simple choice tests involving infected maize plants (MBSP+) with different disease incubation periods and healthy maize plants were evaluated for its settlement and oviposition to healthy or infected *D. maidis* adults. A fourth choice experiment considered MBSP+ asymptomatic maize plants at 7, 9, 13d after inoculation and a MBSP- healthy plant in a multiple preference test to detect any vector preference on asymptomatic maize plants. Detection of the phytoplasma MBSP at different disease incubation times was determined using nested PCR. Finally, the effect of the disease incubation period on the vector transmission efficiency was established with insects previously fed on MBSP+ plants at 3, 5, 10, 14, 20 and 27 days after inoculation for a 48h acquisition access period.

### 2.2.1 Plant material

Hybrid maize seed codified as 2B710PW susceptible to *D. maidis* were grown in amount of one seed per 10x10x15 cm plastic pot for bioassays as well as three seeds/pot for the insect mass rearing maintenance. The material used for bioassays was placed in greenhouse conditions ( $27\pm 8$  °C,  $70 \pm 9$  % R.H; 14:10 h) during 10-15 days until two leaves formation.

MBSP plants were obtained by releasing five infective *D. maidis* adults/plants into rearing cages for a 72h Inoculation Access Period (IAP). After the IAP, plants were separated from insects and were placed in an isolated greenhouse until the desired plant stage.

### 2.2.2 Insect material

Four to six maize plants with four to five leaves were infested with *D. maidis* adults in 30x 30 x 50 cm cages during a 96h oviposition time. Postures were isolated until the nymph hatching and fed with healthy maize plants until the adult emergence. Adults of known age were used for experiments involving healthy *D. maidis* individuals.

In order to obtain MBSP infectious adults, plants exhibiting MBSP symptoms were offered to healthy *D. maidis* adults for a 72h Acquisition Access Period (AAP). After the AAP, *D. maidis* adults were released into rearing cages containing three maize healthy plants during a 24 days Latent Period (LP) under a grown chamber ( $25\pm 5$  °C,  $65 \pm 7$ % R.H and 14:10 h photoperiod).

### 2.2.3 Plant choice of healthy *D. maidis* with MBSP infected plants at different disease incubation periods

In a single choice test, two sets of experiments evaluating MBSP+ plants 30-35d of disease incubation period (expressing initial symptoms) or MBSP+ plants 40-45d of disease incubation period (expressing advanced disease symptoms) were compared against health plants of the same age and conditions for each disease plant incubation period.

The experimental unit consisted of two MBSP- plants and two MBSP+ plants at the mentioned disease incubation period, placed individually on each corner of a cage (Bug Dorm® 0.70 x 0.70 x 0.90 m). Posteriorly, 50 individuals of 5-15d coming from the mass

rearing were sucked and disposed ventrally in a pre-freezed petri dish, for obtaining a 0.6 sexual rate. Sexed insects were placed into plastic tubes covered with a vinyl slice, and finally placed in a grid slung at the top of the cage and released.

#### **2.2.4 Plant choice of infectious *D. maidis* adults on MBSP+ plants at one specific incubation time**

To evaluate the plant preference of infectious *D. maidis* (+) adults, IV instar nymph individuals coming from the colony of MBSP infectious individuals were isolated and reared with infested maize plants until the adult emergence. 50 emerged infectious adults of 5-15d were classified by gender in a 0.6 sexual ratio.

Experimental conditions were the same as mentioned for the before tests, introducing two MBSP+ plants of 25-30d of disease incubation time (initial MBSP symptoms) and two MBSP- plants of the same age and conditions in a Bug Dorm® cage (0.70 x 0.70 x 0.90 m). Infectious adults previously sexed, were released at the top of the cage following procedures previously mentioned.

#### **2.2.5 Multiple plant choice of healthy *D. maidis* adults on asymptomatic MBSP+ plants at several disease incubation times**

A multiple free choice test was made to determine the preference of *D. maidis* adults for asymptomatic MBSP+ maize plants at early disease incubation times.

Health maize plants of 11-15d after sowing were exposed to five infectious *D. maidis* adults/plant during a 48h IAP and maintained in a growth chamber (25±5 °C, 65 ±7% R.H. and a 14:10 h photoperiod). After the IAP, inoculated plants were isolated in cloth cages for 7, 9 and 13d as the proposed disease incubation times (DIT). One MBSP+ plant at each DIT as well as one MBSP- plant 30d after sowing (similar time as the 13d DIT treatment) were introduced inside a cloth cage and located on each corner of the cage.

Thereafter, 50 *D. maidis* adults of 5-15d age in a 0.6 sex ratio were placed in a hang up grid at the top of the cage, for freely releasing.

### **2.2.6 Early detection of MBSP asymptomatic maize plants at early incubation disease periods**

An experiment evaluating plants with early MBSP incubation time was developed using maize plants 12-15d after sowing and exposed with five infectious *D. maidis* adults/plant during a 24 h Inoculation Access period (IAP) under controlled conditions ( $25\pm 5$  °C,  $65 \pm 7\%$  R.H and 14h :10h photoperiod). After the IAP, plants were separated from adults and maintained in greenhouse conditions for 2, 5, 10, 15 and 20d as incubation disease times.

For each incubation time, 10 MBSP+ maize plants were sampled taking one inferior and one superior leaf placed in a plastic bag, labeled by treatment and preserved in cooled conditions at 4°C.

In laboratory, nested PCR was followed according to the protocol used by Rapussi et al., (2012) and detailed on item 1.1.2 of this document.

### **2.2.7 Acquisition and transmission efficiency of *D. maidis* adults fed on MBSP infected plants with different incubation periods**

Groups of 12 MBSP- plants at 12-15d after sowing were exposed to five infectious adults/plant during a 48h IAP and maintained in growth chamber under controlled conditions ( $25\pm 5$  °C,  $65 \pm 7\%$  R.H. ; 14:10 h photoperiod). After the IAP insects were separated, plants were cleaned from any posture and isolated into cloth cages under greenhouse conditions. Plants reaching the disease incubation times of 3, 5, 10, 14, 20 and 27d were relocated by treatment in new cloth cages for the *D. maidis* acquisition. As a control, a group of healthy plants was maintained in the same conditions as the mentioned above.

In a transference chamber, healthy IV instar nymphs coming from the insect mass rearing were separated in subgroups of 10-20 individuals and placed into plastic tubes covered by a vinyl slice. Tubes with the immatures were introduced into the cloth cages and vertically placed on the ground of each pot, allowing the insects freely reaching the plant via the stem, and fed on the MBSP+ plant for a 48h acquisition access period (AAP).

Insects complying the AAP, were recovered from each plant and were released into rearing cages (20 individuals/rearing cage) containing healthy maize plants under growth chambers ( $25\pm 5$  °C,  $65 \pm 7\%$  R.H. and a 14:10 h photoperiod) for a 22 days latent period. MBSP- plants were replaced every four days until the (LP) of *D. maidis* was completed.

Infectious adults reaching the mentioned LP were evaluated for its capacity to transmit the phytoplasma at 22, 24 and 26 days of LP. To do so, five infective adults/treatment (insects previously fed on plants with each disease incubation time) were released on a healthy maize plant (12- 15 days after sowing) contained in a pet cage during a 48h IAP at the same controlled conditions as mentioned above. After the IAP, test plants were replaced for a new one, isolating the new inoculated plant in cages under greenhouse conditions during 40 days, time of the symptom visualization.

Infectious adults used to inoculate test plants were preserved in ethanol 70% until its processing at the laboratory, following the protocol developed by Marzachi, Veratti and Bosco (1998).

A sample of 30, 29, 30, 30, 30, 27 and 24 *D. maidis* adults were processed individually for each treatment (control, 3d, 5d, 10d, 14d, 20d and 27d). For DNA extraction each individual was placed in a 0,5 ml Eppendorf tube containing 10 µl of NaOH (1M). Samples were soaked with a weak pin pale and posteriorly added 20 µl HCL (1%). The diluted solution was denatured in heated water (65 °C) during 40 minutes. Posteriorly samples were centrifuged for 10 minutes (1832,73 g.). 30 µl of the supernatant were disposed in a new tube, adding 30 µl of ethanol 95% and placed in cooled conditions (4 °C) during 40 minutes. After the mentioned time, samples were centrifuged during 10 minutes (1832,73 g.) and pellet formed was separated from the liquid solution and dried at room temperature. The dried pellet was re-suspended in a 100 µl buffer extraction solution and maintained in freezer until necessary.

In a nested PCR procedure the samples were initially amplified using the general primers R16mF2 (CATGCAAGTCGAACGGA) and R16mR1 (CTTAACCCCAATCATCATCGAC) for mollicute detection. 2 µl of the first amplification were diluted in 38 µl of milliQ water and re-amplified using the specific primers R16(I)F1 (TAAAAGACCTAGCAATAGG) - R16(I)R1 (CAATCCGAACTGAGACTGT) for detection of group I phytoplasma . Conditions for both amplifications were: 94 °C x 1 minute (1 cycle); 94°C x 45 seconds, 50 °C x 30 sec., 72°C x 1,5 min. (35 cycles); 72°C x 5 min. (1 cycle); 4°C x indefinite. After the last amplification, samples were run in a 1% agarose gel during 1h at 100V. Afterwards, gel was disposed in a dark chamber with UV light for band visualization (as the signal of phytoplasma presence) and its observation through the software eagle®. For each insect stage were registered the amount of positive samples.

### 2.2.8 Statistical conditions

Plant choice tests at a specific disease incubation period were replicated 10 times beginning 9:00h each, with evaluations 2, 4, 8, 24, 28, 32 and 48 h after initiated. On each evaluation time was registered the amount of settled adults per plant, adults outside the plants and adults death. Six days after the last observation were counted the laid eggs /plant. Variables analyzed consisted in the proportion of settled adults (amount of adults landed per plant shared the released total adults minus death adults) and laid eggs.

Data were processed eliminating zeros and confirming normality with Shapiro-Wilk test. For those data showing normality was applied the F-Test, meanwhile non-normal ones were processed with GLM procedure (families poisson, quasipoisson) Chi-square test, and t-test.

Multiple plant choice test of healthy *D. maidis* consisted of four treatments with seven replications and the evaluation times of 2, 8, 24, 32 and 48 h after the beginning of the experiment. On each evaluation was registered the amount of settled adults/plant, adults outside of plants and death adults. Eggs laid were registered six days after the end of the final observation. Variables analyzed consisted in the proportion of settled adults (amount of adults landed per plant shared the released total adults minus dead adults) and laid eggs. Data were processed applying the GLM procedure (families Poisson, quasipoisson) and Chi-square test for the proportion of settled adults and eggs laid. Comparison of means was made with Tukey test.

Early detection of MBSP asymptomatic maize plants, was analyzed by comparing plants with positive signal to the phytoplasma MBSP by PCR technique, and for level substrate sample. Procedure GLM (family binomial) was used for determining statistical differences and Tukey test for means comparison.

Finally, acquisition and transmission efficiency experiment consisted on seven treatments, 30 replicates/ treatment, and three evaluation times. Variables registered consisted on plants directly observed with MBSP symptoms and indirectly by PCR detection of adults showing positive signal of the phytoplasma. Proportion of infected plants was determined as the amount of plants showing MBSP symptoms/treatment shared by the total amount of plants/treatment. Data were processed applying the GLM procedure (family binomial) and Chi-square test for the proportion of infected plants and positive MBSP adults. Comparison of means was made with Tukey test.

## 2.3. Results

### 2.3.1 Plant choice of healthy *D. maidis* with MBSP infected plants at different disease incubation times

Chi-square test for proportion of settled adults on MBSP infected maize plants (MBSP+) 40-45 days of disease incubation time or health plants (MBSP-) showed significant differences between treatments ( $\chi^2= 5.776$ ;  $df=229, 227$ ;  $P< 5.8e-14$ ) with the higher proportion of settled adults on MBSP- plants (Figure 1A). No statistical difference was evidenced between treatments for settled adults at each observation time ( $\chi^2= 0.064$   $df= 228, 227$ ;  $P= 0.430$ ).

The amount of laid eggs on MBSP+ plants with advanced disease symptoms or MBSP- plants showed statistical differences ( $t=4.459$ ;  $df= 36$ ;  $P< 7.5e-05$ ) with the higher amount of laid eggs on MBSP- plants (Figure 2A).

The experiment evaluating host preference of healthy *D. maidis* adults to MBSP+ plants 30-35 days of disease incubation time did not exhibit statistical differences between treatments for the mean settled adults ( $\chi^2= 0.010$ ;  $df=236, 233$ ;  $P=0.722$ ) (Figure 1B). When analyzed the settled adults on each evaluation time, results showed no significant differences ( $\chi^2= 0.0830$   $df= 236, 233$ ;  $P= 0.314$ ).

The amount of laid eggs on MBSP+ plants 30-35 days of disease incubation time or MBSP-plants showed statistical differences between treatments ( $t= 2.039$ ,  $df= 25$ ;  $P= 0.052$ ) (Figure 2B).

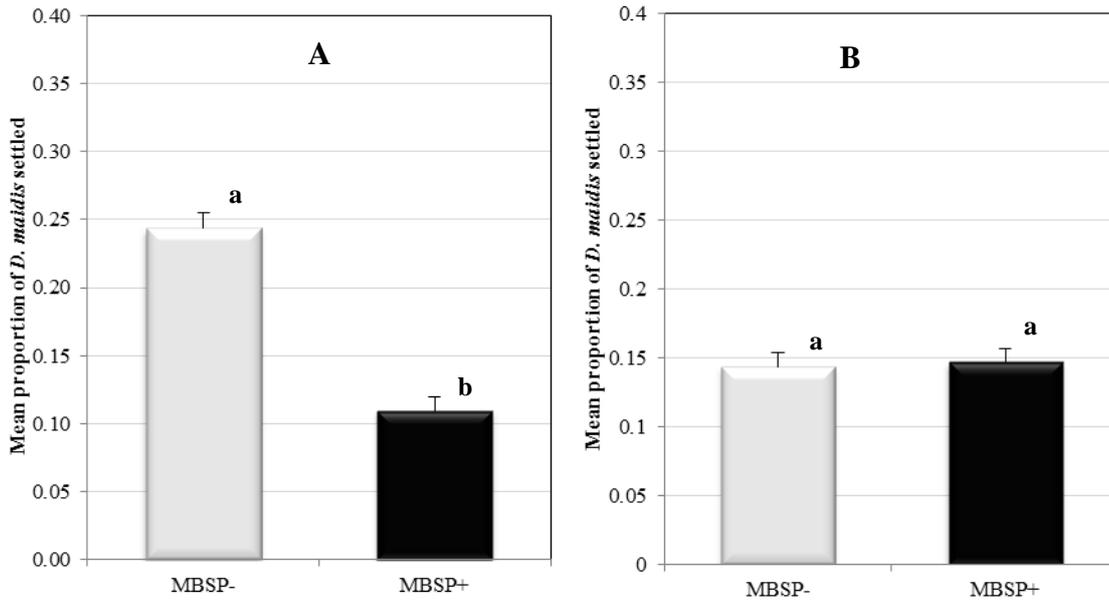


Figure 1 - Mean proportion of healthy *D. maidis* adults settled on healthy (MBSP-) or maize bushy stunt phytoplasma infected (MBSP+) maize plants with A) 40-45 days of disease incubation period, and B) 30-35 days of disease incubation period. Bars on the columns represent the standard error of the mean. Means with the same lowercase letters within the same incubation time, do not differ statistically

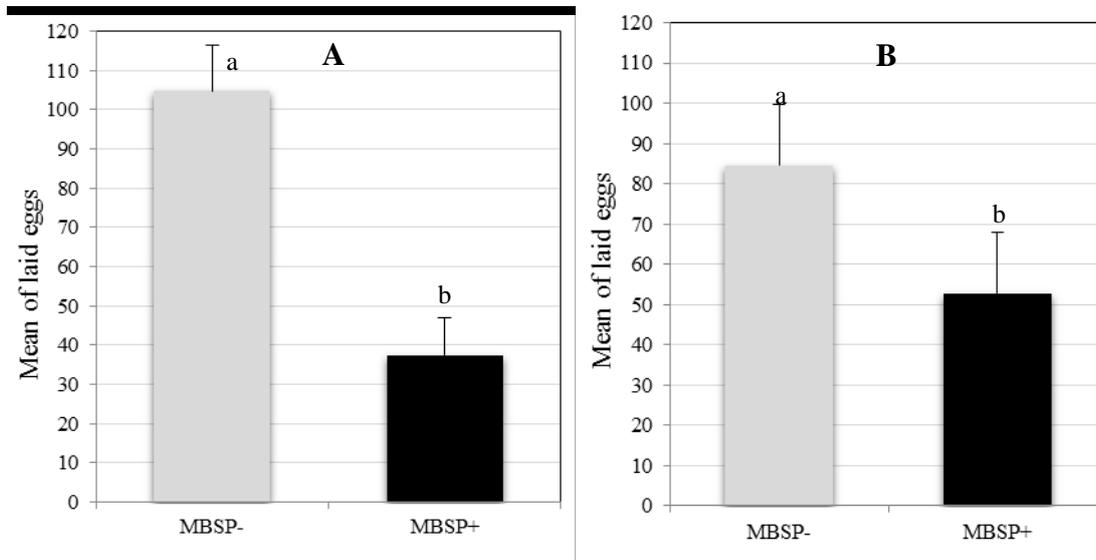


Figure 2 - Mean number of eggs laid by healthy *D. maidis* on healthy (MBSP-) or maize bushy stunt phytoplasma (MBSP+) maize plants with A) 40-45 days of disease incubation period and B) 30-35 days of disease incubation period. Bars on the columns represent the standard error of the mean. Means with the same lowercase letters within the same incubation period do not differ statistically

### **2.3.2 Plant choice of infectious *D. maidis* adults on infected MBSP plants at one specific incubation time**

The statistical analysis for infectious *D. maidis* adults and their preference to settle on MBSP- or MBSP+ plants with 25-30 days of disease incubation time (initial disease symptoms), showed differences between treatments ( $\chi^2 = 8.246$ ;  $df = 240, 237$ ;  $P < 2.2e^{-16}$ ) with a notorious preference of the infectious adults for MBSP- plants ( $0.26 \pm 0.01$ ) than for MBSP+ plants ( $0.10 \pm 0.01$ ).

Oviposition measured as laid eggs of infectious *D. maidis* showed statistical difference between treatments ( $t = 4.875$ ;  $df = 25$ ;  $P < 5.2e^{-05}$ ) with preference for MBSP- plants ( $80.80 \pm 10.95$ ) than on MBSP+ plants ( $23.25 \pm 3.94$ ).

### **2.3.3 Multiple plant choice of healthy *D. maidis* adults on MBSP infected plants at several disease incubation times**

When evaluated asymptomatic MBSP+ plants with different incubation times, statistical analysis for the proportion of settled adults showed statistical differences between treatments ( $\chi^2 = 3.930$ ;  $df = 136, 134$ ;  $P < 2.01e^{-14}$ ) with the higher proportion of settled individuals on MBSP+ plants 13d and 9d with respect to MBSP+ plants 7d and MBSP- plants (Figure 3A).

Chi-square test for the amount of laid eggs exhibited statistical differences between treatments ( $\chi^2 = 214.55$ ;  $df = 16, 15$ ;  $P < 4.5e^{-4}$ ) with the higher oviposition on MBSP+ plants 13d of disease incubation time, followed of MBSP+ plants 9d and 7d, as well as MBSP- plants (Figure 3B).

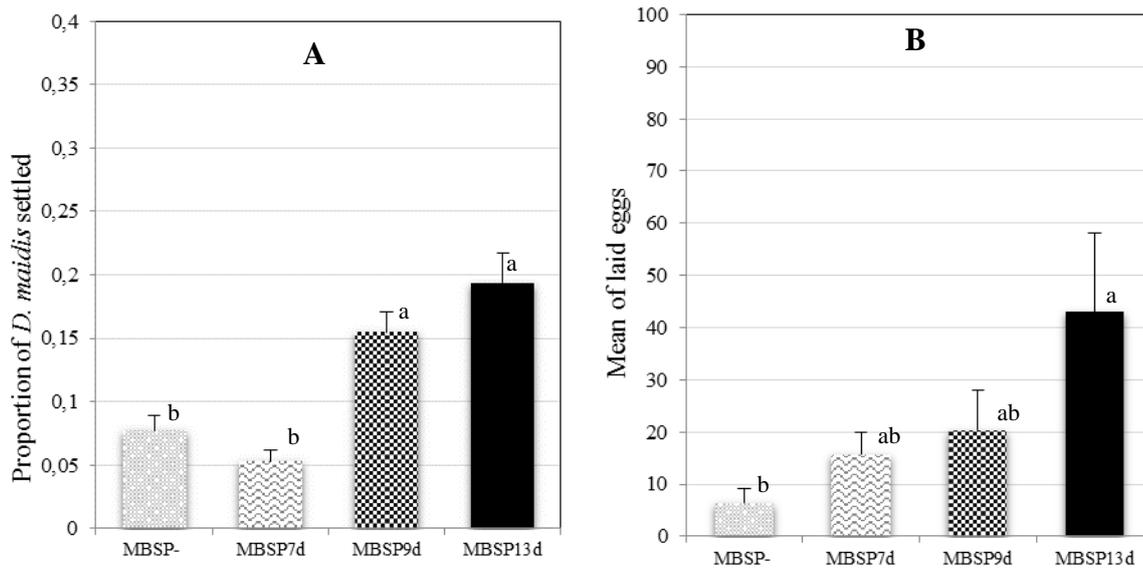


Figure 3 - Mean Proportion of healthy *D. maidis* adults settled on (A) health (MBSP-) or maize bushy stunt phytoplasma infected plants (MBSP+ 7d, 9d, 13d) of disease incubation period (B). Bars on the columns represent the standard error of the mean. Means with the same lowercase letters with in the same incubation time, do not differ statistically by the Chi-square test

### 2.3.4 Early detection of MBSP infected maize plants with early incubation disease period

Statistical analysis for the proportion of MBSP+ plants detected by nested PCR evidenced differences between treatments ( $\chi^2 = 10.352$  df= 95, 93  $P < 0.035$ ) with the higher detection on MBSP+ plants 15d and 20d of disease incubation time ( $0.40 \pm 0.11$  **a**), followed by MBSP+ plants 2d of disease incubation time ( $0.20 \pm 0.09$  **b**), and both MBSP+ plants 5d and 10d of disease incubation time ( $0.10 \pm 0.07$  **b**)

### 2.3.5 Acquisition and transmission efficiency of *D. maidis* adults fed on MBSP infected plants with different incubation periods

Transmission efficiency of adults acquiring the phytoplasma on source plants at different incubation times, showed statistical differences between treatments at the three evaluation times ( $\chi^2$  40d= 3.9308 ; df= 201, 200;  $P < 2e-16$ ); ( $\chi^2$  50d = 163.98 ; df= 201, 200;  $P = 2e-16$ ); ( $\chi^2$  60d = 145.82; df= 201, 200;  $P < 2e-16$ ).

At the first evaluation (40 days after MBSP incubation time), *D. maidis* acquiring the MBSP from source plants 27d and 14d after disease incubation period exhibited the higher

proportion of infected plants with no differences with treatment 20d .Treatments 10d and 5d did not showed statistical differences with treatment 20d neither with treatment 3d . Control treatment did not exhibit infected plants (Table 2).

For the second evaluation (50d after disease inoculation) adults acquiring the phytoplasma on source plants 10d, 14d, 20d and 27d after MBSP incubation period showed statistical differences with those adults acquiring the phytoplasma 3d. Individuals acquiring the phytoplasma on source plants 3d after MBSP incubation time did not evidenced statistical differences with those of 5d. Control did not evidenced symptomatic plants (Table 2).

At the final evaluation (60d after disease inoculation) adults acquiring the phytoplasma on source plants 14d, 27d, 20d, 10d and 5d after incubation period showed differences with treatments 3d and control (Table 2).

Table 2 - Mean proportion of maize test plants expressing MBSP symptoms 40, 50 and 60 days after phytoplasma inoculation with *D. maidis* adults previously fed on source maize plants at six disease incubation times

Incubation time (days) of maize plants used as source to acquire the MBSP by <i>D. maidis</i> adults <sup>1</sup>	Proportion of maize test plants (mean $\pm$ sem) expressing phytoplasma symptoms after inoculated with infectious <i>D. maidis</i> adults <sup>2</sup> . Experiment evaluations at 40, 50, and 60 d after inoculation		
	40	50	60
3	0.40 $\pm$ 0.09 c	0.43 $\pm$ 0.09 b	0.47 $\pm$ 0.09 b
5	0.43 $\pm$ 0.09 bc	0.67 $\pm$ 0.08 ab	0.73 $\pm$ 0.08 a
10	0.43 $\pm$ 0.09 bc	0.77 $\pm$ 0.07 a	0.77 $\pm$ 0.08 a
14	0.93 $\pm$ 0.05 a	0.90 $\pm$ 0.05 a	0.93 $\pm$ 0.03 a
20	0.73 $\pm$ 0.08 ab	0.87 $\pm$ 0.06 a	0.90 $\pm$ 0.06 a
27	0.93 $\pm$ 0.05 a	0.93 $\pm$ 0.05 a	0.97 $\pm$ 0.03 a
C-	0.00 $\pm$ 0.00 d	0.00 $\pm$ 0.00 c	0.00 $\pm$ 0.00 c

<sup>1</sup>Source plants at each incubation time came from 12-15d maize health plants exposed to five infectious *D. maidis* adults/plant during 48h of inoculation access period (IAP) under controlled conditions (25 $\pm$ 5 °C, 65  $\pm$ 7% R.H. ; 14:10 h photoperiod). <sup>2</sup> 10-20 *D. maidis* V instar nymphs were allowed to feed on each source plant during a 48h acquisition access period (AAP). Individuals reaching the AAP were recovered from each source plant and maintained under controlled conditions (25 $\pm$ 5 °C, 65  $\pm$ 7% R.H. and a 14:10 h photoperiod) for a 22 days latent period (LP). Five infectious adults/ test plant were released during a 48h IAP. After the IAP, Inoculated plants were maintained in greenhouse conditions for symptom visualization

Besides the incidence of the disease severity of the MBSP infection allowed to differentiate three groups.

A first group with severe disease symptoms was easily identified because of the reddening mature leaves observed in the middle and inferior plant substrate, the yellowing upper superior leaves, the straightened-up foliage position, and the stunt of plants with a debilitated general appearance. Test plants infested with adults previously fed on MBSP+ maize plants 27d of disease incubation time and some plants infested with adults previously fed on MBSP+ plants 14d of disease incubation time, evidenced the mentioned symptoms (Figure 4).

A second group evidenced a reduced symptom expression with a characteristic reddening band observed at the upper side of the central nervure of the inferior leaves substrate, stunted plants compared with the non-infected plants, and yellowing leaves with a marked and smooth lines crossing the superior foliage level (Figure 5). Test plants infested with adults previously fed on MBSP+ plants 20d, 14d and 10d of disease incubation time were observed with the before symptom description.

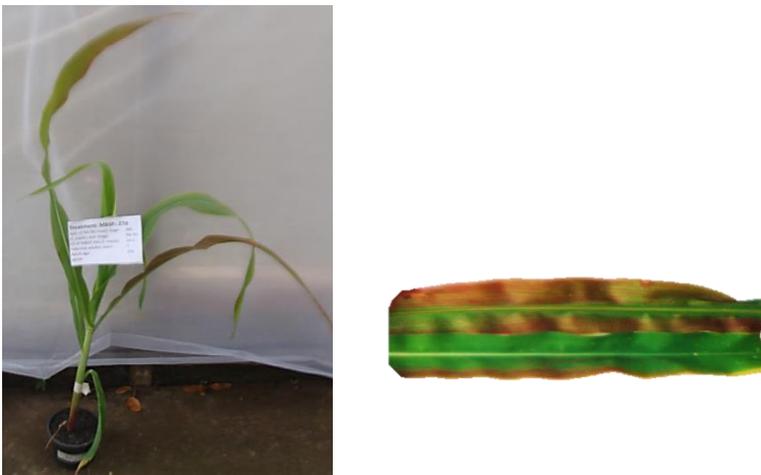


Figure 4 - Symptoms observed on test maize plants at 40d of disease incubation time, when inoculated with *D. maidis* adults (IAP= 48h) previously fed on infected source maize plants (MBSP+) at 27d of disease incubation time. Plants are observed stunted, weaken and with strong reddening leaves.

Photo: J. García-González



Figure 5 – Symptoms observed on test maize plants at 40d of disease incubation time, when inoculated with *D. maidis* adults (IAP= 48h) previously fed on infected source maize plants (MBSP+) at 10, 14, and 27d of disease incubation time. Plants are observed stunted, weakened and with strong reddening leaves.

Photo: J. García González

On a third group were observed test plants with poor expression symptoms corresponding to weakened plant constitution, stunted plants, and in some of them yellowing leaves at the upper substrate. To this description were observed test plants infested with adults previously fed on MBSP+ plants 3d, 5d and 10d of disease incubation time (Figure 6).

These observations allowed confirming the relation of disease incubation time and symptoms incidence observation. Moreover, these results confirm the previous one found on this research, where asymptomatic MBSP+ plants with the higher disease incubation time is positively related with higher transmission efficiency. Legrand and Power (1994), evaluating in separated experiments the effect of the Acquisition Access Period (AAP) and the Inoculation Access Period (IAP) on the *D. maidis* MBSP transmission (defined as MBSM in the original paper), found a positive relation of MBSP+ maize infected plants with increased AAP and IAP. Moreover, the authors demonstrated a higher effect of the IAP (30/59 at 72 h of IAP) than the AAP (11/58 at 72h of AAP) on the *D. maidis* transmission efficiency.

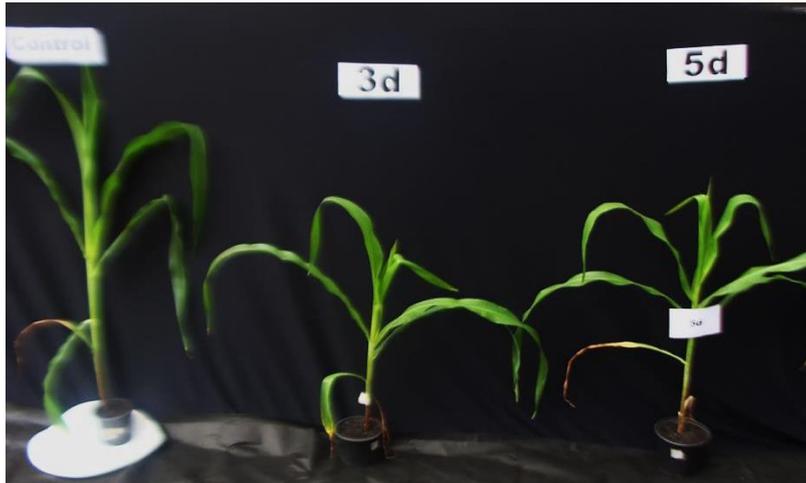


Figure 6 - Disease symptom expression of MBSP+ maize plants infested with *D. maidis* adults previously fed on MBSP+ source plants 3d and 5d of disease incubation time. Comparison of symptom expression can be made between MBSP+ plants with the MBSP- plant (control)

Photo: J. García-González

Results of MBSP detection via PCR for *D. maidis* adults acquiring the phytoplasma on source plants at different disease incubation times, and subsequently used in the transmission efficiency experiment, showed the higher phytoplasma detection in adults fed on source plants 5d, 14d, and 27d after disease incubation time (Table 3). Contrary, the lesser MBSP detection was observed in adults fed on source plants at 3d, and 10d after disease inoculation period (Table 3).

Table 3 - Mean fraction of PCR positive *D. maidis* adults for MBSP after acquisition on source plants with different incubation periods and posterior inoculation of test maize plants

<b>Incubation time (days) of maize plants used as source to acquire the MBSP by <i>D. maidis</i> adults</b>	<b>Fraction of +adults/total evaluated adults (proportion) expressing MBSP signal by nested PCR<sup>1</sup></b>
<b>3</b>	7/29 (0.24)
<b>5</b>	13/30 (0.43)
<b>10</b>	8/30 (0.27)
<b>14</b>	12/30 (0.40)
<b>20</b>	10/27 (0.37)
<b>27</b>	10/24 (0.42)
<b>Control</b>	0/0

<sup>1</sup>A sample of *D. maidis* coming from those adults used in the transmission efficiency experiment were individualized and processed by nested PCR in order to confirm MBSP acquisition from different source plants at different phytoplasma incubation times

## 2.4 Discussion

The hypothesis of the phytoplasma effect of the host plant on the vector settlement and oviposition, evidenced an interesting plant choice behavior depending on its quality and the degree of host plant modification due to the phytoplasma infection (MAYER, VILCINSKAS; GROSS, 2008a, 2008b).

When offered to healthy *D. maidis* health maize plants (MBSP-) or maize plants 40-45d of disease incubation time (MBSP+ expressing advanced disease symptoms), the vector evidenced a strong preference to settle and to oviposit on MBSP- maize plants. Also, when

offered to healthy *D. maidis* adults, MBSP- or maize plants 30-35d of disease incubation time (MBSP+ plants with initial disease symptoms) *D. maidis* did not prefer to settle on any evaluated plant, but an insect tendency to oviposit on MBSP- plants.

The effect of the phytoplasma MBSP on the host preference by infectious *D. maidis* adults when evaluated MBSP+ plants with early disease symptoms or MBSP- plants showed a strong preference to settle and to oviposit on MBSP- plants.

General results evidenced an effect of the plant disease development on the *D. maidis* choice to settle and to oviposit, with a potential visual and tactile plant influence on the insect preference. MBSP+ plants expressing advanced disease symptoms were observed weakened, size reduced, and with mature leaves of redding colour (Figure 4). Additionally, MBSP+ plants showing early disease symptoms, expressed lighted symptoms specifically observed on the inferior leaves and differentiated as a fine redding band present in the central nervure position, and some yellowish coloration at the new formed leaves level.

As shown in previous studies with Maize Bushy Stunt Micoplasma (previous denomination for MBSP) the phytopathogen can produce different disease symptoms in maize depending on the variety and temperature. First disease symptoms at 27 °C : 18 °C (day: night) were observed  $18,9 \pm 1,3$  days of disease incubation time, meanwhile at 31°C : 25°C symptoms were observed earlier at  $9,5 \pm 0,9$  days (day: night), consisting in chlorosis of margins of whorl leaves following by reddening of the tips of older leaves (NAULT, 1980).

When offered asymptomatic MBSP+ plants with different incubation time, the vector preferred to settle and to oviposit on plants with 13 d and 9 d of disease incubation time than on healthy plants or asymptomatic plants with 7 days of disease incubation time. Probably on asymptomatic maize plants 13d of incubation time, the patho-system reaches an equilibrium in which the MBSP infection on maize plants is the adequated for the vector attraction, settlement and oviposition. As demonstrated, the vector attraction on this host plant is visually induced, but some olfactive stimulus would be considered and studied in more detail.

A unique study measuring the visual and olfactive signals on the *D. maidis* stimuli by artificial means, showed that a combination of both the green light and hexane volatile compounds significantly increased the stationary insect time and evidencing the interaction between these stimuli during the host-finding for a leafhopper and for olfactory mediation of post contact behaviors not associated with feeding (TODD; PHELAN; NAULT, 1990).

Exploitation process by a phytophagous insect is divided in searching, finding, recognition, acceptance and host availability (JONES, 1991; KÜNHLE; MÜLLER, 2012).

After host recognition from the distance, tarsi and mouth organs will give the insect the chemical leaf signals for its selection or rejection. If host signals are accepted the insect will begin its sustained feeding and oviposition (MÜLLER; ROSEMBERG, 2006; KÜNHLE; MÜLLER, 2012).

Of interest the early MBSP detection on infected maize plants, since 2d of disease incubation time, with a tendency to detect more MBSP plants with increasing disease incubation times. Indeed, this treatment displayed higher positive detection samples when compared with plants of 5 days or 10 days disease incubation time. In a similar study the presence of the onion yellows phytoplasma in garland chrysanthemum into the main stem, apex, and roots of chrysanthemum plants was confirmed after 2 days of inoculation, but the bacteria required about three weeks infecting all leaves (WEI et al., 2004).

Positive MBSP samples detected by nested PCR were observed lower than plants showing MBSP symptoms (data not shown), evidencing an underestimation of the positive MBSP samples detected by the molecular technique. It is evident that sample quality for diagnose a disease in early stages of phytopathogen is a fundamental factor affecting the precision and sensitivity of any technique.

In the pathosystem MBSP – maize crop there is no research showing the movement of the phytoplasma and its relation with the plant stage as a guide to take a sample of quality for the disease diagnostic. A recent research attempting to find the better place to take a sample for early infections of the Huanglongbing bacteria in asymptomatic citrus crops, found more bacteria titers in roots than on the traditionally sampled foliage (JOHNSON et al., 2014). Wei et al. (2004), using the quantitative PCR technique also demonstrated the higher total concentration of the onion yellows phytoplasma in garland chrysanthemum roots when compared with the leaves, suggesting these organs not only as a storage point but as a cue point for the phytopathogen multiplication.

It is expected a direct relation between the higher disease incubation time and the higher titer of the phytopathogen into the plant, facilitating the disease detection. But factors such as the plant size, movement of the phytoplasma into the plant structures, the amount of infectious individuals in the vector population and the inoculation access period (IAP) of the insect vector will determine the phytopathogen concentration at the moment to take the sample. Related with the IAP on the *D. maidis* transmission efficiency Legrand and Power

(1994) demonstrated an early MBSP phytopathogen transmission by the vector (4/60) after 30 minutes of IAP with the higher transmission efficiency (24/34) after nine days of IAP.

With the interest to confirm the importance of asymptomatic MBSP+ plants on the disease epidemiology a final experiment evaluating adults previously fed as IV instar nymph during a 48h of AAP, maintained in controlled conditions during a 23d LP, and infecting healthy maize plants (12-15 days after planting) during a 2d IAP, intended to determine the effect of the MBSP disease incubation time in asymptomatic plants on the *D. maidis* transmission efficiency.

Expected outcome as the amount of visual infected plants, showed two associated response components related with the symptom visualization time and the severity of symptoms observed as a result of the evaluated treatments.

Related with the visualization time, a positive relation between the earlier symptom observation and the major in planta disease incubation time was evidenced. Treatments using adults fed on MBSP plants 27d, 20d, and 14d after disease incubation time exhibited the earlier symptom observation (28/30; 22/30; 27/30 respectively) for the first evaluation time.

For the rest of the evaluation times the treatments were adults fed on MBSP infected plants of 27d and 14d of disease incubation time maintained the higher amount of tested plants. Contrary to the mentioned, the transmission efficiency of *D. maidis* adults fed on asymptomatic infected plants of the treatment 3d of disease incubation time, exhibited the lower transmission efficiency for each observation time (12/30, 13/30, 14/30).

Important to highlight the results obtained on the experiments evaluating asymptomatic MBSP+ maize plants 13-14d of disease incubation time, in which *D. maidis* preferred to settle and to oviposit (experiment 5), and evidenced one of the higher transmission efficiency (28/30) (experiment 6). Further studies should determine the potential effect of MBSP+ plants 14d of disease incubation time on the phytoplasma epidemiology, presumed as the phytoplasma titer at this disease stage, that facilitates its acquisition by *D. maidis* without interfering on both feeding and oviposition behavior.

When analyzed the positive infectious adults (by nested PCR) used on the MBSP transmission efficiency (experiment 6), data showed a mean of  $0.43 \pm 0.08$  positive adults, evidencing the difficulties for MBSP to invade and to multiply within the vector and possibly due to the phytoplasma movement restrictions, the low titer of bacteria acquired, the reduced

titer of the phytoplasma in the host plant, and the MBSP location into the host plant at the moment of the vector feeding.

A study of Crysanthemum yellow Phytoplasma (CY) transmission efficiency by its vector *Euscelidius variegatus* (Hemiptera: cicadelidae), was obtained a 17-22% of non-transmitter vector individuals, attributing the result to the phytoplasma difficulty to invade organs and cross the physiological insect barriers, especially the midgut, or the vector feeding on plant tissues other than the phloem (GALETTO et al., 2009).

In the present study the Transmission Electronic Microscopy (TEM) technique made for both leaf and root samples (Figures 9B,C,D in chapter 4), coming from MBSP+ plants with advanced disease symptoms (Figure 9A chapter 4) as contrary expected, revealed a higher presence of the phytoplasma bacteria in roots, with no observations in the redding leaves.

This result suggest an active MBSP movement to different plant structures after inoculation of infectious *D. maidis*, and not necessarily reflect a large presence and concentration on the plant organs with visualized disease symptoms, as predicted for redding leaves.

## 2.5 Conclusions

Overall, the evidences gathered in the present study indicates the essential role of asymptomatic MBSP+ maize plants and the disease incubation time on both MBSP acquisition and transmission efficiency by *D. maidis*.

When conceived the early attack of *D. maidis* to maize plants at the initial crop stages, the chances for the vector infection from asymptomatic MBSP+ plants increases the possibility of *D. maidis* secondary disease dissemination.

Insects feeding on asymptomatic MBSP+ plants are effective vectors of the disease expressing high transmission efficiency of new host plants.

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### 3 EFFECT OF MAIZE BUSHY STUNT PHYTOPLASMA MAIZE INFECTION ON THE BIOLOGY OF *Dalbulus maidis* (DELONG & WOLCOTT) (HEMIPTERA: CICADELLIDAE)

#### Abstract

Maize bushy stunt phytoplasma (MBSP), a stunting pathogen in Latin America, presumably has an extended co-evolutionary interaction with its leafhopper vector, *Dalbulus maidis*, perhaps influencing this vector fitness. This research aimed to determine the effects of MBSP infection on the biology of *D. maidis*. Life table and life stage experiments were developed on healthy vs. MBSP-infected maize plants at 45 and 14 days of disease incubation time, respectively. Plants of each treatment were exposed to 30 healthy *D. maidis* couples, during a 24-h oviposition period. Survival and duration time of each insect stage or stadium were recorded at 2-day intervals and at six specific dates, respectively. Real fecundity in each experiment was estimated from a sample of 14 and 11 adult couples emerged from the first adult generation. The net reproductive rate ( $R_0$ ), intrinsic rate of increase ( $r_m$ ), annual growth rate ( $\lambda$ ), and generation time ( $T$ ) were estimated for the experiment with 45 days of incubation period). Samples of three to five N1, N2, N3, N4, N5 nymphs, and of individual female and male genitalia of *D. maidis* growing on asymptomatic MBSP-infected plants were processed through nested-PCR, in order to determine if the vector acquired the phytoplasma. The results showed a neutral effect of MBSP on *D. maidis* biology, with a sexual ratio adjustment and a reduction in development time of some vector stages when reared on MBSP plants. *D. maidis* showed effective acquisition of MBSP by all nymphal stadia, and the phytoplasma was detected in adult male and female reproductive organs, suggesting MBSP horizontal transmission may occur between infective adults.

Keywords: Acquisition; Life table. MBSP; Population parameters

#### 3.1 Introduction

Phytoplasmas are phytopathogenic bacteria commonly transmitted by leafhoppers and planthoppers of the Deltocephalinae subfamily. These insects are effectively phytoplasma vectors because either immature or adult stages feed on the same plant tissue, because of their persistent propagative transmission and because their feeding behavior is nondestructive promoting successful inoculation of the plant vascular phytopathogens (WEINTRAUB; BEANLAND, 2006; WILSON; WEINTRAUB, 2007). After completion of the acquisition access period on infected plants generally an increase in the phytoplasma titer on the vector is observed as an evidence of the phytoplasma multiplication. Such multiplication in the vector points to highly evolved interrelationships and explains the high level of specificity of transmission (BOSCO et al., 2007).

The multiplication of a microbe within the body cavity of an insect often results in a pathological reaction for the vector (PURCEL; NAULT, 1997), but its invasion on the vector

can also generate positive effects, neutral effects or insect fitness reduction (BEANLAND et al., 1999; BRESSAN; GIROULAMI; PADIEU, 2005).

As examples, the generalist planthopper *Macrostelus quadrilineatus* Forbes (Hemiptera: Cicadellidae) fed with the phytoplasma AY-WB infected plants, survive longer and produce more progeny when compared with individuals fed on healthy plants (BEANLAND et al., 1999; SUGIO et al., 2011). In contrast the specialist leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) shows reduced fecundity, life span and reduced nymphs emergence when feed on infected plant of the Flavescence Doreée phytoplasma in grapevines compared with adults fed on healthy plants (BRESSAN; GIROULAMI; PADIEU, 2005).

The leafhopper *Dalbulus maidis* is a monophagous insect colonizing maize crops in the Americas, being identified as vector a of three maize phytopathogens: ‘Corn Stunt spiroplasma’ (CSS; *Spiroplasma kunkelii*), ‘Maize bushy stunt phytoplasmaa’ (MBSP, or Maize phytoplasma) and *Maize rayado fino virus* (MRFV) (AMMAR et al., 2011; ÖZBECK et al., 2003; WAQUIL et al., 1999). These pathogens produce economically important diseases in several countries of Latin America as the pale stunt, reddish stunt and corn streak virus, respectively (WAQUIL et al., 1999).

*D. maidis* at 23.4 °C, 83%R.H., spend eight days in the embryonic stage, 12.5 days in the nymph stage passing generally for five instars and an adult stage where the female survive longer than the male, 42.1 days and 16.3 days of longevity respectively (MARIN, 1987). Studies on biology of the insect has shown a strong effect of the temperature and plant genotype (ZURITA; ANJO; WAQUIL, 2000) on the insect biology in which embrionary stage, amount of nymphal instars and adult longevity are reduced with increasing temperatures, reflecting the populations observed in field during contrasting time seasons were maize is grown (WAQUIL; VIANA; SANTOS, 1999).

Of particular interest the variation in vector biology originated by the phytopathogen presence during insect life. Microorganisms like spiroplasma and phytoplasma favors some insect biological characteristics as adult longevity or survival of insects feeding on infected plants (EBBERT; NAULT, 2001). Certainly, *D. maidis* populations fed and grown on infected plants with the mollicutes corn stunt spiroplasma (CSS) and the MBSP phytoplasma showed a 37% of survival increase when compared with healthy populations present in maize crops during the warm season with a differential effect of the MBSP isolates on the *D. maidis* adult survival (EBBERT ; NAULT, 2001).

In this sense, a longer evolutionary interaction between the phytoplasma and its vector will determine the potential pathogenicity and the negative effects of the phytopathogen on the insect (BOSCO et al., 2007). For example the phytoplasma CYP exhibited a differential pathogenesis degree on the vector species *Macrostelus quadripunctulatus* (Campbell & Purcell) (Hemiptera: Cicadellidae) and *Eusculidius variegatus* (Kirschbaum) (Hemiptera: Cicadellidae), being more pathogenic to the first species and supposing a lesser coevolution degree for this species when compared with *E. variegatus* (BOSCO; LEONCINI; MARZACHI, 2002).

The objective of the present research was to establish the effect of the MBSP maize plant infection on the biology of its vector *D. maidis* and the early phytoplasma acquisition.

### **3.2 Materials and Methods**

To determine the effect of maize plants infected with the phytoplasma MBSP, two experiments were conducted in order to establish the effect of the phytoplasma incubation time in-planta on the biology of *D. maidis*. A third experiment tested the effect of MBSP incubation time on asymptomatic maize plants on the *D. maidis* immature phytoplasma acquisition.

#### **3.2.1 Obtention of healthy maize plants**

Hybrid maize seeds codified as 2B710PW susceptible to *D. maidis* were grown in amount of 3 to 4 seeds per pot every four days during whole the research. The material was placed in a greenhouse exclusively used for the material maintenance. Healthy maize plants used for the experiments were treated in the same conditions as the infected ones, with the difference of infesting them with healthy *D. maidis* adults.

#### **3.2.2 Obtention and maintenance of the MBSP infected plants**

Field plants with typical phytoplasma symptoms were collected from an experimental field at the genetic department of the University of Sao Paulo ESALQ, and carried to the insect vector laboratory. In laboratory, infected plants were covered with a cloth cage and infested with 150-200 *D. maidis* adults for a 96h Acquisition Access Period (AAP). After the AAP, insects were separated from the plants and released in rearing cages containing healthy

plants in amount of 20 insects/cage. Infectious insects were maintained for a 23 days Latent Period (LP) in controlled conditions ( $25 \pm 3$  °C y  $55 \pm 10$  %HR e 14:10 h).

Once completed the LP, five infectious adults per rearing cage containing maize plants of 15 d after sowing were released during a 48h inoculation access period (IAP) and maintained in the same controlled conditions mentioned.

After the IAP plants were isolated from the insects and kept separated into cloth cages for the incubation periods to test.

### **3.2.3 Rearing of health *D. maidis***

A *D. maidis* population collected from “Jardinópolis” (SP, Brazil) and reared in greenhouse conditions since 5 years, was maintained following protocols of Oliveira and Lopes (2004). Insects were fed with maize plants of 40-45 days after sowing contained in cages of 0.40 x 0.40 x 0.60 m. Every four days the plant material was awarded to maintained quality food to the insect.

Two cages with insects of known age were weekly offered with maize 25-30 days maize plants after sowing during a 96 h oviposition period, time after which plants with ovipositions were isolated in a new cage for the development of the immature stages. Ecloded nymphs were fed with healthy maize plants until adult emergence continuing the process.

### **3.2.4 Rearing of infectious *D. maidis* adults with the phytoplasma MBSP**

An insect population with MBSP was maintained in greenhouse conditions, releasing groups of nymph IV, nymph V and adults in cages of 0.3 x 0.3 x 0.9 m size fed with MBSP infected plants (40-50 days after inoculation). Maize infected plants were renewed every 15 days, providing the insect with new infected plants as food substrate. In greenhouse, and following the before conditions an infectious *D. maidis* cohort was periodically maintained.

### **3.2.5 Experiment 1. Effect of symptomatic maize plants with advanced MBSP+ disease incubation period on the *D. maidis* biology**

In order to test the effect of MBSP+ maize plants with advanced disease symptoms (45 days of disease incubation period (IP)) on the vector biology an initial experiment was

developed under greenhouse conditions ( $26.1 \text{ }^{\circ}\text{C} \pm 3.3$ ;  $78.7\% \pm 20.5\%$ , 14h photo-phase). Eight infected maize plants at the IP mentioned and eight healthy maize plants at the same physiological stage, were exposed to 30 health *D. maidis* couples per plant for a 24h oviposition time.

After the oviposition time, plants with postures were separated from adults and placed in amount of four plants per cage of  $0.75\text{m} \times 0.75\text{m} \times 1.15\text{m}$ , considering this time as the initial time of the observations.

Each plant was evaluated in a two days interval period registering the total amount of laid eggs, the amount of each of the five instar nymphs alive and the amount of adults emerged by gender. With the data obtained, a survival life Table was constructed.

Fecundity and adult stage longevity of *D. maidis* were measured placing two couples (a total of 14 replications per treatment) coming from the emerged adults into a rearing cage containing two healthy plants of 10-15 days after sowing. Plants of the same condition were renewed every 48h until death of the last adult, meanwhile plants with ovipositions were relocated in greenhouse condition for first instar nymph counting as an estimation of the real fecundity.

Collected data was processed manually by both excel table and survival analysis (SAS software) estimating the population parameters for each treatment of Reproductive rate ( $R_0$ ), intrinsic rate of increase ( $r_m$ ), annual growth rate ( $\lambda$ ), generation time (T), doubling population time (Td). Comparison of population parameters was made with using t-test using the R software.

In order to determine any possible effect of the phytoplasma infection on the sexual ratio, 30 *D. maidis* couples emerged from health plants (MBSP-) and MBSP infected maize plants (MBSP+) at the mentioned IP, were released in a cage containing eight plants of each plant during a 96h oviposition period. After the time adults were separated from the plants, maintaining plants and ovipositions in isolated conditions. Ecloded nymphs were allowed to growth freely until the adult emergence. Adults emerged were sucked and placed in eppendorf tubes containing ethanol 70% for preservation.

In laboratory, adults were labeled by gender and the sexual rate was estimated by treatment. Treatments belonged to: healthy insects Vs healthy plants (Control treatment);

healthy insects Vs MBSP infected maize plants at 45d of IP; MBSP infectious insects Vs MBSP infected maize plants at 45d of IP. The experiment was replicated two times. Data analyses of sexual rate were made by Chi-Square test comparing the MBSP treatments against the control treatment.

### **3.2.6 Experiment 2. Effect of asymptomatic maize plants with early MBSP incubation period on the *D. maidis* biology**

The effect of asymptomatic maize plants infected with MBSP 14d of disease incubation period (MBSP+ 14d) and healthy maize plants (MBSP-) of the same age, was determined on the *D. maidis* biology.

Ten MBSP- maize plants and ten MBSP+ 14d, were infested with 30 healthy *D. maidis* couples/plant during a 24h oviposition time. Afterwards, plants were separated from insects and shared in two groups of five plants per cage (0.75m x 0.75m x 1.15m). This time was considered as the initial experimental time. In the evaluation times of 7d, 11d, 19d, 26d and 31d after oviposition were registered per plant the total amount of eggs laid, I, II, III, IV and V instar nymph.

V instar nymph coming from each plant were removed and placed in a rearing cage containing three healthy maize plants 10-15 days after sowing for adult evaluation by gender and estimation of the sexual rate.

*D. maidis* fecundity was evaluated at 2, 5, 8, 11, 14 and 15 days after adult emergence, placing two couples of emerged adults (F<sub>1</sub>) into a rearing cage containing two healthy maize plants 10-15 days after sowing during a 24h oviposition period. Plants with ovipositions, were maintained under greenhouse conditions for 11 days until eclosion of first instar nymph, variable registered as fecundity. A total of 11 replicates per treatment were evaluated registering fecundity.

Data were processed using the GLM procedure (family Poisson) for each treatment and time evaluated using the software R.

### 3.2.7 Experiment 3. Acquisition of MBSP phytoplasma by *D. maidis* immature stages when grown on maize plants with early MBSP incubation time

In order to establish phytoplasma MBSP acquisition by the immature *D. maidis* stages when fed on asymptomatic maize plants, ten MBSP maize plants of 14-20 days of disease inoculation period (MBSP+) were offered to 60 healthy *D. maidis* adults as feeding and oviposition substrate during 48h. After the oviposition period, plants were separated from insects and were maintained isolated into cloth cages under greenhouse conditions ( $26.1\text{ }^{\circ}\text{C} \pm 3.3$ ;  $78.7\% \pm 20.5\%$ , 14h: 10h) for insect development. A group of 100-150 individuals of each *D. maidis* stage growing on the MBSP+ plants, was taken and preserved in eppendorf tubes containing 70% ethanol for posterior processing in laboratory.

In laboratory MBSP detection was determined on each *D. maidis* group through nested PCR following Marzachi, Veratti and Bosco (1998) protocol. From each collected group were evaluated 3-5 individuals for N-I (n=17), N-II (n=16), N-III (n=17), N-IV (n=17), N-V (n=17), and individual female genitalia (n=21) as an indirect measure of the egg infection.

For DNA extraction each sample group was placed in a 0.5 ml eppendorf tube containing 10  $\mu\text{l}$  of NaOH (1M). Samples were soaked with a fine pin pale and posteriorly adding 20  $\mu\text{l}$  HCL (1%). The diluted insect solution was denatured in heated water ( $65\text{ }^{\circ}\text{C}$ ) during 40 minutes. Posteriorly samples were centrifuged 10 minutes (1832,73 g.). 30  $\mu\text{l}$  of the supernatant were disposed in a new tube, adding 30  $\mu\text{l}$  of ethanol 95% and placed in cooled conditions ( $4\text{ }^{\circ}\text{C}$ ) during 40 minutes. After mentioned time, samples were centrifuged during 10 minutes (1832,73 g.) and pellet formed was separated from the liquid solution, for DNA drying at room temperature. The dried pellet was suspended in a 100  $\mu\text{l}$  buffer extraction solution and maintained in freezer until necessary.

In a nested PCR procedure samples were initially amplified using the general primers for mollicutes F2 - R1. 2  $\mu\text{l}$  of the first amplification were diluted in 38  $\mu\text{l}$  of milliQ water and re-amplified using the specific primers R16(I)F1 - R16(I)R1 for detection of group I phytoplasma. Conditions for both amplifications were:  $94\text{ }^{\circ}\text{C} \times 1\text{ minute}$  (1 cycle);  $94^{\circ}\text{C} \times 45\text{ seconds}$ ,  $50\text{ }^{\circ}\text{C} \times 30\text{ sec.}$ ,  $72^{\circ}\text{C} \times 1,5\text{ min.}$  (35 cycles);  $72^{\circ}\text{C} \times 5\text{ min.}$  (1 cycle);  $4^{\circ}\text{C} \times \text{infinite}$ .

After last amplification, samples were run in a 1% sacarose gel during 1h at 100V. Afterwards, gel was disposed in a dark chamber with UV light for band visualization (as the signal of phytoplasma presence) using the software eagle®. For each insect stage were

registered both the amount of positive and negative samples. Data analysis was made with GLM procedure (family=Binomial) and mean comparisons by test Tukey.

### 3.3 Results

#### 3.3.1 Experiment 1. Effect of maize plants with advanced MBSP incubation time on the *D. maidis* biology

Insect survival ( $lx$ ) measured from egg to the last adult dead did not evidenced statistical differences between treatments ( $\chi^2= 0.143$ ;df=43, 42; P= 0.71), but biologically showed higher values of *D. maidis* reared on health maize plants (MBSP-) ( $0.29\pm 0.04$ ) than on MBSP+ infected plants with advanced disease symptoms ( $0.23\pm 0.03$ ).

Fecundity measured as the I instar nymph emerged/female/day at the first generation did not show statistical differences between treatments ( $\chi^2= 0.294$ ; df= 43, 42; P= 0.59) with similar values for MBSP- plants ( $25.2 \pm 4.9$  ecloded nymphs/female/day) and MBSP+ plants ( $24.3 \pm 4.3$  ecloded nymphs/female/day).

Sexual rate of *D. maidis* did not show statistical differences between treatments ( $\chi^2= 0.099$ ; df= 13, 12; P< 0.059), but biologically exhibiting an increase on MBSP+ plants when compared with MBSP- (Table 1).

Total adult longevity showed no statistical difference between treatments ( $\chi^2= 1.128$  df =25, 26 P= 0.289) with similar mean values for adults reared on MBSP+ ( $42.9 \pm 1.60$ ) or MBSP- ( $40.3 \pm 1.68$ ). When analyzed by gender, longevity did not show statistical differences between treatments neither for male ( $\chi^2= 0.227$  df =25, 26 P= 0.633) nor female ( $\chi^2= 0.934$ ; df =25, 26 P= 0.334) with values of ( $39.0 \text{ d} \pm 1.73$ ) and ( $40.1 \text{ d} \pm 1.72$ ) for males reared on MBSP- and MBSP+ maize plants respectively. Meanwhile longevitiy for female was ( $40.2 \text{ d} \pm 1.53$ ) and ( $42.5 \text{ d} \pm 1.63$ ) MBSP- and MBSP+ infected maize plants respectively.

When analyzed the population parameters of the two *D. maidis* cohorts reared on MBSP- or MBSP+ maize plants with advanced disease symptoms, t-test evidenced statistical differences for Reproductive rate (Ro) parameter (t= 18.98; df= 1; P< 0.03), and generation time (T) parameter (t= -75.77;df= 1; P< 0.004) (Table 2).

No statistical differences were observed either for intrinsic rate of increase ( $r_m$ ) (t= 2.91; df= 1; P= 0.211), nor for annual growth rate ( $\lambda$ ) (t= 0.90; df= 1; P= 0.532) (Table 2).

Table 1 - Survival and sexual rate of *D. maidis* when fed and grew on MBSP- or MBSP+ plants with advanced disease symptoms

Stage or Insect	MBSP-			MBSP+		
	N	Survival Probability	Eclosion (%)	N	Survival Probability	Eclosion (%)
<b>Egg</b>	433	0.87	86	160	0.97	78
<b>Nymph I</b>	378	0.78		155	0.57	
<b>Nymph II</b>	296	0.92		88	0.93	
<b>Nymph III</b>	273	0.92		82	1.00	
<b>Nymph IV</b>	250	1.00		82	0.84	
<b>Nymph V</b>	270	0.91		69	0.99	
<b>Adult</b>	246	0.91		65	1.00	
<b>Egg- Adult</b>	246	0.57		68	0.43	
<b>Sexual Rate*</b>	<b>0.40 (b)</b>			<b>0.50 (a)</b>		

\* Estimated as the proportion of females/ females+males

Table 2 - Population parameters estimated for two *D. maidis* cohorts grown on healthy or MBSP maize infected plants

Population Parameter	<i>D. maidis</i> grown on MBSP- maize plants	<i>D. maidis</i> grown on MBSP+ maize plants	t- student test
Reproductive rate (Ro)	90.95	82.05	0.03 *
Intrinsic rate of increase ( $r_m$ )	0.05	0.05	0.21
Annual growth rate ( $\lambda$ )	1.05	1.05	0.53
Generation time (T)	41.02	41.33	0.004**

\* Parameter values with statistical differences with t-test for P= 0.05; \*\* Parameter values with statistical differences with t-test for P= 0.01

To reconfirm the potential effect of MBSP+ plants on the *D. maidis* sexual rate, the specific experiment evaluating *D. maidis* sexual rate obtained from MBSP- plants Vs health

*D. maidis*(-), MBSP+ plants with advanced disease symptoms Vs health *D. maidis* (-) and MBSP+ plants with advanced disease symptoms Vs infectious *D. maidis*(+) showed statistical differences between treatments ( $\chi^2 = 0.021$ ; df= 5, 3;  $P < 8.2e-05$ ) being MBSP+ Vs *D. maidis*+ the treatment with the high mean sexual rate, followed by MBSP(+) Vs *D. maidis*(-) and MBSP(-) Vs *D. maidis* (-) (Table 3).

Table 3 - Sexual rate of *D. maidis* adults at the first generation emerged from parents at either plant or vector MBSP conditions

Parents rearing history	Experiment 1				Experiment 2				mean sexual ratio $\pm$ sem
	N	♀	♂	Sex. Rate *	N	♀	♂	Sex. Rate *	
Healthy <i>D. maidis</i> Vs Healthy maize plants	204	78	126	<b>0.40</b>	257	114	143	<b>0.44</b>	0.42 $\pm$ 0.001 <b>c</b>
Healthy <i>D. maidis</i> Vs MBSP infected maize plants	601	276	325	<b>0.46</b>	217	100	117	<b>0.46</b>	0.46 $\pm$ 0.0 <b>b</b>
MBSP infectious <i>D. maidis</i> Vs MBSP infected maize plants	272	148	124	<b>0.54</b>	735	365	370	<b>0.50</b>	0.52 $\pm$ 0.001 <b>a</b>

\* Estimated as the proportion of female/ female+male

### 3.3.2 Experiment 2. Effect of symptomless maize plants with early MBSP incubation period on *D. maidis* biology

The amount of insect stages obtained for two *D. maidis* cohorts reared on healthy maize plants (MBSP-) or asymptomatic maize plants at early phytoplasma (MBSP+) incubation time (14d of disease incubation period), evidenced statistical differences between treatments at evaluation time 19 d after oviposition for both second instar nymph ( $\chi^2=20.393$ ;df= 19, 20 ; P< 6.3e-06) and IV instar nymph ( $\chi^2=223.55$  ;df=1, 18; P<2.2e-16) (Figure 1. C, H); 26d after oviposition for IV instar nymph ( $\chi^2=13.717$ ; df= 1, 18; P< 2.1 e-4) (Figure 1. D, I); and 31d after oviposition for V instar nymph ( $\chi^2=21.12$ ; df= 1, 18; P=4.3e-6) (Figure 1. E, J).

Remaining individuals were observed on treatment MBSP- at 19d (Figure 1C) for I instar nymph, and at 26d (Figure 1D) for III instar nymph. The importance, on treatment MBSP+ at 26d evaluation time was observed early adult emergence (Figure 1I).

No statistical differences between treatments were evidenced 7d (Figure 1. A, F) neither for 11d after oviposition (Figure 1. B, G) for eggs laid (F= 0.107; df= 1, 36; P=0.75); 11d after oviposition for I instar nymph (F=1.371; df=1, 18; P= 0.257), 19d after oviposition (Figure 1. C, H) for III instar nymph (F= 4.13; df= 1, 18; P= 0.06), neither at evaluation time 26d (Figure 1. D, I) for V instar nymph ( $\chi^2=1.529$ ; df= 1, 18; P=0.216), nor for evaluation time 31d after oviposition (Figure 1. E, J) for adult stage (F= 2.244; df= 1, 18; P=0.152).

Sexual rate of individuals obtained on *D. maidis* reared on MBSP+ 14d after incubation period and MBSP- plants expressed the same amount (0.5), meanwhile no statistical difference was evidenced between treatments for the survival proportion ( $\chi^2=0.009$ ; df= 1, 18; P= 0.924), being  $(0.57 \pm 0.04)$  on MBSP+ plants and  $(0.54 \pm 0.04)$  MBSP- plants.

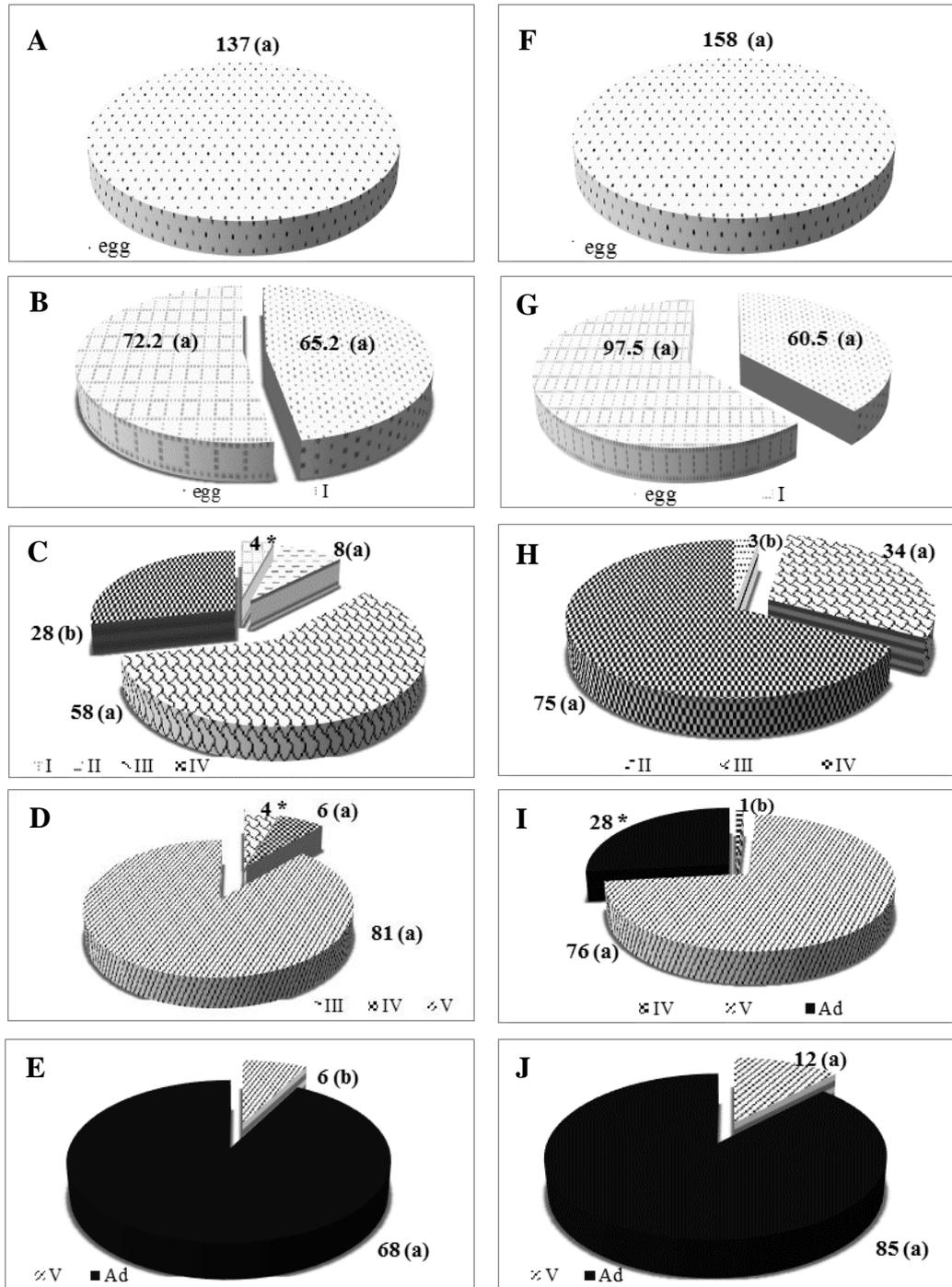


Figure 1 - *D. maidis* population composition present on MBSP- plants (left column plots) and MBSP+ asymptomatic plants with early incubation time (right column plots) at different evaluation times after oviposition. (A-F) insect stages 7d after oviposition. (B-G) insect stages 11d after oviposition. (C-H) 19d after oviposition. (D-I) insect stages 26d after oviposition. (E-J) insect stages 31d after oviposition. Each plot shows the mean amount of individuals/ insect stage and the statistical significance of the stage-stage comparison between treatments as a lowercase into parenthesis. Asterisks represent presence of one specific stage just in one of the treatments. Experimental conditions:  $26.1^{\circ}\text{C} \pm 0.04$  ;  $78.4 \pm 0.3$

When analyzed fecundity (measured as the I instar nymph eclosed) of emerged adults at F<sub>1</sub> generation for the populations reared on MBSP+ and MBSP- plants, results showed statistical differences between treatments ( $\chi^2= 63.466$ ; df= 1, 130;  $P < 1.6e-15$ ), time ( $\chi^2= 747.72$ ; df= 5, 125;  $P < 2.2e-16$ ), and treatment x time ( $\chi^2= 943.54$ ; df= 5, 120;  $P < 2.2 e-16$ ). Statistical differences were observed at 2d after adult emergence on MBSP+ ( $41.9 \pm 2.7$ ), and at 11d after adult emergence on MBSP- ( $68.8 \pm 3.9$ ) when compared with MBSP+ ( $26.8 \pm 4.9$ ) (Figure 2).

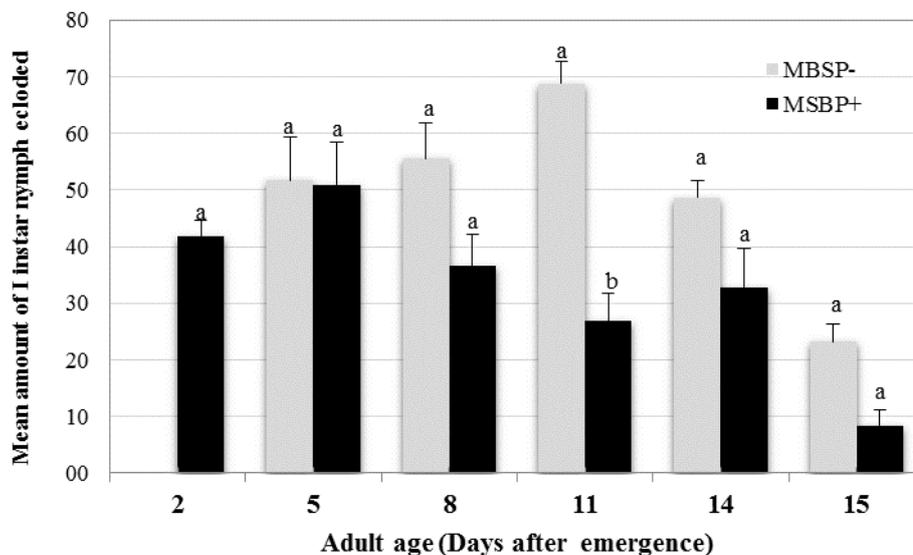


Figure 2 - Fecundity of two adult cohorts reared on MBSP- and MBSP+ asymptomatic maize plants (14 days of disease incubation time). Comparisons are made between treatments into each evaluation time and are represented as a lowercase. Treatments with different letters are statistically different at  $P = 0.01$

### 3.3.3 Experiment 3. Acquisition of MBSP phytoplasma by *D. maidis* immature stages when grown on maize plants with early MBSP incubation time

Results showed statistical differences between treatments ( $\chi^2=$  ; df=6, 114;  $P < 1.4e-06$ ), with differences for MBSP detection on III instar nymph, ovariole, testis, IV instar nymph, and V instar nymph with the stadiums I instar nymph and II instar nymph (Table 4).

No statistical differences for MBSP detection were observed for I instar nymph, II instar nymph and V instar nymph, being the less amounts of infected individuals (Table 3.4). Neither differences of MBSP detection were found in testis, ovariole, III instar nymph, IV instar nymph and V instar nymph (Table 4).

Table 4 - MBSP acquisition of *D. maidis* immatures and adults when fed on asymptomatic maize plants with the phytoplasma MBSP (14 – 20 days after disease incubation period)

<b>Treatment</b>	<b>Fraction of <i>D. maidis</i>+ / total evaluated (mean ± sem) *</b>
<b>I instar nymph (&lt; 24h)</b>	2/17 (0.12 ± 0.08 <b>bc</b> )
<b>II instar nymph</b>	1/16 (0.06 ± 0.06 <b>c</b> )
<b>III instar nymph</b>	12/17 (0.71 ± 0.11 <b>a</b> )
<b>IV instar nymph</b>	9/17 (0.53 ± 0.12 <b>ab</b> )
<b>V instar nymph</b>	7/17 (0.41 ± 0.12 <b>abc</b> )
<b>Ovariole</b>	15 /21 (0.71 ± 0.10 <b>a</b> )
<b>Testis</b>	12/16 (0.75 ± 0.11 <b>a</b> )

\* Values with different lowercase are statistically different at P=0.01

### 3.4 Discussion

In the present study the effect of the MBSP plant infection on its vector *D. maidis* evidenced minimum implications on the insect fitness, basically due to the vector arrangements in some of its biological parameters when reared on MBSP+ maize plants.

Neither the insect survival, fecundity of the first adult generation nor the adult longevity differed between the two insect cohorts when the vector reared on MBSP+ with advanced disease symptoms.

This is in contrast to the observed in other studies, where maize bushy stunt phytoplasma (maize bushy stunt mycoplasma or MBSM in the original document) reduced the survival, life span of adults, fecundity and net reproductive rate ( $R_0$ ), but not the intrinsic rate of increase ( $r_m$ ) of *D. maidis* when compared with the corn stunt spiroplasma and control treatments at temperatures from 20 to 29 °C (MADDEN et al., 1984).

Other studies evaluating *D. maidis* adult longevity of populations coming from Colombia, Perú and Brazil were unaffected by the exposure to MBSP (MBSM in the document), unlike the Mexican *D. maidis* population (NAULT, 1985).

Along the association vector – phytopathogen should result in a selection of vectors and pathogens that are highly compatible, that is, plant pathogens not pathogenic to their vectors (NAULT, 1985).

Studies related with *D. maidis* biological parameters possibly explain the differential mutualistic interaction between the insect species and the plant pathogens vectored (EBBERT; NAULT, 2001). During the warm season in Mexico, Corn stunt spiroplasma (CSS) and Maize bushy stunt phytoplasma (MBSP) isolates increased *D. maidis* survival (EBBERT ; NAULT, 2001).

On the other side, a study evaluating the effect of both CSS and MBSP (MBSM mentioned in the document) isolates on the survival of seven leafhopper species coming from Mexican fields, demonstrated the negative effect of the MBSP for all the species with a lesser detrimental level on *D. maidis*, but showing a survival decrease when compared with the control and the CSS treatment (MADDEN; NAULT, 1983).

Interestingly, the light effect of MBSP on the *D. maidis* biology found in this study, can be explained by some biological parameter arrangements of the vector when reared on MBSP+ plants. In the experiment using MBSP+ plants with advanced disease symptoms, evidences showed a *D. maidis* sexual rate adjustment. *D. maidis* adults reared on the mentioned plants resulted in an increased sexual rate for its population (0.50 in Table 3.1) when compared with MBSP- plants (0.40 in Table 3.1).

The modification of the sexual rate for *D. maidis* was confirmed in the experiment 3, with an increase of its value when infectious *D. maidis* (+) were reared on MBSP+ plants favoring the female emergence ( $0.52 \pm 0.001$ ), when compared with healthy *D. maidis* (-) reared on MBSP-plants the control ( $0.42 \pm 0.03$ ) (Table 3.3). Studies of phytoplasma transmission have demonstrated the greater transmission efficiency of females when compared with males, as reported for the aster yellow phytoplasma transmission by its vector *Macrostelus quadrilineatus* (BEANLAND et al., 1999).

Presumably this result is influenced by both the increased amount of the endosymbiont *Wolbachia* and the decreased amount of the endosymbiont *Rickettsia* observed with the incremental phytoplasma incubation period (Chapter 4). Contrary to the observed on healthy *D. maidis*, insects with presence of MBSP increases the amount of *Wolbachia*, favored with MBSP increased incubation periods.

It is known the effect of *Wolbachia* and *Rickettsia* on the insect host reproduction modification causing feminization, incompatibility, male killing or parthenogenesis (SANCHEZ-CONTRERAS; VLISIDOU, 2008; MORAN; MCCUTCHEON, NAKABACHI, 2008; ALMA et al., 2010). So it is presumed that the higher pressure of MBSP infection in both vector and host plant, the higher vector microbiota composition favoring the female production.

Modification of the *D. maidis* sexual rate was observed in maize fields where changes in sex ratio of trapped leafhoppers over different seasons were related with migratory activity (TERAGUCHI, 1986). During the maize crop season in Anastacio (MS-Brasil), males were generally collected in higher numbers than females, especially during the courtship (DE OLIVEIRA et al., 2004).

However, in periods before planting and toward the end of the maize crop season, as well as at population peaks following soil preparation, the proportion of females trapped increased significantly and was generally higher than that of males (OLIVEIRA; LOPES; NAULT, 2013).

Interestingly, *D. maidis* reared on MBSP+ plants 14 days of disease incubation period showed a different vector arrangement, linked with an early adult emergence (28 adults in Figure. 1I) at 26d after oviposition when compared with no adult emergence observed on MBSP- plants, subsequently producing a first oviposition ( $41.9 \pm 8.9$ ) in the absence of healthy adult population emerged at 2d after adult emergence (Figure 2). Fecundity for the rest of the evaluation times (Figure 2) was statistically different at 11d after adult emergence on MBSP- plants ( $69 \pm 3.90$ ) when compared with MBSP+ plants ( $27 \pm 4.86$ ).

MBSP acquisition of *D. maidis* immatures and adults reared on asymptomatic MBSP+ 14-20d of disease incubation period, corroborate and highlight the importance of the asymptomatic maize plants infected with the phytoplasma on the plant pathogen epidemiology. All the insect stages evidenced positive signal for MBSP, with the higher detection in testis (12/16), ovariole (15/21), and III instar nymph (12/17) (Table 3.4).

As demonstrated in Chapter 2, *D. maidis* prefers to settle and to oviposit on healthy or “apparently” healthy maize plants to rear its next generation. The mentioned result linked with the actual result demonstrating the effective MBSP acquisition by *D. maidis* whatever the

vector stage, reinforce the need to sample the insect vector at the different stages, and at the maize plants focusing the efforts on the initial stages of the crop season.

A second interesting result rising from the positive *D. maidis* acquisition on the several stages, is the MBSP detection on both ovarioles and testis of the vector, result strongly suggesting an MBSP transmission via mating. This is the first demonstration of the phytoplasma MBSP detection on both the adult genitalia and I instar nymph of *D. maidis*, opening the chance of a new MBSP route for its persistence and transmission via the immature vector.

This kind of horizontal transmission would be facilitated by the huge amount of MBSP cells observed in the midgut, salivary gland and especially in the malpighian tubules of the insect vector (chapter 4). Anatomically the malpighian tubules are connected at the posterior portion of the hindgut in the final part of the abdomen (TSAI; PERRIER, 1996), facilitating the adult genitalia infection.

Transovarial transmission of phytoplasmas from one generation to the next have been confirmed by experimental results in *Scaphoideus titanus* and the Flavescence dorée phytoplasma in grapevine (ALMA et al., 1997) as well as in *Matsumuratettix hiroglyphicus* (Matsumura) vector of the of the sugarcane white leaf phytoplasma (WEINTRAUB; BEANLAND, 2006).

Nested-PCR detection in the vector reared for two generations on free sugarcane grown from tissue cultures, confirmed the phytoplasma presence in eggs, nymphs and adults of the first and second generations, demonstrating transovarial transmission and confirming via *in-situ* PCR technique, the widely distribution of the phytopathogen throughout the insect body, especially in the female genitalia (HANBOONSONG et al., 2002).

Further specific studies of the MBSP transovarial transmission in *D. maidis* will confirm the present result, and will be able to demonstrate the phytoplasma infection to the egg stage since the early pre-embryonic moment.

### 3.5 Conclusions

A weakened effect of the maize bushy stunt phytoplasma on the biology of its vector the leafhopper *Dalbulus maidis* was evidenced when the vector grows and feed on MBSP infected plants.

Early adult emergence of *D. maidis* and a consequently early vector oviposition when reared on MBSP+ maize with early disease symptom plants increases the chance of a secondary crop infestation under field conditions.

Increased female emergence of the vector population on a higher phytoplasma presence, shows the importance of the phytopathogen titer in planta, on the gender population composition.

Asymptomatic maize plant with early disease incubation period confirmed the importance of this material as source plant for the phytoplasma epidemiology

High probability of transovarial transmission will benefit the phytoplasma persistence in the populations originally affected.

Interaction of maize bushy stunt phytoplasma (MBSP) and its vector *D. maidis*, probably gets more transmission routes than the interaction host plant- vector,

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#### 4 ULTRASTRUCTURE STUDIES APPLIED TO THE MOVEMENT OF MAIZE BUSHY STUNT PHYTOPLASMA WITHIN ITS VECTOR *Dalbulus maidis* (DELONG&WOLCOTT) (HEMIPTERA: CICADELLIDAE)

##### Abstract

In the system *Dalbulus maidis* (Hemiptera: Cicadellidae) vector of the maize bushy stunt phytoplasma (MBSP) the interactions occurring during the passage, invasion, and multiplication of the phytoplasma in the vector body are unknown. With the aim to understand the MBSP movement within the its vector, adults previously fed on infected plants for a 96h acquisition access period (AAP) and maintained 23 days of latent period (LP) in controlled conditions ( $25\pm 3$  °C;  $58\pm 7\%$ ; 14h:10h) were dissected and processed for visualization to the Transmission Electronic Microscope (TEM). Morphology of cells previously observed in the insect structures were confirmed as the phytoplasma cells through processing and observation to the TEM of reddish leaves and root samples of plants with advance disease symptoms. External images of the insect structures evaluated were obtained with the Scanning Electron Microscopy technique (SEM). The phytoplasma cells were observed in the digestive tube, epithelium of the mesenteron and hemocele of the insect vector forming a mass of cells protected by a matrix like a biofilm. In both the epithelium of the mesenteron and salivary gland were observed mitochondria and bacteria like endosymbionts joining the same spaces with the MBSP cells. In both epithelium of the intestine and salivary gland were observed mitochondria and bacteria like endosymbionts joining the same space with the phytoplasma cells. In plants, cells similar to those observed in the insect intestine were visualized in maize sample roots but not in the reddish leaf samples. For the first time the MBSP movement within the *D. maidis* body is evidenced with original images showing the presence of a matrix protecting the phytoplasma mass cells, and demonstrating the interaction between this mass with bacteria like endosymbionts facilitating the phytoplasma passage into the insect.

Keywords: Biofilm; Endosymbionts; MBSP movement; Ultrastructure; Microvilli

Phytoplasmas are obligated phytopathogenic bacteria living in both plants and insects depending on them for its acquisition and inoculation (CHRISTENSEN et al., 2005; DURET et al., 2014). They are acquired by its vector passively during feeding on infected plants with consumption times being as short as few minutes but generally measured in hours (WEINTRAUB, 2007). Geographical isolation of some phytoplasma seems to be correlated with the distribution of their host plants and the insect vectors that are native in the particular region. For instance, maize bushy stunt phytoplasma [16SrI-B(rp-L)] is restricted to Central and South America and part of North America. These regions correspond to the geographical range of the insect vectors *Dalbulus maidis* (Delong & Wolcott) (Hemiptera: Cicadellidae)

and *Dalbulus. elimatus* (Ball) (Hemiptera: Cicadellidae) (LEE; DAVIS; GUNDERSEN-RINDAL, 2000).

The maize leafhopper *D. maidis* is a monophagous insect of maize crops considered an important pest because of its transmission efficacy of ‘Corn stunt Spiroplasma’ (*Spiroplasma kunkelii*), ‘Maize bushy stunt phytoplasma’ (MBSP) and *Maize rayado fino virus* (MRFV) being dominant MBSP (WAQUIL et al., 1999; ÖZBECK et al., 2003; AMMAR et al., 2011).

A general and poor understanding of the phytoplasma movement into the insect has been documented for some pathosystems (WEINTRAUB, 2007). The generally accepted model proposes the phytoplasma acquisition by the insect vector starting with the stylet penetration and sustained food on the infected plant, entering into the insect food channel, passaging for the digestive tube, adhering to the midgut epithelium cells, passaging between or through the midgut cells, and moving to the corporal cavity and hemolymph with transport to different organs and tissues including the salivary gland (BOSCO et al., 2007).

Phytoplasmas as other plant mollicutes have been shown to heavily infect and colonize insect midgut epithelial cells and salivary gland of the vector (GALLETO et al., 2009). To infect new plants, the phytoplasma must colonize the salivary gland of its vector before being inoculated via saliva during the insect plant consumption on new plants in a persistent propagative transmission process (BAI et al., 2006; HOGENHOUT et al., 2008). Failure to penetrate the midgut wall or to enter and infect the salivary glands has been indicated as intrinsic factors preventing vector competence of mosquitoes for arboviruses (GALLETO et al., 2009).

In the case of the Spiroplasmas, mollicutes transmitted by leafhoppers, they invades insect organs before being released along with the saliva into a new plant through insect feeding. During this process, internalization of Spiroplasmas into insect cells is an obligate step which requires intimate interactions, for both crossing the gut epithelium (acquisition) and invading the salivary gland cells (transmission) (DURET et al., 2014).

In the interaction of *Spiroplasma citri* and its vector *Circulifer haematocaps* (Mulsant & Rey, 1855) (Hemiptera: Cicadellidae) the Spiroplasma ability to undergo morphological changes from helical, in the phloem sap and insect hemolymph, to rounded shape when internalized into insect cells has been demonstrated (KWON; WAYADANDE; FLETCHER, 1999; OZBEK et al., 2003), with robust interactions between Spiroplasmal and host cell membrane proteins mediated by phosphoglycerate kinase (PGK), the adhesin ScARP3d, and the lipoprotein spiralin serving for attaching to the insect cells (DURET et al., 2014).

In the plant the phytoplasma is transferred into the pierced sieve elements, from which they disperse systematically using the continuous sieve tube system not as a merely passive process via the savia, with the adhesion capacity of the bacteria to the host cells and growth there, a factor influencing the phytoplasma movement (CHRISTENSEN et al., 2005). Then the bacteria cells can be found in plant structures other than those tissues originally infected, as young leaves and root system, and external observed symptoms in plant are a good evidence of the disease presence but are not necessary correlated with the presence of the phytoplasma in a given tissue (SIDDIQUE et al., 1998).

Like other mollicutes, phytoplasma possesses a minimal genome and lack of genes for the synthesis of some aminoacids, fatty acids, nucleotides, proteins involved in cell division and filament formation and the lack of genes coding some subunits for the TPA TPsynthesis (CHRISTENSEN et al., 2005).

Perhaps of the agriculture importance of *D. maidis* as vector of the MBSP in maize crops, a limited amount of information on the interaction insect vector – phytoplasma transmission is available with no related evidence of the possible interactions between insect microbiota and phytoplasma transmission. The research aimed to get in detail about the MBSP movement into the vector cavity during the latent period and to establish diversity and abundance of *D. maidis* microbiota potentially interacting with the phytoplasma MBSP.

## **4.2 Materials and Methods**

Biological material was maintained and initially processed at the insect vector laboratory belonged to the entomology and acarology department, Universidade de São Paulo – Escola Superior de Agricultura Luiz de Queiroz (Piracicaba- Brazil). Insect and plant infection by the phytoplasma were studied using both Scanning Electronic Microscopy (SEM) and Transmission Electronic Microscopy (TEM) presents at the Núcleo de Apoio a Pesquisa NAP-MEPA of the phytopathology department of the Universidade de São Paulo-ESALQ.

### **4.2.1 Obtention of maize plants and *D. maidis* insects infected with the phytoplasma MBSP**

A *D. maidis* colony was maintained from adult ovipositions allowed to mate during a 96h period in an entomological cage containing maize plants 35d after sowing. Endophitic

ovipositions close to hatch, were isolated from the maize plant and were relocated on healthy plants. Immature stages of the vector were allowed to feed on healthy maize plants until reached the adult stage.

A MBSP isolate coming from a maize crop grown in a field at the genetic department of the University of Sao Paulo –ESALQ (Piracicaba-Brazil), was maintained and replicated on maize plants via infectious vectors. Plants expressing disease symptoms were used for adult feeding during a 96h acquisition access period (AAP). After the AAP, adults were isolated and maintained during a 23d latent period (LP) in rearing cages containing healthy maize plants ( $25\pm 3$  °C;  $58\pm 7\%$ ; 14h:10h photoperiod).

MBSP infectious adults completing the LP mentioned were used to infest new maize plants for a 96h inoculation access period (IAP). Plants fulfilling the IAP were separated and maintained isolated in greenhouse for disease symptom expressions. Plants showing symptoms were used as a feeding substrate for new adults to be used in the experiments, as well as to maintain the phytoplasma isolate.

#### **4.2.2 MBSP movement within the insect vector *D. maidis* during the phytoplasma latent period**

Healthy *D. maidis* adults were allowed to feed on maize infected plants with the phytoplasma MBSP during a 96h (AAP). Then adults were relocated in pet cages containing healthy maize plants during a 23d (LP) in controlled conditions ( $25\pm 3$  °C;  $58\pm 7\%$ ; 14h:10h photoperiod). Adults completing the LP mentioned were separated from the plants and preserved in Eppendorf tubes containing a fixed solution of glutaraldehyde 4% + saccharose.

Head and abdomen of seven healthy and infected *D. maidis* adults were gently dissected and processed for its observation to the Scanning Electronic Microscope (SEM) and the Transmission Electronic Microscope (TEM) following protocols of the NAP-MEPA laboratory. Samples for SEM observation were dehydrated in acetone gradient of 30%, 50%, 70%, 90% and 100% during 10 minutes each, with three final washes in 100% acetone during 10 minutes. Samples were placed in optical paper, labeled and disposed in small receipts for the critical point step. In Belzers® a critical point equipment, samples were washed five times with CO<sub>2</sub>, and then placed in a pin receipt with a sticky slide for the sample retention. In a sputter machine, samples were covered with a thin glide of gold staining during 2 minutes. Samples were preserved in boxes containing silica gel, until the observation time to the Scanning Electronic Microscope.

For TEM analysis intestine and salivary gland of five healthy and infectious *D. maidis* adults were dissected as mentioned before and preserved in solution glutaraldehyde 4% + saccharose. Samples were vacuum three times during five minutes each and maintained at 10°C for 24h. After the mentioned time, fixed solution was throw out and a CaCod 0,1 M was added three times during 10 minutes each. Last solution was replaced with a mixture of an equal proportion of CaCod 0,1 + OsO<sub>4</sub> 2% liquid solution during 1.5h. After the time, samples were washed five times with water and immediately contrasted with uranile acetate 0.5% overnight. Coming day samples were dehydrated in an acetone gradient of 30%, 50%, 70%, 90% and 100% concentration (15 minutes each), with three splashed in the last acetone concentration. After the final splashed, samples were suspended and maintained overnight in a 1:1 mix solution of acetone 100% + spurr® resine. Following day mix solution was replaced by pure spurr® resine for at least 12h. Samples were blocked in boxes containing spurr® resin and maintained in heating conditions for three days.

Resine blocks containing the samples were gently polished for initiating fine cuts. Ultrathin sections of 70- 80 µm were obtained with the aid of a diamond knife in an ultramicrotome, then collected in gilder grids of 200 mesh holes. Grids with samples were stained in uranile acetate for 15 minutes, washed in sterile water and immediately placed in citrate jumbo for other 15 minutes. Samples were again washed in sterile water, dried in a filter paper and preserved for its observation to the Transmission Electronic Microscope (Zeiss900EM).

For maize plant observation to the TEM, small pieces of leaf and roots were taken from plants with advanced disease symptoms and preserved in glutaraldehyde 4% as fixed solution. For processing of the plant material the same protocol as that of the insect organs was followed.

### 4.3 Results

Images of the *D. maidis* stylet to the Scanning Electron Microscope (SEM) showed the maxilar and mandibular stylets (Figure 1A) with a particular drill shape (Figure 1B) facilitating insect leaf cuticle penetration, generally positioned at the lower side of the plant.

Once *D. maidis* feeds on maize infected plants, phytoplasma cells access the insect body through the food canal of the stylet and passes to the esophagus until reaching the filter chamber, point of connection between the foregut and the midgut (Figure 2). *D. maidis*

intestine is placed in the middle part of the body cavity at the abdomen position protected by the fat tissue at the upper part side.

Images obtained to the Transmission Electronic microscopy (TEM) for the anterior midgut portion of infectious *D. maidis*, shows a phytoplasma cell mass moving as a “snowslide” of bacteria for the lumen of the digestive tube (Figure 3A). At this point the phytoplasma mass was observed “encapsulated” by a protein like a film clearly defined (Figure 3B) in front of the mass, possibly protecting and condensing the phytoplasma cells during its movement for this structure.

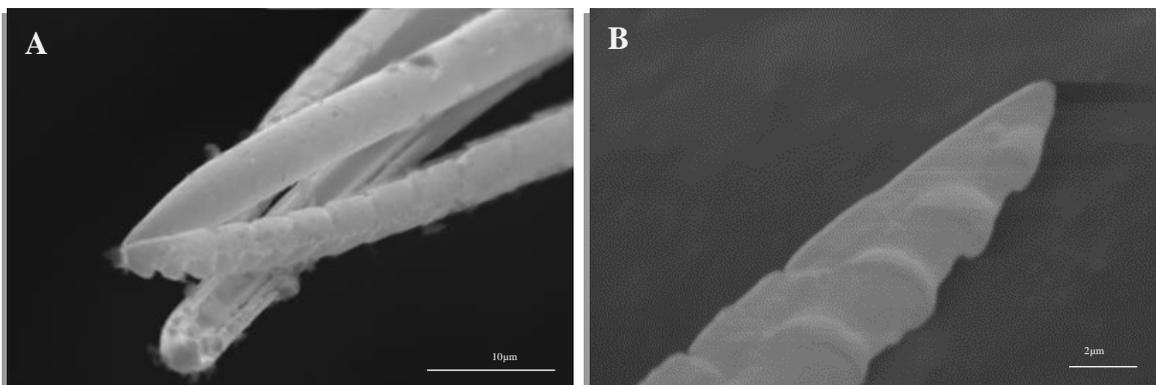


Figure 1 - Mouth parts of *D. maidis* observed to the Scanning electronic microscope. **A)** The maxilar and mandibular stylets are differentiated. **B)** A detail of the stylet shows the drill form, facilitating the leaf penetration and consumption by *D. maidis*

Images: J. García González

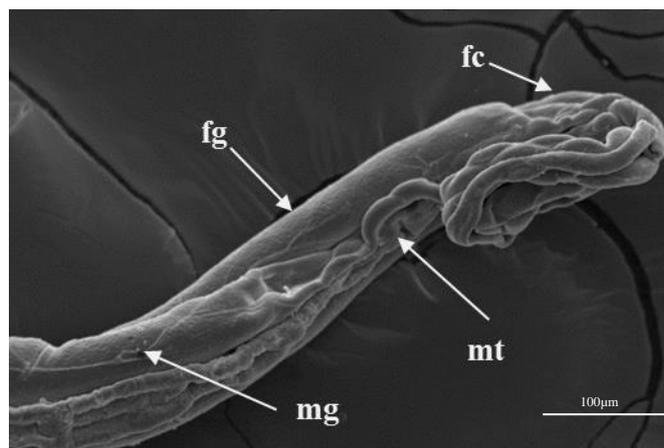


Figure 2 - Intestine of *D. maidis* observed to the Scanning electronic microscope. showing the filter chamber (*fc*), foregut (*fg*), midgut (*mg*) and some malpighian tubules (*mt*)

Image: J. Garcia González

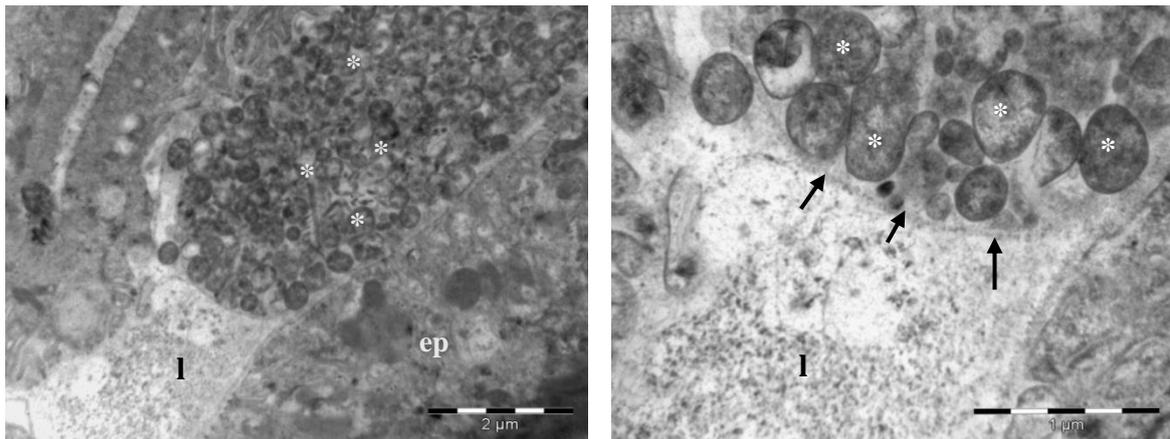


Figure 3 - Transmission electronic microscopy images obtained at the foregut of *D. maidis*. Adults were previously fed on maize plants infected with the phytoplasma MBSP (AAP= 96h; LP= 23d). A) A mass of phytoplasma (\*) is observed crossing the lumen (*l*) of the digestive tube. B) A detail of the matrix (arrows) in which the MBSP mass is moving through the lumen of the digestive tube of its vector. (*ep*) epithelium of the intestine

Images: J. Garcia González

The midgut of *D. maidis* was easily differentiated by the presence of microvilli contiguous to the superior border of the epithelium intestine and separating it from the lumen of the digestive tube (Figure 4A). Access of the phytoplasma cells to the midgut was observed either directly through the phytoplasma cells trying to access the microvilli (Figure 4B-C) or through by an enzyme like protein production altering the microvilli wall (Figure 4B-D) as a concave hole made in between the aligned microvilli wall (Figure 4E). The microvilli wall is broken by the enzyme production and an accessing space is opened for the phytoplasma cells entrance (Figure 4F). At this point, a group of phytoplasma cells congregate on the lumen side, and again release a biofilm protein (Figure 4F) to form the matrix previously observed in the lumen of the digestive tube, for continuing its movement as a mass through the epithelium of the midgut (Figure 4G).

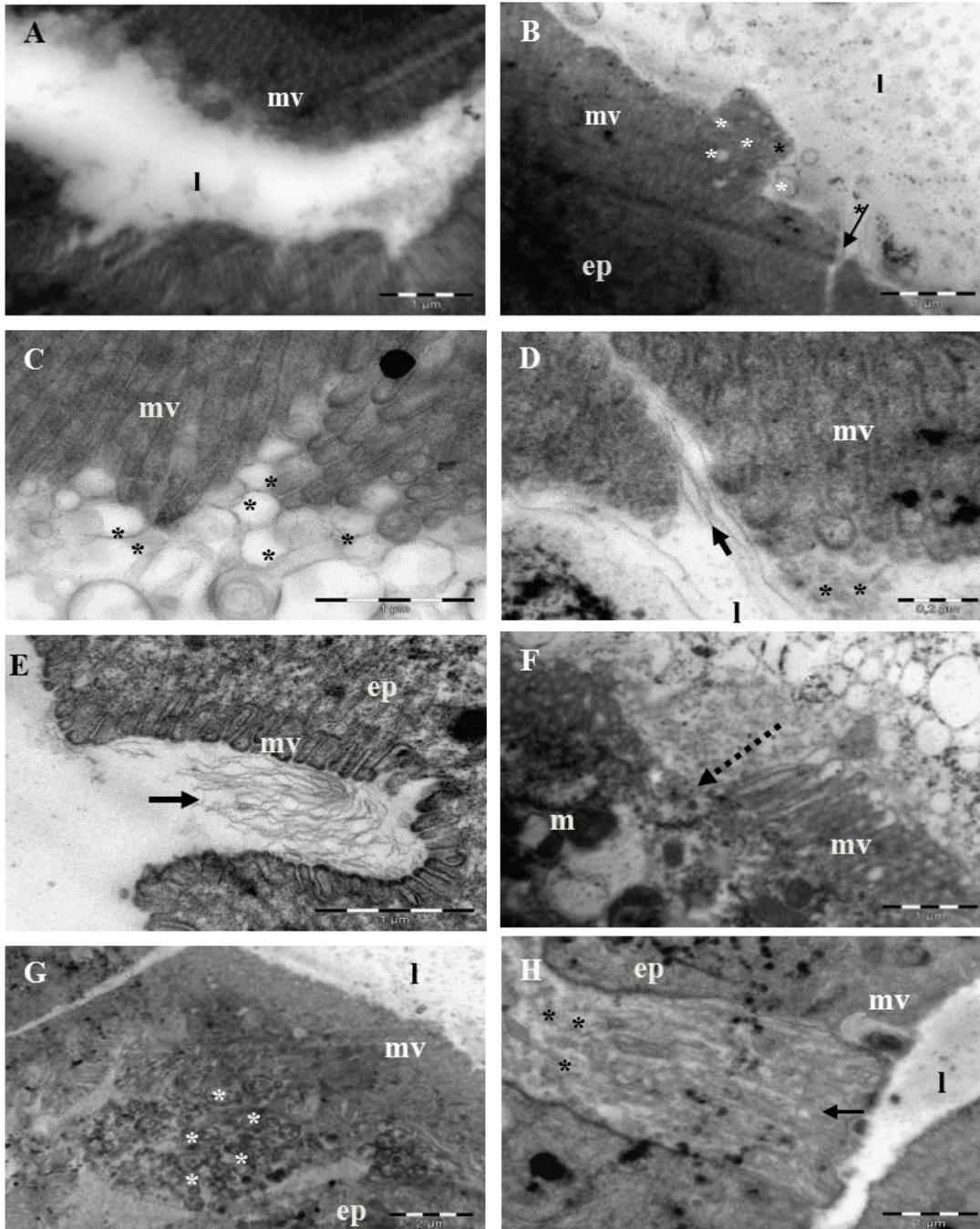


Figure 4 - General steps observed in the MBSP entrance and movement through the epithelium of the *D. maidis* midgut. **A)** Health intestine of *D. maidis* showing a cleaned lumen of the digestive tract and the well organized microvilli hairs. **B)** a small group of MBSP cells (\*) reaching the microvilli wall (*mv*) with some cells accessing directly through the *mv* (**C**) or by an enzyme production (dark arrow in **D**). The produced protein (dark arrow) generates a concave indentation at the *mv* zone (**E**) leaving an open space used by the phytoplasma cells for accessing the epithelium of the intestine (**F**). After the phytoplasma entrance, the mass cell of MBSP produce a biofilm for its movement (**G**), leaving behind on the *mv* wall a layer (arrowhead) as a signal of its entrance (**H**)

Images: J. Garcia González

As an evidence of the phytopathogen access to the epithelium a path leaved behind the phytoplasma mass (Figure 4H) is observed, with the microvilli wall recovering the intestine isolation from the digestive tube, probably with a cap produced by the phytoplasma protein production.

To highlight, the phytoplasma passage through the epithelium of the intestine was shown convoyed with a variety of insect microbiota (Figure 5) occupying the same spaces as the phytoplasma cells, contrary as observed in the healthy *D. maidis* samples processed, were neither the phytoplasma cells nor the insect bacteria were visualized.

As a general observation the insect microbiota are bacteria of 0.5 to 1.0  $\mu\text{m}$  size, with two defined wall cells, internal content characterizing each of the 12 morphotypes observed with oval (Figures 5A-B-C-D-L), rounded (Figures 5E-F-G-H-J-K) or enlarged non defined forms (Figure 5I). Some of the bacteria morphotypes were observed frequently in the samples examined, being the morphotypes (5A-B-C) those with the higher presence in the intestine with the phytoplasma.

The lamina basal (*lb*) of healthy *D. maidis* intestine is observed aligned at the epithelium border, isolating this organ with the hemolymph (Figure 6A). The intestine of the infected individuals, showed as a first way some MBSP cells leaving the intestine to the hemolymph, by pushing out the basal lamina and leaving an internal vacuole between this wall and the epithelium, for the posterior protein release in which the bacteria cells goes into and moves by the hemolymph (Figure 6B).

In a second way for leaving the epithelium, some MBSP cells are observed broken the basal lamina (Figure 6C) followed by a protein production (Figure 6D) probably coming from the phytoplasma cells, producing the matrix protecting and carrying the MBSP mass cell through the hemolymph.

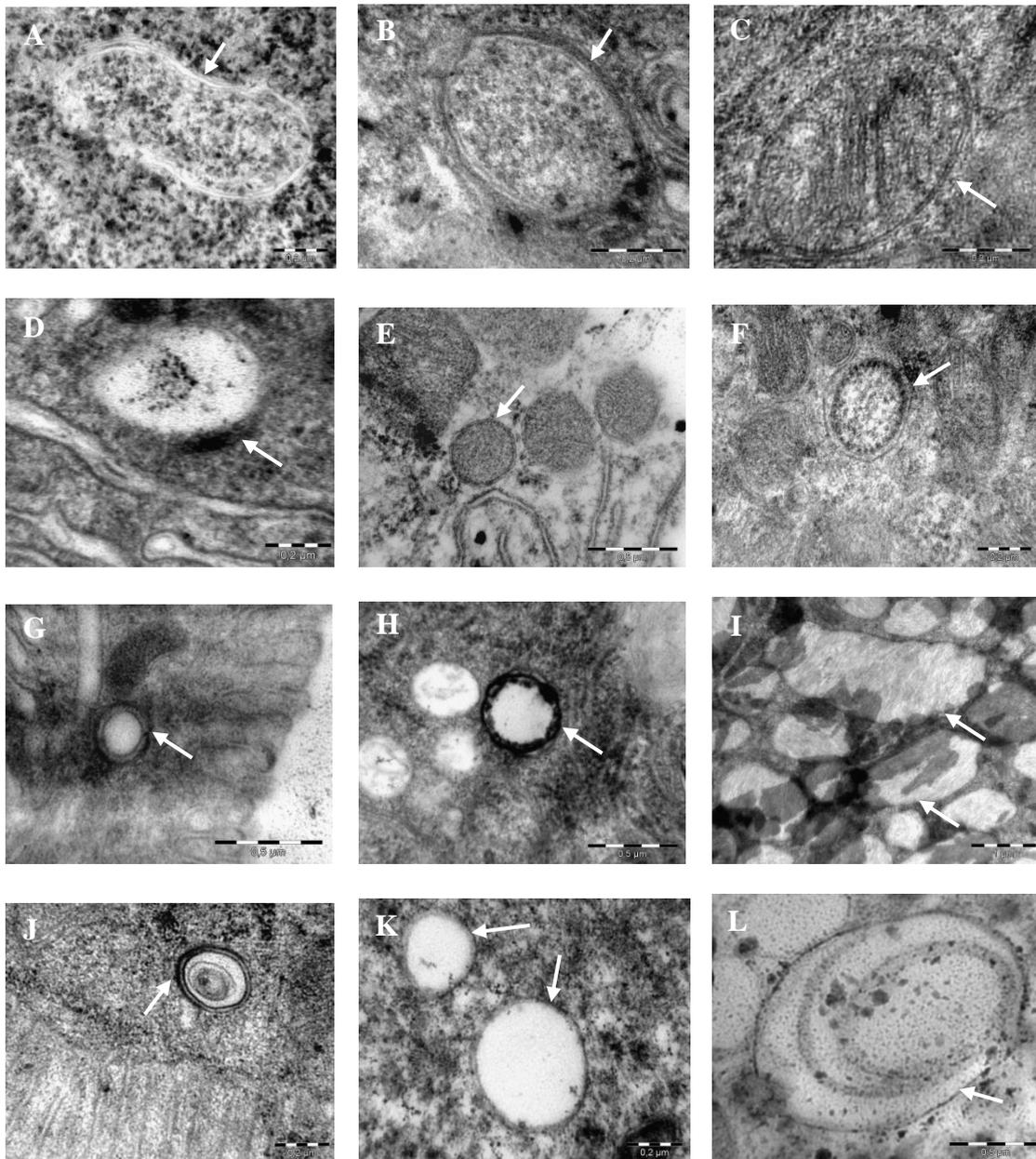


Figure 5 - Different types of bacterial cells resembling endosymbionts present in the epithelium of the *D. maidis* midgut infected with the phytoplasma MBSP as indicated by arrows

. Images: J. García González

Interestingly, with the phytoplasma protein production released at the border lane of the intestine, bacteria like endosymbionts previously observed in the epithelium of the intestine were seen two possibly waiting for joining the phytoplasma movement through the hemocele (Figures 6E-F). The phytoplasma cells are packed into the matrix in conjunction with some intestine components like nucleus and mitochondria (Figure 6F) and some endosymbiont like bacteria (Figures 6G-H), and begin its journey to attain other insect structures.

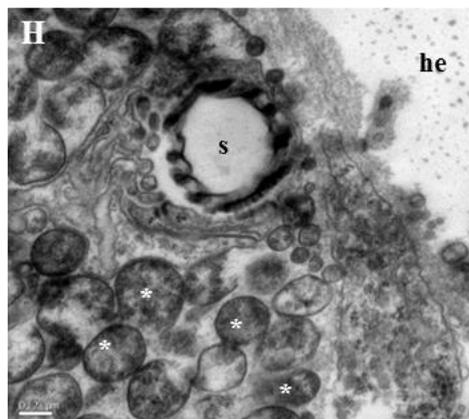
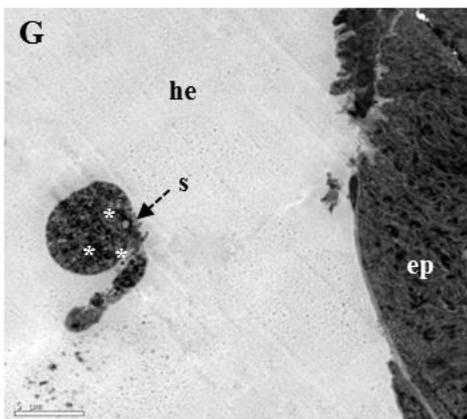
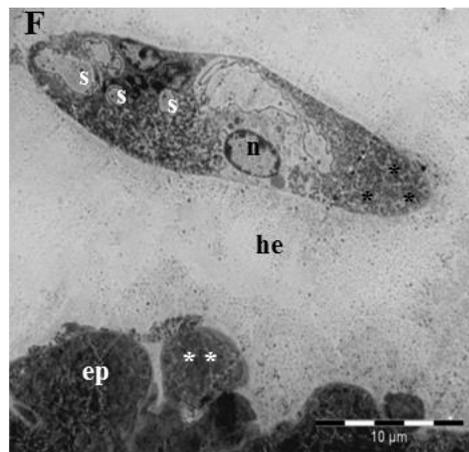
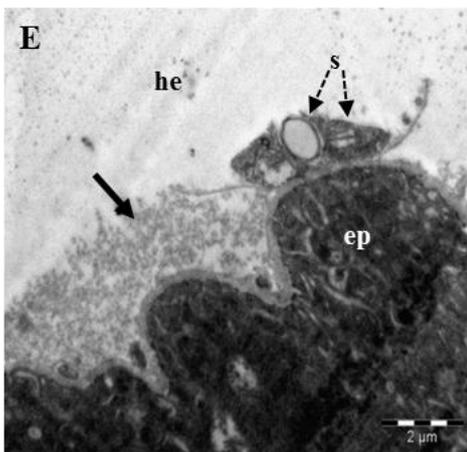
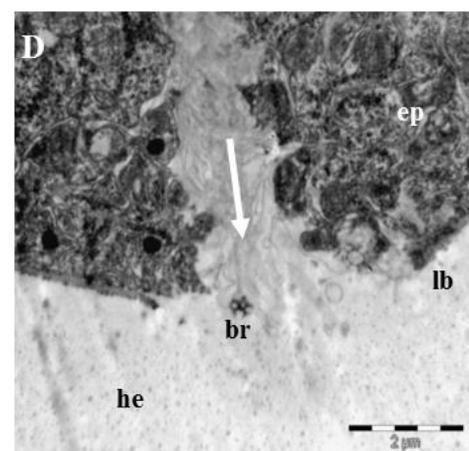
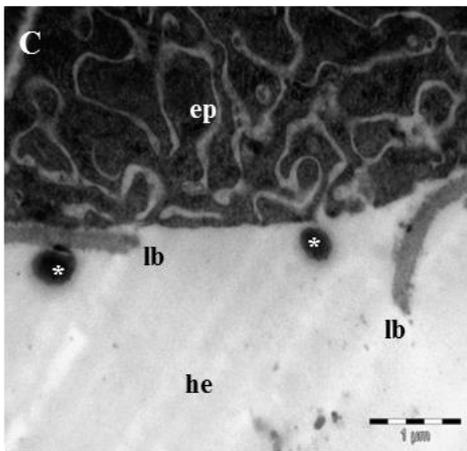
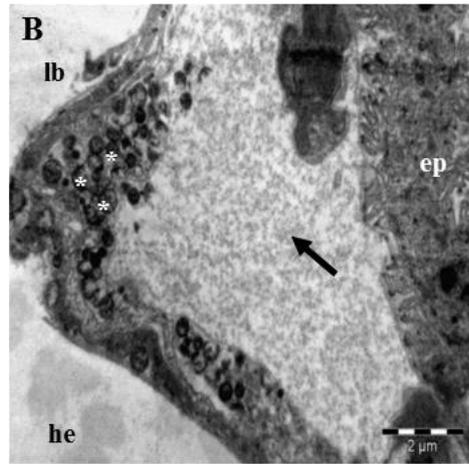
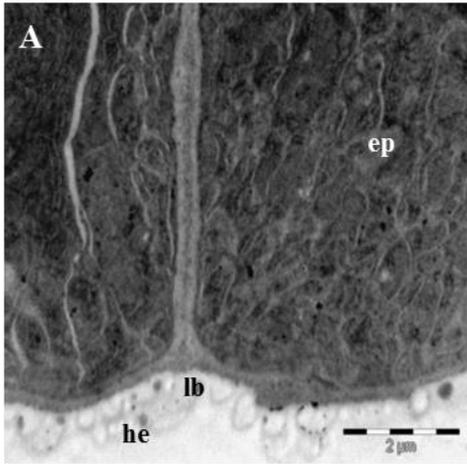


Figure 6- Steps followed at the phytoplasma MBSP cell escape from the midgut. A healthy intestine of *D. maidis* shows the epithelium (*ep*) and the basal lamina (*lb*) clearly differentiated when the phytoplasma MBSP cells are absent (A). When the MBSP cells are present, two different mechanisms of the MBSP cells for reaching the hemolymph (*he*) were observed. In the first one, the phytoplasma cells (\*) push out the *lb* leaving a space between the epithelium and this wall, for releasing the biofilm (dark arrow) in which the MBSP cells will move into the *he* (B). In the second mechanism, the *lb* is broken by some MBSP cells (\*) (C), posteriorly occurring a biofilm production (dark arrow) which reaches the hemolymph (D), for locating at the exterior side of the *lb* presumably waiting for the phytoplasma cells movement into the biofilm matrix, in conjunction with some bacteria like endosymbionts (*s*) (E). Once the MBSP mass cells (\*) are packed into the biofilm, the phytoplasma movement through the hemolymph will begin interestingly joined with some nucleus (*n*) presumably coming from the epithelium of the midgut (F), as well as some (*s*) (G) previously observed in the epithelium of the midgut, as shown in detail in (H). Images: J. Garcia González

Besides the mass cells of phytoplasma leaving the epithelium of intestine, phytoplasma activity was observed in malpighian tubules with both protein activity and MBSP cells leaving the structure.

Phytoplasma cells reaching the insect salivary gland were observed arriving to the external wall of the structure in the same way as observed when the phytoplasma mass left the intestine, it means, protected and encapsulated in a biofilm matrix (Figure 7A). Interestingly the phytoplasma mass cell getting the salivary gland at the terminal end, contained a portion of nucleus presumably belonged to the intestine (Figure 7B).

Phytoplasma entrance to the salivary gland began with some bacteria cells getting contact with the external wall and again a protein releasing presumably served to break the protecting wall (Figure 7C). Once inside the phytoplasma cell multiplies in high quantity (Figure 7D) and moves for the acini to reach the vesicles, point for continuing the movement until reach the salivary ducts (Figure 7E).

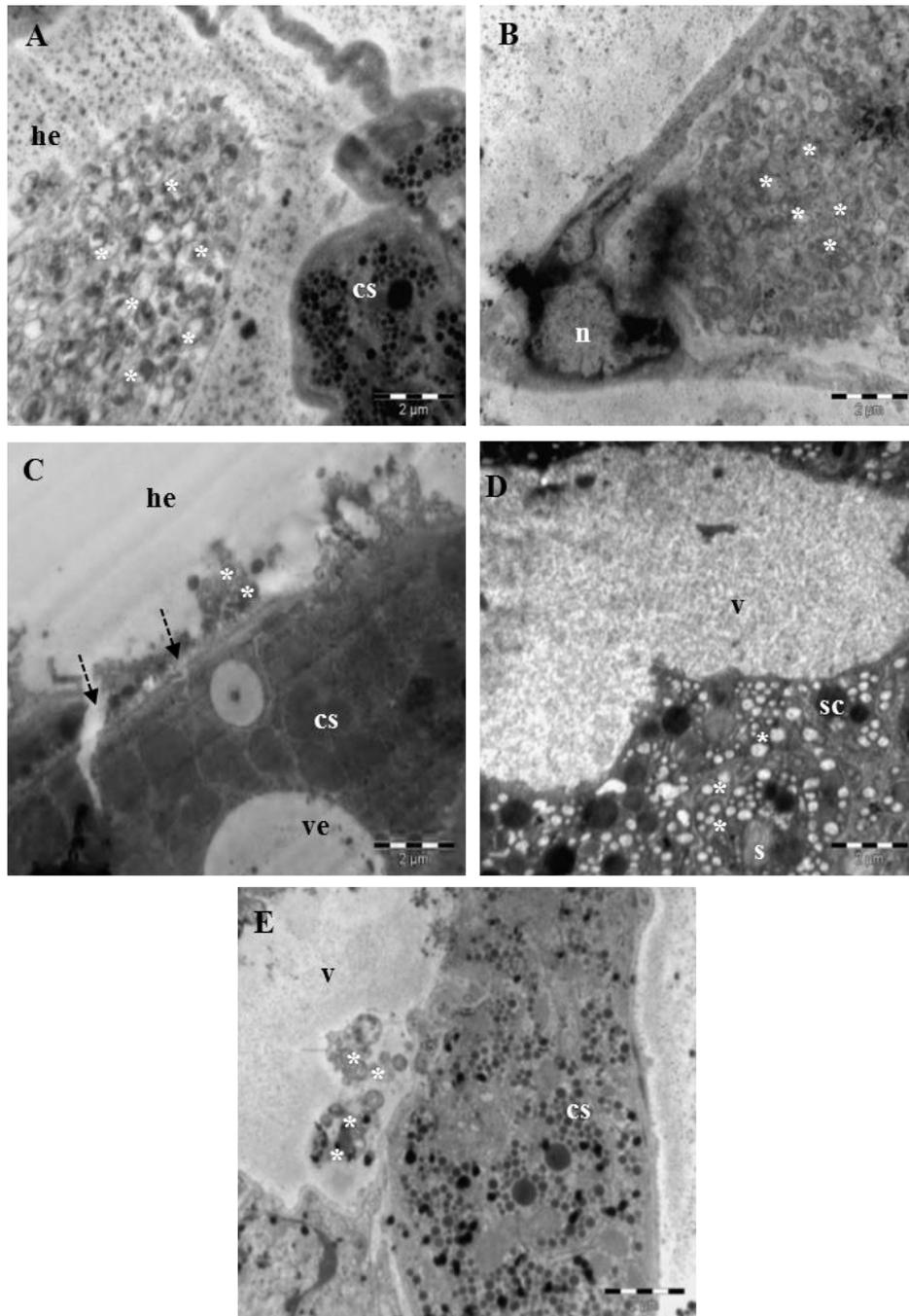


Figure 7- Arrival, access, invasion and leaving of the *D. maidis* salivary gland by the MBSP cell mass, coming from the intestine. **A)** the MBSP mass cell (\*) arrives to one of the salivary gland acini, encapsulated into the biofilm (arrow) and **B)** with some nucleus content placed in the posterior side of the mass cell. **C)** Some of the phytoplasma cells interact with the basal lamina causing rupture (dotted line) and opening the spaces for the cells access to the structure. **D)** the MBSP cells (\*) invade the epithelium of the salivary gland with a high amount of cells formed into the structure, and observed close to the vacuoles were the salivary content is deposited. **E)** Phytoplasma cells access the vacuoles of the salivary gland, moving with the salivary content toward the salivary duct, passing through the salivary canal of the stylet and entering to the plant via food consumption

Images: J.García González.

In the phytoplasma movement for the acini of the salivary gland, different bacteria like endosymbionts previously observed at the epithelium intestine, were observed interacting with the phytopathogen in most of the cases where secretory cells of the salivary gland were present (Figure 8). To highlight the bacterium of Figure (8F), characterized by a two strong and well defined external and internal wall with dark spots at the cytoplasmic bacterium content, which was observed in higher abundance in this structure as well as in the epithelium of intestine, giving strong evidences of interactions with the phytoplasma.

Once the phytoplasma multiplies into the salivary gland in the enough amount, the bacteria cells leave the insect via the salivary canal at the moment of the *D. maidis* feeding on a new host plants (Figure 9A).

In this way the transmission process begins again with the easily observed symptoms (Figure 9A). TEM analysis of roots and leaves of MBSP infected plants with 45 days of disease incubation period, evidenced the phytoplasma bacteria invading the root cells with no infection evidences in the reddish leaf samples (Figures 9B-C-D ) suggesting a multiplication point at the radical system, where bacteria cell grows forming a mass protected by a biofilm in such a way that completely blocks the crivated cells (Figure 9D).

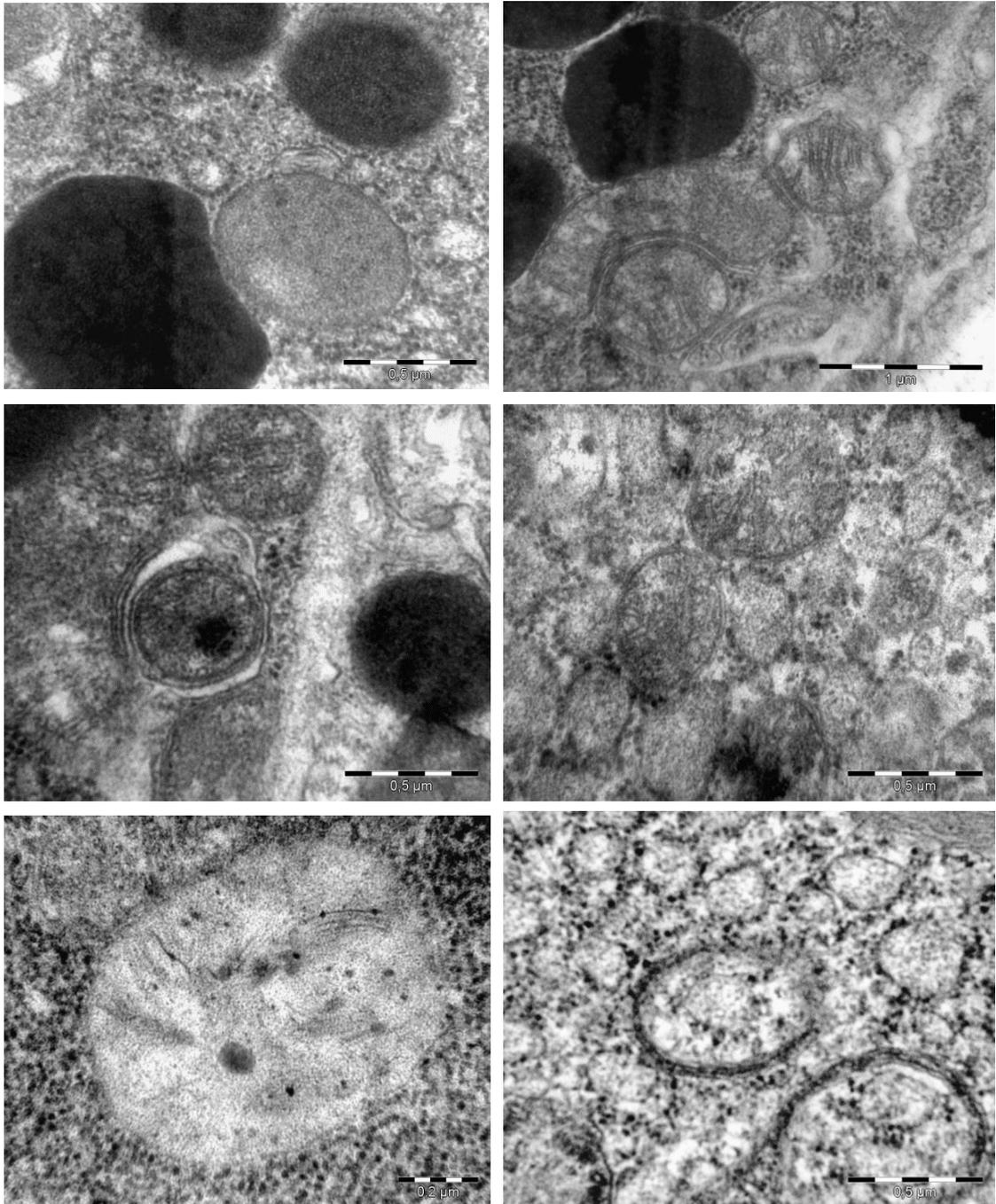


Figure 8 - Bacteria like endosymbionts joining spaces in the salivary gland with the phytoplasma cells. Almost all of the bacteria observed in the salivary gland were observed in the intestine infected with the phytoplasma cells as well

Images: J. García González.

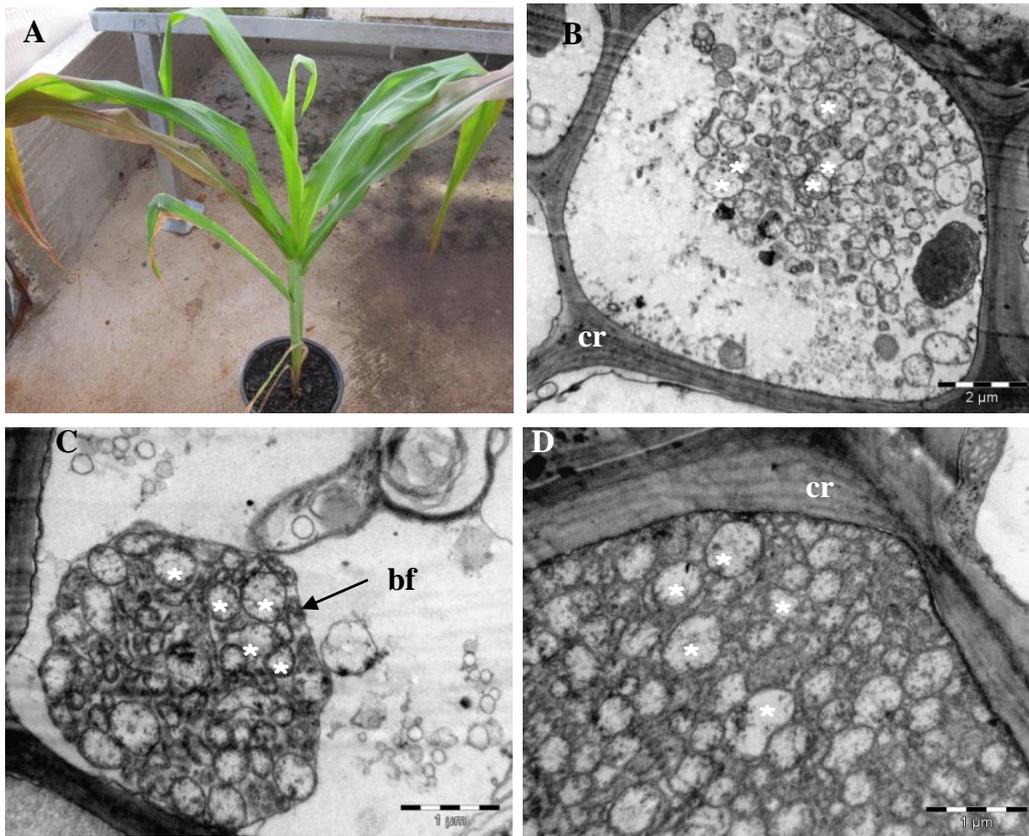


Figure 9 - Infection *in-planta* of the phytoplasma MBSP. A) Advanced symptoms of the phytoplasma infection in maize plants with 45 days of disease inoculation period. From the infected plant were taken samples of both leaves and roots, for its observation to the TEM, interestingly found the phytoplasma in the roots but not in the leaves B, C, D). Images revealed the phytoplasma presence (\*) in the crivated cells (*cr*) of the root system, forming a mass and interestingly being encapsulated into the biofilm (*bf* indicated with an arrow) as previously observed in the phytoplasma movement into the vector structures

Images: J. Garcia González

#### 4.4 Discussion

For the first time is presented a detailed study of the mayze bushy stunt phytoplasma movement and passage for the *D. maidis* physiological barriers.

The phytoplasma MBSP in the passage for the food canal, epithelium of intestine, and hemocele arriving to the salivary gland, showed almost the same pattern in which its movement is made as a cell group protected for a matrix composed of protein acting as a biofilm produced for the phytopathogen cells. For some gram-positive bacteria species, it has been demonstrated the existence of a regulation gene expression in response to fluctuations in the bacteria cell-population density in a pattern named “quorum sensing” ruling different bacteria physiological activities such as symbiosis, virulence, competence, conjugation,

antibiotic production, motility, sporulation, and biofilm formation (MILLER; BASSLER, 2001; ZAVILGELSKY; MANUKHOV, 2001; GALETTO et al., 2009).

An interesting phytoplasma production of enzymes for moving, entering and leaving the intestine as well as for accessing the salivary gland, shows the importance of the enzyme production by the phytopathogen in the insect structures. As observed in the specificity of luteoviruses protein formation in the aphid transmission (GRAY; GILDOW, 2003), and for the onion yellows phytoplasma transmission (WEINTRAUB, 2007) some genes possessing an immune-dominant membrane protein (Amp) determine to interact with microfilament complexes in muscle cells surrounding the intestinal tract seeming to be responsible for vector phytoplasma specificity.

In the phytoplasma entrance and leaving both the intestine and salivary gland of *D. maidis* a negative effect on the insect fitness is expected as a result of the rupture of the structures by the phytopathogen. The invasion mechanism observed for MBSP on its vector appears to be more detrimental and aggressive when compared with that of the mollicute *Spiroplasma kunkelli* also transmitted by *D. maidis*. The spiroplasma bacteria access the microvilli of intestine prudently protected by endocytic vesicles, then the spiroplasma moves for the intestine in individually cells protected by endosomes, and reach the hemocele broken carefully the lamina densa, maintaining the lamina rara of the basal lamina (ÖZBEK et al., 2003).

For the first time it is confirmed the MBSP phytoplasma movement by the hemocele joint with some bacteria like endosymbionts as well as with some intestine nuclear compounds like nucleus and mitochondria. Of interest, phytoplasma mass reaching the salivary gland were observed with a deformed and despoiled nucleus located at the posterior side of the phytopathogenic mass arriving to the structure.

The gene loss of phytoplasma for synthesizing amino acids, fatty acids, nucleotides or ATP synthases, (CHRISTENSEN et al., 2005; BAI et al., 2006) allows to presume that nucleus and mitochondria joining the phytopathogen movement through the hemocele for attaining the salivary gland, serves to supplement the phytoplasma with the nutrient and energy required in that passage.

In order to leave both the intestine and salivary gland, the phytoplasma must multiply and invade the structures in such a way to facilitate the cells assembly within the biofilm for protecting and moving to the tissues of both insect and plant.

When infecting a new maize plant, the phytoplasma probably moves in group via salivary canal of the *D. maidis* stylet. Within the phloem leaf the phytopathogenic bacteria probably moves downward to the root system for invading these structure and multiplying there. This behavior was demonstrated in garland chrysanthemum where the infection of the phytoplasma inoculated in a middle leaf of an eight- leafed plant migrated into the main stem, apex and roots in a two days period but required about 3 weeks to infect all leaves (WEINTRAUB; BEANLAND, 2006).

For the first time, TEM images confirm the interactions between the insect microbiota and the phytoplasma cells during its passage in both the intestine and salivary gland of MBSP infected individuals, where some bacteria like endosymbionts were observed occupying the same spaces of the phytoplasma cells, with no evidences of these bacteria in the same organs of the healthy adults, as demonstrated also for Alma et al. (2010) studying the phytoplasma doree vectored by *Scaphoideus titanus*. In my study the effect of the phytoplasma MBSP on its vector *D. maidis* allowed to observe a microbiota diversity and abundance joining the intestine and salivary gland of infected insects, with at least 12 morphologically differentiated bacteria, did not observed in the healthy individual organs analyzed. This result reveals the dramatic physiological alteration suffered by *D. maidis* when the phytopathogenic bacteria invades the insect body for its, movement, passage and multiplication.

#### 4.5 Conclusions

It is evident the aggressive phytoplasma governance of the insect cavity when acquired by the vector, because of the mass cell movement observed in the digestive tube, epithelium of the mesenteron, hemolymph and e salivary gland

Interestingly, the phytoplasma was observed promoting a “quorum sensing” behavior during its movement for the insect organs with a protein mass production protecting the bacteria cells.

An enzyme production coming from the phytoplasma cells is probably the main responsible for the bacteria access through the microvilli of the mesenteron, and basal lamina rupture on both the intestine and salivary gland.

A specific visualization of the insect microbiota on the intestine and salivary gland when the phytoplasma is present within *D. maidis*, will give insights for detailed studies of the role and status of the bacteria joining the phytoplasma MBSP.

It will be important to recognize and name each one of them and understand its function during the phytoplasma mass passage. Coming studies will be focused in the bacteria like endosymbiont joining the phytoplasma movement by the hemolymph, otherwise this bacteria can be included in strategies of the disease management through para-transgenesis programs as made by human disease control vectored by insects.

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## **5 MICROBIOTA ASSOCIATED WITH MAIZE BUSHY STUNT PHYTOPLASMA (MBSP) IN THE INSECT VECTOR *Dalbulus maidis* (DELONG & WOLCOTT) (HEMIPTERA: CICADELLIDAE)**

### **Abstract**

Considering the potential benefit of endosymbionts to the insect vector – phytoplasma interactions during the phytopathogen passage into its insect vector, the research aimed to identify the *Dalbulus maidis* (Hemiptera: Cicadellidae) microbiota vector of the maize bushy stunt phytoplasma and to determine any microbiota variation associated with the MBSP infection. *D. maidis* microbiota identification and variation as result on the phytoplasma latent period were determined using *D. maidis* adults previously fed during 72h on MBSP infected plants were maintained on healthy plants for 3, 5, 10 and 20 days of latent period (LP), at  $25 \pm 3^\circ\text{C}$ ,  $55 \pm 10\%$  RH, 14:10 (L:D) photoperiod. The whole adult body, dissected intestine and salivary gland for each LP insect group, as well as female and male genitalia were analyzed using PCR-Denaturing Gradient Gel Electrophoresis (DGGE) and Fluorescence In-Situ Hybridization (FISH). PCR-DGGE revealed a simple endosymbiont community with a beta-proteobacterium present in the whole body and female genitalia. FISH technique determined a variation in diversity and abundance of the microbiota present in intestine and salivary glands of *D. maidis* adults with different LP, occupying the same spaces of the MBSP. TEM observations confirmed the microbiota diversity in intestine and salivary glands of MBSP infected adults when compared with non-infected ones. Overall the results indicate the presence of *D. maidis*-associated microbiota in the same organs occupied by MBSP, with an interesting effect of the phytoplasma residence time on both symbiont abundance and diversity, suggesting a potential and differential interaction between the insect microbiota and phytoplasma transmission.

Keywords: FISH; Leafhopper microbiota; Primary endosymbionts

### **5.1 Introduction**

The success of insects in nature in part is due to a great extent to the symbiotic associations established between microorganisms and insect hosts (ISHIKAWA 2003; FELDHAAR; GROSS 2009; CLARK et al., 2010), which have not only contributed to the evolution of both partners, but have also made important contributions to the success of the insect lifestyle (KIKUCHI; HOSOKAWA; FUKATSU, 2007; ALMA et al., 2010). Endosymbionts might affect important insect physiological traits including nutrition (JANSON et al., 2008; GIBSON; HUNTER, 2010), reproduction (ALMA et al., 2010; CLARK et al., 2010) and some other processes in order to improve the benefits obtained from the symbiosis (SKALJACK et al., 2010).

Hemiptera as specialized insect feeding in xylem and phloem sap content require of symbiotic bacteria for complementing its diet with essential amino acids needed for growth

and reproduction (DOUGLAS, 2009; GOSALBES et al., 2010). Some species suborder Auchenorrhyncha is an important agricultural pest of Hemiptera and considered an efficient vector of phytopathogenic microorganisms, with a symbiosis generally represented between the insect group with the bacteria ‘*Candidatus Sulcia muelleri*’ and ‘*Candidatus Baumannia cicadellinicola*’ (MORAN; TRAN; GERARDO, 2005). This constantly increased interaction between insect vectors and its endosymbionts should be extended with phytopathogenic microorganisms and can lead to an increased ability of the disease transmission (AMMAR, 1997).

Phytoplasma are obligated phytopathogenic bacteria living in both plants and insects and depend on them for its acquisition and inoculation. Phytoplasma acquisition begins with stylet penetration and sustained food process of the insect vector on infected plants, phytoplasma entrance in the insect food canal, passage for the digestive tube and movement to the corporal cavity or hemocoel and hemolymph. To infect new plants the phytoplasma must colonize salivary glands of the insect vector and later being inoculated via saliva in the insect plant consumption on new plants, in a persistent propagative transmission process (CHRISTENSEN et al., 2005; BAI et al., 2006; HOGENHOUT et al., 2008).

In some pathosystems some proteins produced by the primary endosymbiont in the aphid hemolymph has been identified directly binding to the virus particles acting as protein folding, translocation across membranes, and recovery from stress in the insect hemolymph (FERRARI; VAVRE, 2011; GRAY; GILDOW, 2003).

In the case of the phytoplasma responsible for the grapevine yellows vectored by the leafhopper *Scaphoideus titanus* (Ball) (Hemiptera: Cicadellidae), the phytopathogenic microorganism share spaces with the P-endosymbiont *Cardinium* speculating that the same localization, multiplication and increasing titers inside the insect by both endosymbiont and phytoplasma are evidences for the interaction between these organisms (ALMA et al., 2010).

The maize leafhopper *Dalbulus maidis* (Delong & Wolcott) (Hemiptera: Cicadellidae) is a monophagous insect of maize crops considered an important pest because of its transmission efficacy of ‘Corn stunt spiroplasma’ (*Spiroplasma kunkelii*), ‘Maize bushy stunt phytoplasma’ (MBSP) and *Maize rayado fino virus* (MRFV) being dominant MBSP (WAQUIL; VIANA; SANTOS, 1999; ÖZBECK et al., 2003; AMMAR et al., 2011).

Perhaps of the agriculture importance of *D. maidis* as vector of the MBSP in maize crops, a limited amount of information on the interaction insect vector – phytoplasma transmission is available with no related evidence of the possible interactions between insect

microbiota and phytoplasma transmission. The research aimed to establish the diversity and abundance of *D. maidis* microbiota potentially interacting with the phytoplasma MBSP.

## 5.2 Materials and methods

Biological material was maintained and processed for Fluorescence In Situ Hybridization -FISH in both the insect vector and laboratory and the department of genetics at the 'Universidade de São Paulo – ESALQ (Piracicaba- Brazil). Microbiota studies using PCR-Denature Gradient Gel Electrophoresis PCR-DGGE and FISH studies were done and replicated in the department of food, environmental and nutritional Science of the 'Universita degli Studi di Milano (Milan- Italy)'.

### 5.2.1 Obtention of maize plants and *D. maidis* insects infected with the phytoplasma MBSP

A *D. maidis* colony was initialized with ovipositions coming from adults mating during a 96h period in an entomological cage containing maize plants 35 days after sowing. Endophytic ovipositions near to hatch were isolated from the maize plant and were relocated on healthy plants to facilitate the feed consumption of the new eclosed nymphs. The insect immature stages were allowed to feed on healthy maize plants until reaching the adult stage.

A MBSP isolate coming from a maize crop grown in the Genetic department at the University of Sao Paulo –ESALQ (Piracicaba-Brazil) was maintained and replicated on maize plants via infective vectors. *D. maidis* adults were fed on maize plants expressing disease symptoms during a 96h acquisition access period (AAP). After the AAP, adults were isolated and maintained during a 23 days latent period (LP) in rearing cages containing healthy maize plants at  $25\pm 3$  °C ;  $58\pm 7\%$ ; 14h:10h photoperiod conditions.

MBSP infective adults reaching the mentioned LP were used to infest new maize plants for a 96h inoculation access period (IAP). Fulfilled the IAP plants were separated and maintained isolated in greenhouse for disease symptom expression. Plants showing symptoms were used as a substrate feeding for the adults used in the experiments, for obtaining plant material to do some observations, as well as for preserving the phytoplasma isolate.

### 5.2.2 *D. maidis* microbiota identification using PCR-Denature Gel Gradient Electrophoresis Technique

Pools of 40-50 infective adults were allowed to feed on MBSP maize plants and complying 3, 5, 10, 20 days of LP, as well as healthy fed on healthy plants for 20 days were gently dissected in order to obtain intestine and salivary gland for each treatment. In the analysis were included male and female genitalia and five complete adults infected with MBSP, all maintained in ethanol 70%, to be used in PCR-Denature Gradient Gel Electrophoresis (PCR-DGGE) protocol.

The total DNA of whole insects and dissected organs was extracted according to a method previously described by Doyle and Doyle (1990) and followed by GONELLA et al. (2011). Samples maintained in PBS 1x were centrifuged for 20 seconds (2.000rpm) in order to place the mini-structures at the base of the eppendorff. PBS was separated, then added 20  $\mu$ l of extraction buffer, macerated and completed with 30  $\mu$ l of the same buffer. Samples were placed three times for ten minutes at  $-80^{\circ}\text{C}$  and then at  $+70^{\circ}\text{C}$ . After the last heating, 5  $\mu$ l of lysozyme were added to the structures and incubated at  $37^{\circ}\text{C}$  under stirring for 30 minutes. 1  $\mu$ l of K-proteinase was added to the samples for a posterior incubation at  $37^{\circ}\text{C}$  in agitation for 30 min. 3 $\mu$ l of SDS (10%) were added and incubated at  $37^{\circ}\text{C}$  under stirring during 30 minutes. 10  $\mu$ l of NaCl 5M were added and posteriorly 8 $\mu$ l of CTAB/NaCl solution was included, mixed gently and again incubated in agitation at  $65^{\circ}\text{C}$  for 10 min. In extraction chamber an equivalent volume (about 70  $\mu$ l) of FIAC (phenol-chloroform-isoamyl alcohol) was added, mixed gently and then centrifuged at 1832,73 g. during 10 min. The aqueous phase was transferred to a new tube. About 70  $\mu$ l volume of chloroform-isoamyl alcohol were added to the sample, mixed and centrifuged at 1832,73 g. for 10 min. The supernatant was transferred to a new tube adding 42  $\mu$ l of isopropanol during 1,5 h. After the mentioned time samples were centrifuged at 1832,73 g. during 15 min. and the supernatant produced was carefully removed to a new tube. 50  $\mu$ l of ethanol were added to the sample, doing a new centrifugation at 1832,73 g. during 15 min. The ethanol was totally removed and the pellet was dried in a thermo-mixer at  $37^{\circ}\text{C}$ . The pellet was re-suspended in 30-50  $\mu$ l of TE and stored at  $4^{\circ}\text{C}$  until its uses.

An initial PCR was made for 50  $\mu$ l solution reaction using 48  $\mu$ l of mix solution (Gen16S, primers GC357F+907R) and 2  $\mu$ l of DNA sample in the following conditions:  $94^{\circ}\text{C}$  for 4 minutes; 10 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $61^{\circ}\text{C}$  for 1 min and  $72^{\circ}\text{C}$  for 1 min.; 20 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $56^{\circ}\text{C}$  for 1 min and  $72^{\circ}\text{C}$  for 1 min; with a final extension at

72°C for 10 min. Amplified samples were run in a polyacrylamide gel of 60% to 40% denaturant gradient placed in a vertical box containing a solutions of TAE 50% + Acrylamide and running conditions of 60°C, 90V during 17h.

When the running time was completed, gel was stained in a cyber-green solution for 20 minutes and three washes in milli-Q water during 5 minutes each. Band observation was made in dark conditions placing the gel in a BIO-RAD® visualization box.

The gel with bands was transferred to a darken room and exposed to UV light, where the obtained bands were excised and eluted in 50µl of milli-Q water contained in eppendorf tubes. Eluted bands were centrifuged during 15 seconds, and heated at 37°C during 3h. DNA samples were re-amplified with primers 357F and 907R in order to re-confirm DNA presence.

Obtained PCR products were purified and sent for sequencing (Macrogen®). Sequences for each band were compared with those existing in the National Center for Biotechnology Information (NCBI) sequence database by using the tool BLAST ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)).

### **5.2.3 *D. maidis* microbiota localization and variation using Fluorescence In-Situ Hybridization**

Following protocols of Gonella et al. (2011) and Crotti et al. (2009) endosymbiont localization was done in both *D. maidis* intestine and salivary gland, using the Fluorescence In-situ Hybridization (FISH) technique. Pools of 15-20 insect organs coming from infective *D. maidis* adults complying the latent periods (LP) of 3, 5, 10, 20 and 25 days as well as insect individuals coming from healthy adults with the LP of 5, 10 and 20 days were gently dissected and preserved in eppendorf tubes containing PBS + ethanol (50% : 50%).

In a first experiment the probes obtained from the planthopper *Hyalestes obsoletus* (Hemiptera: Cixidae) main vector of phytoplasma doree in grape-vines were tested in the vector *D. maidis* at the food, environmental and nutritional sciences (DeFENS) Department of the university of Milan. Probes belonged to *Sulcia* (S1150), *Cardinium* (card172 and card1069), *Wolbachia* (W1), *Rickettsia* (Rox), Phytoplasma group II (ph1107) *Purcellliella* (P820) and Eubacteria (EUB338).

Probes card172, card1069, S1150, AAB and W1 were labeled at their 5\_ ends with the fluorochrome Cy3 (indocarbocyanine) (absorption and emission at 550 nm and 570 nm, respectively) or Cy5 (indodicarbocyanine) (absorption and emission at 650 nm and 670 nm, respectively). Probe ph1107 was labeled with DIG (absorption and emission at 495 nm and

519 nm, respectively); probe P820 was labeled with HEX (4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein) (absorption and emission at 535 nm and 556 nm, respectively), and EUB338 were labeled with fluorescein isothiocyanate (FITC) (absorption and emission at 494 nm and 520 nm, respectively).

In a second set of experiments done at the USP-ESALQ (Brazil) the previous results were confirmed evaluating the probes for *Sulcia* (Sulcia16R1-A647), *Cardinium* (Card172) and *Rickettsia* (RickB1) obtained from the microbiota sequencing for *D. maidis*, and the probe for the phytoplasma MBSP group I (R16PhytIa) coming from the primer sequence used in the nested PCRs for detecting this mollicute.

Probes Sulcia16RI-A647, Card172, RickB1 and R16PhytIa were labeled at their 5' ends with the fluorochromes Cy5 (indodicarbocyanine) (absorption and emission at 650 nm and 670 nm, respectively), FAM (5-Carboxyfluorescein) (absorption and emission at 492 nm and 518 nm, respectively), Cy3 (indodicarbocyanine) (absorption and emission at 550 nm and 570 nm, respectively) and Texas Red (absorption and emission at 596 nm and 615 nm respectively) respectively.

FISH processing began with a PBS 1x washing during 10 minutes at run temperature. Then, a pepsin solution (0.1%) was added to the samples for a partial tissue digestion during 10 min at 37°C. Samples were two times washed in PBS 1x solution during 10 minutes each at run temperature. A 100µl volume of mix hybridization buffer (SSC 0.2x + Formamide 50% + probe 40ng + milliQ 36 µl water) was added to the samples and maintained them at 37°C in dark conditions overnight.

Following day, samples were eluted in a mix buffer (SSC 0.2x + Formamide 50% + milliQ water 36 µl water) without the probes, for 30 minutes at 37°C. Samples were washed two times in SSC 0.1x for 10 min. (room temperature), two times in SSC 1x for 10 min. (37°C), and two times in PBS 1x for 5 min (room temperature). After the last washing, samples were placed in glass slides containing a glicerine drop. Samples were protected with a cover slide and preserved at 4°C under dark conditions. Samples were observed to the microscopy confocals Leyca® system SP2 (Italy) and Olympus® Flwoview FV1000 (Brasil).

The abundance of each bacteria showing signal at the confocal microscopy was measured with the aid of free downloaded software ImageJ, estimating the covered area occupied by each bacteria with respect to the total area of the analyzed insect structure and obtaining the percentage of covered area.

## 5.3 Results

### 5.3.1 *D. maidis* microbiota identification using PCR-Denature Gel Gradient Electrophoresis (PCR-DGGE)

Samples of intestine and salivary gland infected with the phytoplasma MBSP at different latent periods, as well as female genitalia, male genitalia and complete adults were processed by PCR-DGGE, sequencing and blasted with the tool blast of the gene bank® database.

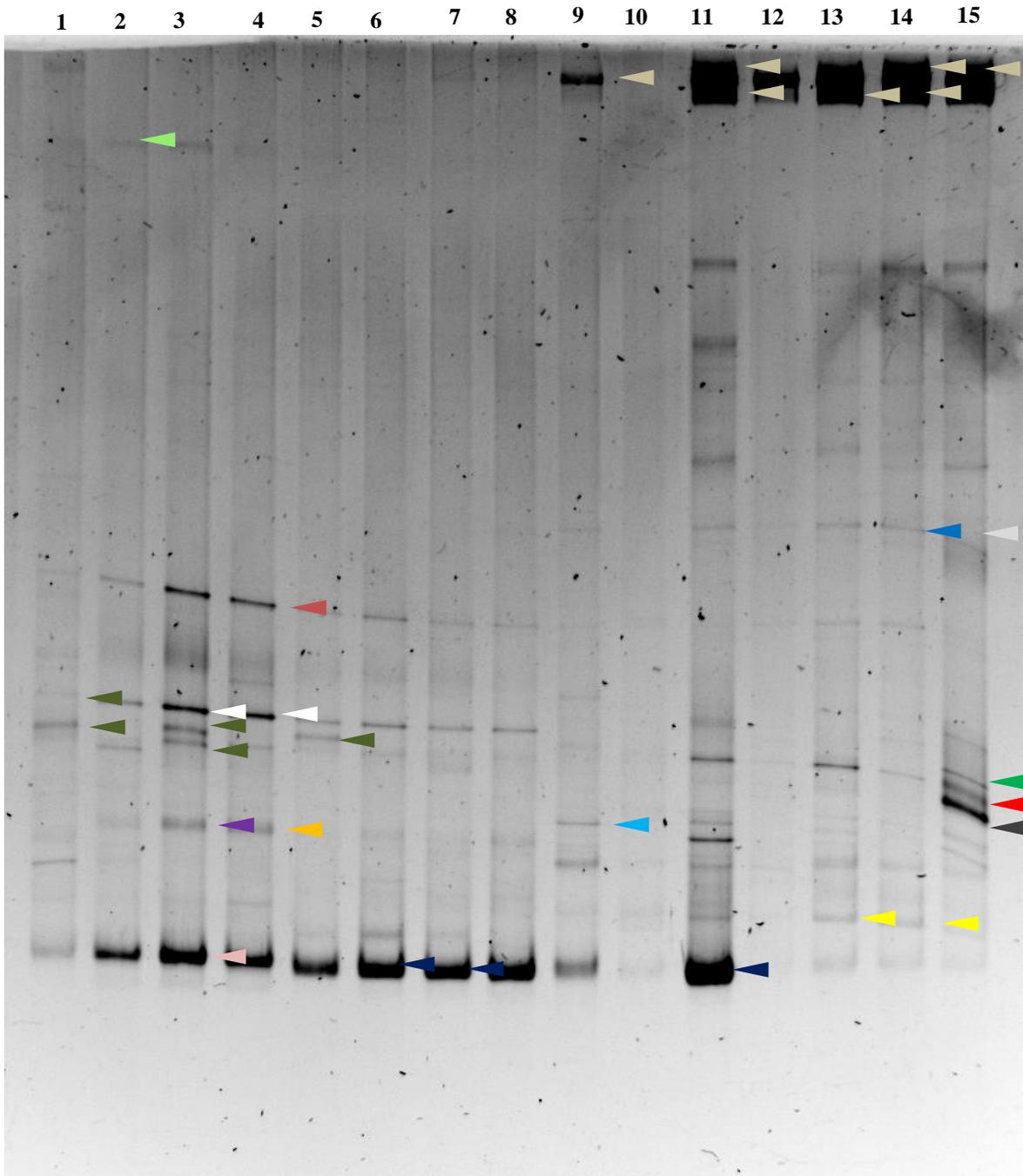
Results of the PCR-DGGE determined strong bands with a low molecular weight located in the upper part of the running gel identified in samples 11 to 15 belonged to adult samples (Figure 1). Sequencing and blasting processes identified these bands as *Candidatus Nasuia deltocephalinicola* (88% identity) respect to the compared sequence (560 base pairs).

Other bacteria identified for the adult samples belonged to *Bacteroides graminisolvens* (99% identity, 572 bp), *Lactobacillus plantarum* (85% identity, 196 bp), *Acetobacter cibinongensis* (99% identity, 554 bp), *Acetobacter persicus* (99 % identity, 554 bp), *Rhizobium* sp. (91% identity, 555 bp) and *Curtobacterium* sp. (98% identity, 557 bp) (Figure 1).

When analyzed the female genitalia (sample 9 in Figure 1), PCR-DGGE results showed strong signal for the bacterium *Candidatus Nasuia deltocephalinicola* as observed for the adult samples and the bacterium *Ideonella* sp. (95% identity, 575 bp), and *Curtobacterium* sp. Contrary Male genitalia (sample 10 in Figure 1) did not evidenced signal for bacteria with an incipient band at the same position of the bacterium *Curtobacterium* sp. (Figure 1).

Results for salivary gland (samples 1 to 4 in Figure 1) exhibited signal for the bacteria *Agrobacterium* sp.(99% identity, 537 bp), *Ralstonia* sp. (98% identity, 572 bp), *Delftia tsuruhatensis* (95% identity, 571 bp), *Staphylococcus* sp. (99% identity, 582 bp), *Microbacterium* sp. (90% identity, 199 bp), *Acidovorax wautersii* (97% identity, 649bp), *Acidovorax soli* (97% identity, 560 bp), and *Curtobacterium* sp.

Finally, intestine microbiota (samples 5 to 8 in Figure 1) showed a lesser diversity than the salivary gland microbiota with the bacteria *Ralstonia* sp., *Delftia tsuruhatensis*, *Staphylococcus* sp., and *Curtobacterium* sp.



- |   |  |   |
|---|--|---|
| <span style="color: red;">■</span> <i>Ralstonia</i>                   | <span style="color: yellow;">■</span> <i>Leptothrix</i> sp.        | <span style="color: olive;">■</span> <i>Staphylococcus</i> sp.  |
| <span style="color: blue;">■</span> <i>Bacteroides graminisolvens</i> | <span style="color: cyan;">■</span> <i>Ideonella</i> sp.           | <span style="border: 1px solid black; display: inline-block; width: 10px; height: 10px;"></span> <i>Delftia tsuruhatensis</i>         |
| <span style="color: green;">■</span> <i>Acetobacter cibinongensis</i> | <span style="color: orange;">■</span> <i>Acidovorax wautersi</i>   | <span style="border: 1px solid black; display: inline-block; width: 10px; height: 10px;"></span> <i>Ca. Nasuia deltocephalinicola</i> |
| <span style="color: red;">■</span> <i>Rhizobium</i> sp.               | <span style="color: purple;">■</span> <i>Acidovorax soli</i>       | <span style="color: darkblue;">■</span> <i>Curtobacterium</i> sp.   |
| <span style="color: pink;">■</span> <i>Microbacterium</i>             | <span style="color: lightgreen;">■</span> <i>Agrobacterium</i> sp. | <span style="border: 1px solid black; display: inline-block; width: 10px; height: 10px;"></span> <i>Lactobacillus plantarum</i>       |
| <span style="color: black;">■</span> <i>Acetobacter persicus</i>      |  |   |

Figure 1 - DNA bands obtained from the PCR DGGE technique which were posteriorly sequenced and blasted for the *D. Maidis* samples processed. **1:** Salivary gland control, **2-3-4:** salivary gland with 3, 5 and 10d of MBSP incubation time, respectively. **5-6-7:** Intestine with 3, 5 and 10d of MBSP incubation time, respectively. **8:** Intestine control. **9:** female genitalia. **10:** Male genitalia. **11-15:** five adults. Arrows indicate bands whose DNA sequence was related to the bacteria identification by Blasting process

### 5.3.2 *D. maidis* microbiota variation using Fluorescence In-Situ Hybridization (FISH)

Replication 1 realized for intestine and salivary gland of *D. maidis* adults reaching 3, 5, 10 and 20d of the phytoplasma inoculation period and processed with the FISH technique, evidenced signal for the endosymbionts *Sulcia*, *Cardinium*, *Wolbachia*, *Rickettsia* and Eubacteria (Figure 2). Neither signal was observed for Phytoplasma group II nor for Purcellielia. In salivary gland without phytoplasma and salivary gland with phytoplasma 5d was observed signal for *Rickettsia* and for *Rickettsia* and *Wolbachia* respectively (Figures 2 I-J).

Intestine with presence of phytoplasma evidenced signal for the bacteria *Sulcia*, *Cardinium* and Eubacteria in the evaluated treatments with an estimated abundance of 6.0%, 4.0%, 3.0% and 96% of covered area for the treatments 3, 5, 10 and 20d of the phytoplasma incubation period respectively (Figure 3).

Salivary gland with presence of the phytoplasma showed a higher microbiota diversity than in intestine with presence of *Sulcia*, *Cardinium*, *Eubacteria*, *Wolbachia* and *Rickettsia*. Presence of *Sulcia*, *Cardinium* and Eubacteria showed increased abundance values of 30%, 30%, 64% and 84% covered area for 3, 5, 10, and 20 days of the phytoplasma incubation period respectively (Figure 3B).

*Rickettsia* was observed in salivary gland as the unique bacteria showing signal in adults without the phytoplasma MBSP, covering 53% of the structure in the control individuals. The same endosymbiont was observed in adults with the phytoplasma at 3d of disease incubation period with a lesser covered area (11%) when compared with the control individuals (Figure 3B).

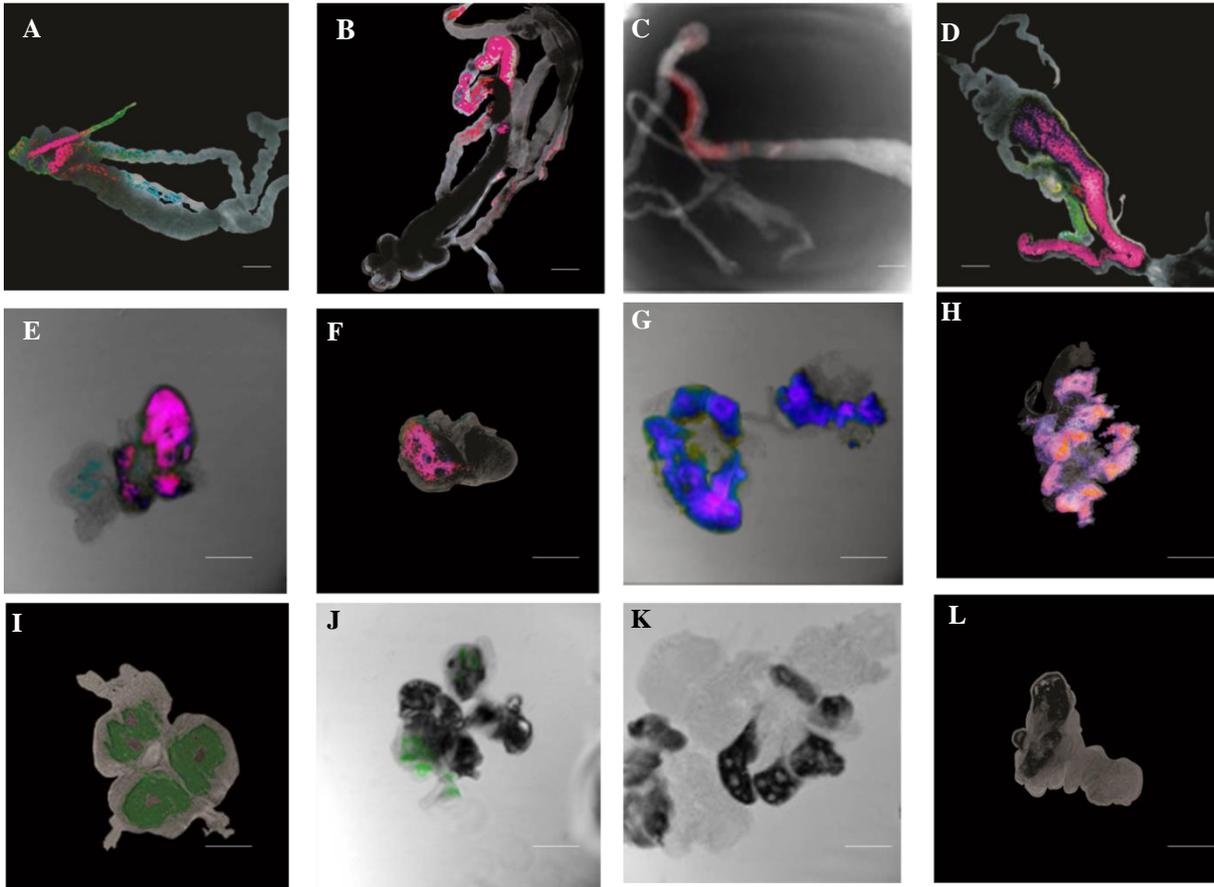


Figure 2 - Intestine and salivary gland samples of *D. maidis* adults processed by FISH technique in replication 1 to determine the bacteria like endosymbiont presence in the samples previously infected with the MBSP at different latent periods (LP). A-B-C-D) Intestine with MBSP. LP= 3, 5, 10, and 20d. *Sulcia* (red - green), *Cardinium* (blue), Eubacteria (white). Line= 100 $\mu$ . E-F-G-H) Salivary gland with MBSP. left to right LP= 3, 5, 10, 20. *Sulcia* (red - green), *Cardinium* (blue), Eubacteria (white), Line= 100 $\mu$  I-J-K-L) Salivary Gland without MBSP (image I) and with MBSP (J-K-L) LP= 5, 10, 20. *Rickettsia* (green), *Wolbachia* (Darken grey). Line= 100 $\mu$

Images: J. García González

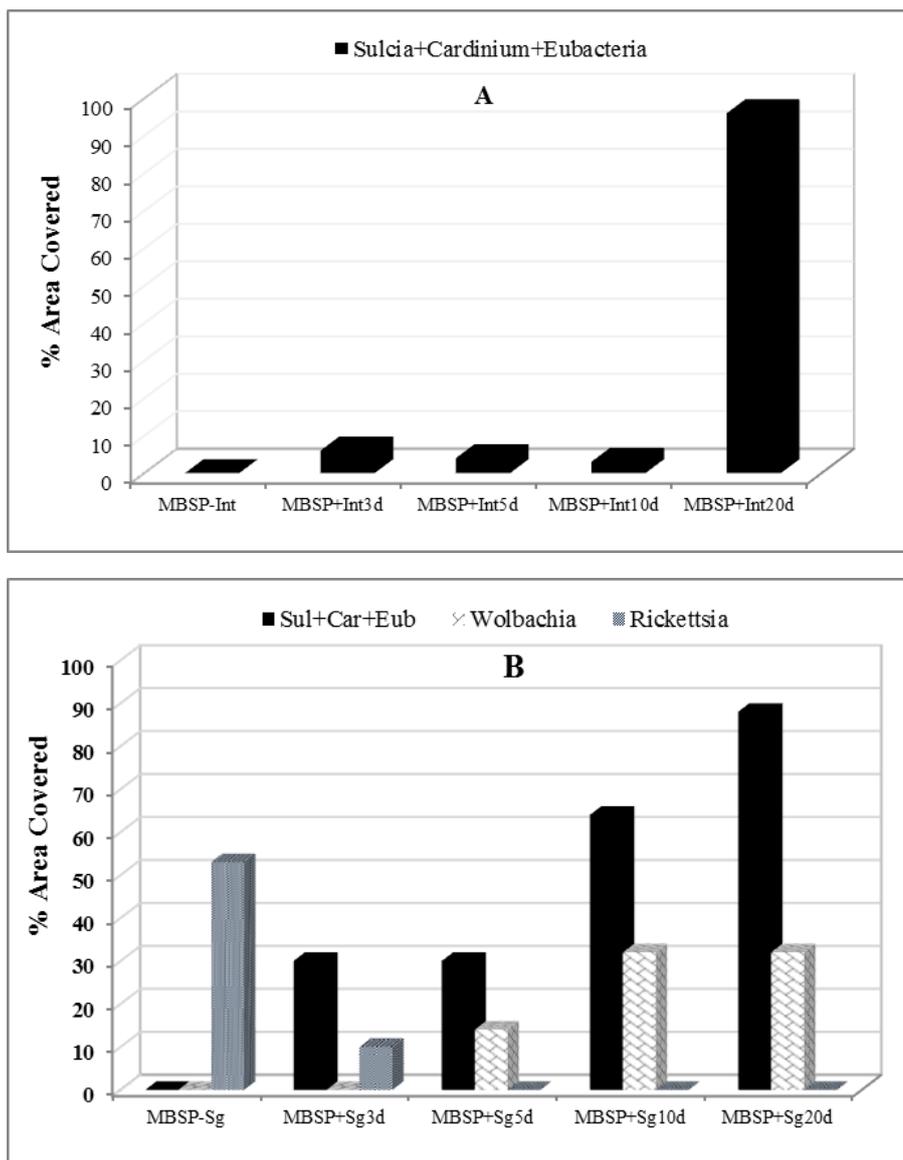


Figure 3 - Abundance measured as covered area (%) of the endosymbionts like bacteria identified in intestine (*Int*) and salivary gland (*Sg*) dissected from *D. maidis* adults without (MBSP-) and with the phytoplasma MBSP (MBSP+) at four latent periods (3d, 5d, 10d, 20d). **A-** Abundance of the bacteria Sulcia+Cardinium+Eubacteria identified through FISH in the *D. maidis* intestine. **B-** Abundance of the bacteria Sulcia+Cardinium+Eubacteria, Wolbachia and Rickettsia identified through FISH in salivary gland without and with the phytoplasma MBSP

On the other side, *Wolbachia* was observed in treatments with presence of MBSP showing a positive relation in the bacteria abundance 1%, 14%, 32% and 32% of covered area with increases of the phytoplasma latent period (3, 5, 10 and 20d respectively) (Figure 3B). Replication 2 of the experiment made for intestine and salivary gland of *D. maidis* with presence of the phytoplasma MBSP at 3, 5, 10 and 25d evidenced signal for the bacteria

*Sulcia*, *Cardinium*, and the phytoplasma MBSP (group I). *Rickettsia* did not evidenced signal in the mentioned structures (Figure 4).

Intestine and salivary gland of *D. maidis* with MBSP+ showed a positive relation between the increased incubation time resulted and the incidence of the bacteria *Sulcia*, *Cardinium*, and the phytoplasma MBSP (Table 1). The presence of MBSP was detected in intestine since 5d of MBSP+ incubation time, with detection in all the salivary gland samples evaluated.

When evaluated the same structures from health adults at 3, 5, and 10d after the test initiation, results did not evidence signal for any bacteria at any disease incubation time in intestine. In salivary gland a light signal was observed for the endosymbiont *Cardinium* at 10d of disease incubation time.

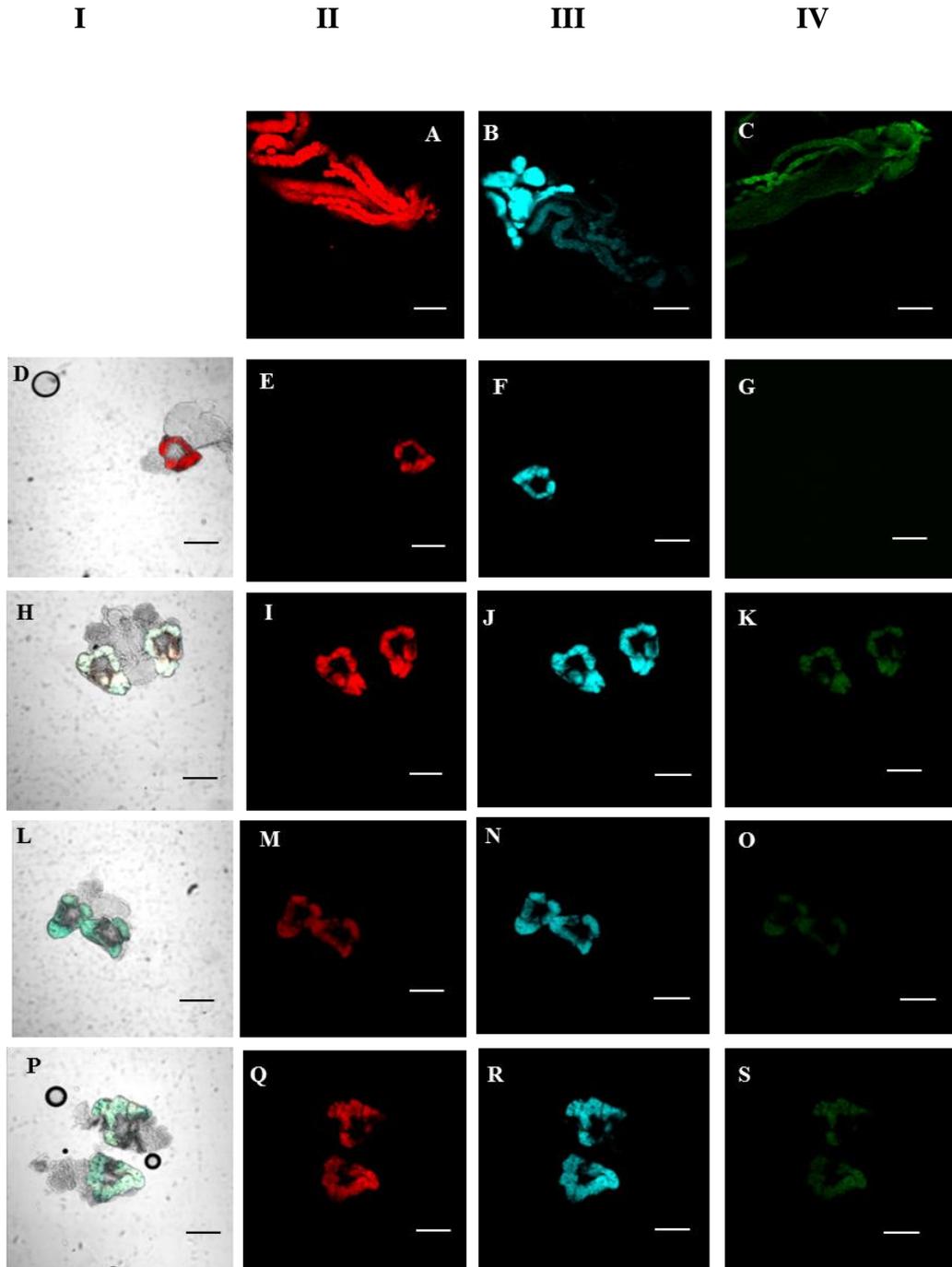


Figure 4 - Intestine and salivary gland samples of *D. maidis* adults processed in replication 2 by FISH technique showing signal for the bacteria like endosymbiont presence in the samples previously infected with the MBSP at different latent periods (LP). A-B-C) MBSP+ Intestine LP= 20d D-E-F-G) MBSP+ salivary gland LP= 3d. H-I-J-K) MBSP+ salivary gland LP= 5d. L-M-N-O) MBSP+ salivary gland LP= 10d. P-Q-R-S) MBSP+ salivary gland LP= 25d. In columns can be observed: I, Mixed image with bacteria signals. II: Images showing signal for *Sulcia* (red) III: Images showing signal for *Cardinium* (blue) IV: Images showing signal for the phytoplasma MBSP (green). Line= 100  $\mu$ m.

Images: J. García González

Table 1 - Detection of the endosymbionts *Sulcia*, *Cardinium* and *Rickettsia* as well as the phytoplasma MBSP with the FISH technique on intestine and salivary gland of *D. maidis* at different disease incubation times. Data obtained for the experiment replication two made at Brazil

Vector Organ	MBSP latent period into the vector	FISH positive Signal / total samples			
		<i>Sulcia</i>	<i>Cardinium</i>	<i>Rickettsia</i>	MBSP+
Intestine	3d	2/4	0	0	0
	5d	4/4	1/4	0	3/4
	10d	6/6	3/6	0	5/6
	25d	5/5	4/5	0	5/5
Salivary gland	3d	5/5	5/5	0	5/5
	5d	5/5	5/5	0	5/5
	10d	5/5	5/5	0	5/5
	25d	5/5	5/5	0	4/5

## 5.4 Discussion

### 5.4.1 *D. maidis* microbiota identification and localization

For the first time is registered the *D. maidis* microbiota coming from an adult mass rearing maintained in greenhouse conditions, with the primary endosymbionts *Ca.Nasuia deltocephalinicola* and *Sulcia* sp. Were identified by PCR-DGGE and FISH techniques respectively. The last bacterium is considered the ancient endosymbiont associated with Auchenorrhyncha and Sternorrhyncha suborders (MORAN; TRAN; GERARDO, 2005; NODA et al., 2012; WANGKEEREE; MILLER; HANBOONSONG, 2012).

The co-symbiosis of *Sulcia* sp. with other primary endosymbionts presumed since the early cicadomorpha origins has been demonstrated with the primary endosymbionts *Nasuia* and *Zinderia*, as a mechanism to complement the dietary nutrients lacked by the plant food to the insects (KOGA et al., 2013; BENNET; MORAN, 2013). In a *D. maidis* relative species the leafhopper *Macrostoteles quadrilineatus*, amino acid synthesis by the symbionts of *M. quadrilineatus* is achieved by the conserved complementation pattern by *Sulcia*-ALF

synthesizing 8 of the 10 amino acids required by the host, whereas *Nasuia*-ALF synthesizes the remaining 2 amino acids (MCCUTCHEON; MORAN, 2010).

FISH and PCR-DGGE techniques identified the secondary endosymbionts *Wolbachia*, *Cardinium*, *Rickettsia*, *Burkholderia picketti*, and *Delftia tsuruhatensis*. The genus *Wolbachia* is prevailing among 40 to 70% of the insect species, and is known for causing various phenotypic effects on their hosts, including parthenogenesis induction, feminization, cytoplasmic incompatibility, and male killing (SANCHEZ-CONTRERAS; VLISIDOU, 2008; MORAN; MCCUTCHEON; NAKABACHI, 2008). *Cardinium* and *Rickettsia* also modify insect host reproduction patterns to facilitate their own spread and in situations where simple fitness effects are extended into actual manipulation with consequences subtle and complex (ALMA et al., 2010; CLARK et al., 2010).

Other bacteria joining spaces with the phytoplasma MBSP in both insect structures were *Agrobacterium* sp., *Ralstonia* sp., *Delftia tsuruhatensis*, *Staphylococcus* sp., *Microbacterium* sp., *Acidovorax wautersii*, *Acidovorax soli*, *Curtobacterium* sp. They are shown as opportunistic bacteria present in maize plants and probably are absorbed with the phytoplasma at the moment of *D. maidis* sieve consumption.

On this respect, it is assumed that many species derive their microbiota from the surrounding environment such as the phylloplane of food plants or the skin of the animal host, but the degree of persistence of strains of the ingested species is unknown with the distinction if a microbial species is able to colonize the gut habitat (DILLON AND DILLON, 2004).

Of importance the signals for *Delftia tsuruhatensis* and *Curtobacterium* observed in all salivary gland and intestine samples with the phytoplasma MBSP. Studies with *D. tsuruhatensis* as well as *Burkholderia* have shown their role as growth-promoting bacteria in rice (HAN et al., 2005) and maize root plant systems respectively (BEVIVINO et al., 1998), but interestingly *Burkholderia* has been registered in association with stinkbugs of the superfamilies Lygaeoidea and Coreoidea as specific and beneficial symbiotic bacteria localized in a specific region of the posterior midgut facilitating the growth and reproduction of the host insects (KIKUCHI; MENG; FUKATSU, 2005; KIKUCHI; HOSOKAWA; FUKATSU, 2007, 2011). Detailed studies of the *D. maidis* identified microbiota should determine its role and potential symbiosis with the phytopathogenic transmission.

The *D. maidis* microbiota identified in the present study was closed related with the microbiota recently identified in Japan for two *Macrosteles* species vectoring phytopathogenic phytoplasmas (ISHI et al., 2013). This fact allows to presume a specific

relationship between the type of the transmitted phytopatogenic agent and the vector microbiota and interaction for facilitating the phytopathogen transmission.

As previously showed by MET images, some bacteria observed in the *D. maidis* midgut infected with the phytoplasma were observed in the root system of maize plants infected with MBSP. This finding allows to assume that the vector would acquire or release other bacteria than the phytoplasma with the insect feeding on infected plants or from individuals infected with the phytoplasma and accompanying bacteria.

#### **5.4.2 *D. maidis* microbiota variation as MBSP phytoplasma presence at different LP using FISH technique**

Vector microbiota variation measured as abundance and diversity was evaluated in intestine and salivary gland of *D. maidis* infected with the phytoplasma MBSP using FISH technique for identification of *Sulcia* (S1150), *Cardinium* (card172 and card1069), *Wolbachia* (W1), *Rickettsia* (Rox), Phytoplasma group II (ph1107), *Purcellielia* (P820) and Eubacteria (EUB338).

Results of experiments realized at Italy and Brazil showed a direct effect of phytoplasma latent period within the vector on both the abundance and diversity of the microbiota accompanying the phytoplasma, with a higher endosymbiont diversity joining the phytoplasma spaces in the salivary gland. In this organ was confirmed signal for *Sulcia*, *Cardinium*, Eubacteria, *Rickettsia* and *Wolbachia* joining the phytoplasma spaces meanwhile for intestine was determined signal just for *Sulcia*, *Cardinium*, or Eubacteria. FISH technique allowed to identify the presence of the primary endosymbiont *Sulcia*, broadly related with leafhoppers of the Deltocephalinae group (BENNET AND MORAN, 2013).

Presence of the endosymbiont *Cardinium* in salivary gland was observed in *Scaphoideus titanus* (SACHI et al., 2008) vector of phytoplasma doree in grapevines, suggesting a metabolic role and giving the chance of a possible transmission to the plant during feeding behavior and confirming its widespread apparition in different insects and other arthropods.

As observed with the MET technique, the phytoplasma MBSP is joining spaces with some bacteria like endosymbionts in a probably synergy for the disease transmission. The increasing amount of endosymbiont presence as a result of the increasing MBSP titer in the

analyzed structures is a remarkable evidence of the interaction between the phytoplasma and insect microbiota, a fact poorly understood for the disease transmission of phytopathogens vectored by pest insects.

An identification of the microbiota for the leafhoppers *Macrostelus striifrons* and *M. sexnotatus* (Hemiptera: Cicadellidae) vectors of *Candidatus* Phytoplasma asteris and *Ca.* Phytoplasma Orizae in Japan (ISHII et al., 2013), exhibited similarities with the microbiota found in this study for *D. maidis* vector of Maize Bushy Stunt Phytoplasma in the American continent. The leafhopper species shared the two co-primary endosymbionts *Sulcia muelleri* and *Nasua deltochepalinicola* and the secondary endosymbionts *Wolbachia* and *Rickettsia*. In healthy *D. maidis* intestine and salivary gland was observed lightly signal for the bacteria *Cardinium* and *Rickettsia*, this last bacteria replaced with the endosymbiont *Wolbachia* when the phytoplasma MBSP began to invade the salivary gland.

*Wolbachia* (WERREN; BALDO; CLARCK, 2008) as well as *Rickettsia* and *Cardinium* (SACHI et al., 2008) are a well-known bacteria modifying insect sexual patterns, but its presence in salivary gland is probably related with any phytopathogen synergy and movement to the plant, as mentioned for the leafhoppers *Hishimonoides sellatiformis* and *Hishimonus sellatus*, vectors of mulberry dwarf- Phytoplasma (MITSUHASHI et al., 2002).

Overall the results showed strong evidences of the synergy existing between the phytoplasma MBSP and some of the *D. maidis* endosymbionts, opening the option to investigate in detail the specific endosymbionts interacting with the phytopathogen and their role in the phytopathogen transmission when the phytopathogen is present within its vector.

## 5.5 Conclusions

For the first time the *D. maidis* microbiota is identified consisting on the primary endosymbionts *Sulcia* sp. and *Nasua deltochepalinicola*, as well as the secondary endosymbionts *Cardinium*, *Rickettsia*, *Wolbachia* and *Delftia tsuruhatensis*. Some other microorganisms identified as *Ralstonia*, *Rhizobium*, *Acetobacter* spp. and *Lactobacillus* are probably present in the maize plant with an interesting uptake at the moment of the vector consumption and with a potential benefit to the phytopathogenic transmission. An evident effect of the latent period for the phytoplasma MBSP on both abundance and diversity of *D. maidis* endosymbionts at the physiological barrier organs required to be ultrapassed by the phytoplasma within the vector. For the first time the synergy of the insect microbiota with the

phytoplasma is demonstrated strongly suggesting for future studies on the role of each one of these microorganisms within the insect vector.

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