HOST RESISTANCE AND DIVERSITY OF

SPIROPLASMA KUNKELII AS COMPONENTS OF

CORN STUNT DISEASE

By

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CHAPTER I

INTRODUCTION

Corn stunt is one of the most important maize diseases in America (Bradfute et al. 1981, Bajet and Renfro 1989, Nault 1990). Since it was first described (Alstatt 1945, Frazier 1945) its prevalence and economic impact have increased both in geographical range and incidence during recent years (Bradfute et al. 1981, Power 1987, Nault 1990, Hruska et al. 1996). This disease has been correlated in field with the presence of three different pathogens, found either alone or in combination: the mollicutes *Spiroplasma kunkelii* and maize bushy stunt phytoplasma (MBSP) (Nault 1980), and the maize rayado fino virus (MRFV) (Gamez 1973). However, disease symptoms were most commonly related to the presence of *S. kunkelii*, which is considered as the most important pathogen causing corn stunt throughout the Americas (Bajet and Renfro 1989).

S. kunkelii is one of three known phytopathogenic spiroplasmas, together with *S. citri* and *S. phoeniceum* (Fletcher et al. 2005); all of them placed in the Spiroplasmataceae family in the class Mollicutes. *S. kunkelii* is transmitted from plant to plant in a propagative manner by some leafhopper (Hemiptera: Cicadellidae) species, mainly by the corn leafhopper, *Dalbulus maidis* (Kunkel 1946). The host range of this insect in nature consists of some *Zea* spp, particularly maize (*Zea mays*) (Nault 1980). Both the geographical range and the length of the growing season of this crop have been increasing, so mor insects harbor the pathogen, and the disease is becoming more prevalent in the field (Hruska et al. 1996, Summers et al. 2004).

One of the most effective ways to reduce disease losses is through the development of resistant crops (Hogenboom 1993, George et al. 1997, Bouzar et al. 1999). Plant resistance is an environmentally friendly alternative for disease control and is compatible

with other integrated pest management components (Sauge et al. 2002). Resistance minimizes negative impacts on non-target organisms (Saxena 1987) and creates a highly favorable ratio between financial input and return. In addition, because genes for different mechanisms of resistance can be introduced into the same host genotype, resistant genotypes obtained using this method could be highly stable (Hogenboom 1993).

Host resistance can be directed to either the insect or to the pathogen. In the case of other insect-transmitted pathogens, insect-resistant plant genotypes showed lower disease incidence than those lacking such mechanisms (Saxena 1987, Rezaul Karim and Saxena 1991, Jimenez-Martinez et al. 2004). However, the role of *D. maidis* behavior in determining maize resistance is not clear. For instance, while some research showed clearly that insect density was reduced on resistant genotypes compared to susceptible ones (Power 1988), other studies found no relationship between insect preference and host resistance to disease (Collins and Pitre 1969, Hruska et al. 1996). Although the latter findings would suggest that these host genotypes might be pathogen- rather than insect-resistant, no definitive studies have been performed to characterize the target organism or the mechanism of resistance.

As the pathogen *S. kunkelii* is transmitted during leafhopper probing, the extent of probing time in phloem sap has a central significance on acquisition and transmission. Indeed, the efficiency of both events rises from about 20 to 80% if the time of insect-plant contact increases from 1 to 48 h, respectively (Alivizatos and Markham 1986). Since *S. kunkelii* is located in phloem and is thought to be unable to transverse plant cell walls (Fletcher et al. 2005), spiroplasmas will be either acquired or inoculated when the insect probes in phloem. In other plant-disease systems, modification of insect probing behavior reduces the chance of pathogen transmission, because probing time in phloem is greatly reduced (Saxena 1987, Rapusas and Heinrichs 1990, Rezaul Karim and Saxena 1991). At present, no studies have been performed to relate probing activity of *D. maidis* and transmission of *S. kunkelii*, nor the role that the insect-plant interaction would have in pathogen transmission.

Success of plant resistance relies on the inability of the pathogen to circumvent such mechanisms of resistance and attack the host (Burdon 1993, George et al. 1997).

Although corn stunt resistant maize genotypes have been obtained by selecting healthy plants in fields affected by corn stunt (Scott and Rosenkranz 1974, 1975, Jeffers 2002), such genotypes have usually been found susceptible in other geographical locations or after a certain period of time (Silva et al. 2003). With other diseases caused by bacteria, the processes of microbial mutation, geographical migration and recombination contribute to generate genetic variation that may lead to pathogenicity on formerly resistant genotypes (Louws et al. 1994). Indeed, this resistance "breakdown" may be caused by two kinds of pathogen strains: those originating in other geographical locations and those that evolved in response to the deployment of formerly resistant germplasm (Leach et al. 1995, George et al. 1997, Bouzar et al. 1999).

At present, little is known about the existence of pathogenic or geographic variants of *S. kunkelii*, in part due to the lack of effective tools for strain discrimination. However, molecular resources recently available, like the genome sequence of *S. kunkelii*, may provide alternate approaches to evaluate spiroplasma variability. Knowledge of natural pathogen population diversity and possible occurrence and distribution of strains would be important in understanding pathogen-host relationships and designing effective plant breeding strategies.

The objectives of this research are to evaluate aspects of the vector and pathogen that may influence plant resistance to the disease:

1 - To determine life history parameters of *D. maidis* on maize hybrids differing in susceptibility to corn stunt in the field.

2 - To analyze probing behavior of *D. maidis* on maize hybrids differing in susceptibility to corn stunt.

3 - To determine natural genomic variability of *S. kunkelii* in local and regional populations.

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CHAPTER II

REVIEW OF LITERATURE

I - Economic importance

Corn stunt is one of the most important maize diseases in America (Bradfute et al. 1981, Bajet and Renfro 1989, Nault 1990). Since the disease was first described (Alstatt 1945, Frazier 1945) its prevalence and economic impact have been considerable (Stoner 1964, Summers et al. 2004). Losses of up to $60 million have been reported in Florida (Bradfute et al. 1981), and in other countries disease outbreaks have caused loss of entire maize crops (Power 1987, Nault 1990, Hruska et al. 1996).

Corn stunt prevalence has been increasing in recent years, due to several crop-management practices and circumstances. These include: expansion in maize producing areas, improper cultural practices, and lack of maize germplasm resistant to the disease (Hruska et al. 1996, Summers et al. 2004). As an example, in the United States, Nicaragua and Brazil the increase in disease incidence is due to the introduction of technologies that allow maize farming almost year round (Hruska et al. 1996, Summers et al. 2004).

II - Disease components

II-a -Pathogen

Three different phloem-restricted pathogens may cause corn stunt: the mollicutes *Spiroplasma kunkelii* and maize bushy stunt phytoplasm (MBSP) (Nault 1980), and the maize rayado fino virus (MRFV) (Gamez 1973). Although careful symptom examination can help to determine the actual causal agent, presence of combinations of pathogens in

the same plant and interaction with environment and host plant (Nault 1980) can make diagnosis of the actual causal agent of the disease difficult. The mollicute *S. kunkelii* is the most important of these three pathogens because of its widespread distribution in different countries of the Americas (Bradfute et al. 1981, Bajet and Renfro 1989).

S. kunkelii is one of three phytopathogenic spiroplasmas known, together with *S. citri* and *S. phoeniceum* (Fletcher et al. 2005). It is found in the phloem sap of maize plants and in the hemolymph of leafhopper insects, where it multiplies to high titers using the nutrients that these tissues provide (Hackett 1990). Koch's postulates were fulfilled (Chen and Liao 1975, Williamson and Whitcomb 1975), showing the etiologic agent to be a wall-less prokaryote, designated corn stunt spiroplasma (CSS). Later, the binomial name *Spiroplasma kunkelii* Whitcomb was designated (Whitcomb et al. 1986), and the microbe was placed in the family Spiroplasmataceae and class Mollicutes of bacteria. Mollicutes are among the smallest and simplest autonomously living organisms (Dybvig and Voelker 1996). They evolved from Gram-positive bacteria through a continuous process of gene loss, relying on many of the anabolic functions in the host (Hackett 1990). Because many of the nutrients they need are provided by the environment that they inhabit, they posses many proteins with catabolic and degradative functions, and fewer anabolic ones (Dybvig and Voelker 1996).

One of the prominent characteristics of the spiroplasmas is their external morphology. They have lost the cell wall typical of bacteria during evolution; hence they are surrounded only by a cell membrane (Davis et al. 1972). Instead of a cell wall, spiroplasmas have an internal cytoskeleton composed of fibrils located immediately under the cell membrane (Trachtenberg 1998). This cytoskeleton contributes to two other typical features of the genus: shape and motility. Spiroplasmas are helical, about 3-15 µm long and 0.2-0.5 µm in diameter, with a regular gyre of 0.4 µm (Davis and Worley 1973). Spiroplasmas use this cytoskeleton to move along chemical gradients (Trachtenberg et al. 2003), which may be a factor in their accumulation in young plant tissues and in insect salivary glands (Daniels et al. 1980).

Spiroplasmas share some genetic characteristics with other mollicutes. For instance, a gradual replacement of G+C to A+T base pairs leads to G+C contents as low as 25-29

% (Dybvig and Voelker 1996, Bentley and Parkhill 2004). A second unusual feature of spiroplasmas is that they read UGA as a tryptophan rather than as a stop codon during translation (Dybvig and Voelker 1996). Additionally, the spiroplasmal genome is augmented by DNAs in addition to the circular chromosome: viruses and plasmids (Mouches and Bove 1983, Ye et al. 1996, Melcher and Fletcher 1999, Melcher et al. 1999). These DNAs usually become integrated into the chromosome in one or more copies, either intact or fragmented (Mouches and Bove 1983, Ye et al. 1996, Melcher et al. 1999, Sha et al. 2000, Bai and Hogenhout 2002).

As in other bacteria, the spiroplasmal genome may be seen as consisting of two parts: the core genome and the accessory genome (Hacker and Kaper 2000). The core sequences encode structures necessary to maintain metabolism, and are consequently greatly conserved. On the other hand, the accessory genome encodes non-vital functions such as antibiotic resistance, microbial fitness, symbiosis, pathogenesis and/or generation of variability (Hacker and Kaper 2000, Hacker and Carniel 2001), and thus is more prone to variation. The spiroplasmal genome may change at high rates, with variations up to 30 % in the genome size in *S. citri* (Carle et al. 1995, Ye et al. 1996, Melcher and Fletcher 1999). Changes in DNA sequence may be divided into three types: small changes in DNA sequence due to failures in DNA replication or mismatch repair, displacement or duplication of DNA fragments along the chromosome, or DNA exchange with other cells.

Small changes in DNA sequence include nucleotide changes or substitutions, small deletions or insertions of one or a few nucleotides (Melcher and Fletcher 1999). This mutation rate is high in mollicutes due to the loss of DNA repairing mechanisms during evolution (Dybvig and Voelker 1996). Although quantitatively small, these mutations may alter gene expression and phenotype. For instance, the resistance of mycobacteria to the antibiotics rifampicin and streptomycin is caused by point mutations in the RNA polymerase and ribosomal genes, respectively (Ziebuhr et al. 1999).

A second mechanism is a rearrangement of genomic DNA sequences by duplications, deletions, insertions, inversions and recombination of DNA segments (Robertson and Meyer 1992, Dybvig and Voelker 1996, Melcher and Fletcher 1999). In

S. citri, viruses and plasmids integrate into the spiroplasma genome, and consequently alter DNA sequence (Nur et al. 1986, Nur et al. 1987, Melcher and Fletcher 1999, Sha et al. 2000).

The third mechanism, cell-to-cell (horizontal) gene transfer, results in the acquisition of large foreign DNA sequences, primarily of the accessory genome (Hacker and Kaper 2000). This strategy of DNA acquisition may result in new capacities in a single step of acquisition and allow a spiroplasma strain to explore new ecological niches (Hacker and Carniel 2001). The mobile elements are usually flanked by small insertion sequences, which are recognized by recombinases, causing DNA rearrangements (Barroso and Labarere 1988, Hacker and Kaper 2000).

In the only strain of *S. kunkelii* sequenced so far, CR2-3X, several features might indicate active mechanisms of genomic variability. For example, DNA sequences of viral origin (Melcher et al. 1999, Bai and Hogenhout 2002) and genes for DNA horizontal movement (Bai et al. 2004, Davis et al. 2005) have been found in *S. kunkelii*, and may be valuable tools to identify not only the generation of variability, but also changes in phenotype, including relationships with the insect and plant hosts.

II-b - Insect Hosts

S. kunkelii is transmitted by leafhopper insects (Hemiptera: Cicadellidae), and not through mechanical contact or through seeds (Kunkel 1946). This may be due to the fact that the spiroplasmas colonize phloem sap (Davis and Worley 1973), and this tissue is accessible only to microorganisms introduced by inoculative phloem-feeding insects (Tonkyn and Whitcomb 1987).

The principal field vector of *S. kunkelii* is *Dalbulus maidis* (Kunkel 1946), although the related species *D. elimatus* (Nault 1980) and *D. guevarai* (Ramirez et al. 1975) are also natural vectors. In addition, other leafhopper species able to transmit the pathogen in controlled conditions include *Graminella nigrifrons*, *Exitianus exitiosus*, *Stirellus bicolor* (Nault 1980), *Euscelidius variegatus*, *Macrosteles sexnotatus* and *Cicadulina mbila* (Markham and Alivizatos 1983). Of all these species, *D. maidis* is the most important epidemiologically due to its ample geographical distribution, from the southern United

States to Argentina (Nault 1990), and to its high efficiency of transmission (Alivizatos and Markham 1986b, Nault 1990).

II-c - Plant Hosts

The plant species affected by this pathogen in natural conditions belong to the genus *Zea*, including maize (*Z. mays* L) and the teosintes: *Z. diploperenis*, *Z. perennis*, *Z. mays* x *Tripsacum floridanum* and *Z. luxurians* (Nault 1980). Plants of the closely related genus *Tripsacum* are apparently immune to spiroplasma infection (Nault 1980). Dicotyledonous plants *Catharanthus roseus* and *Vicia faba* (Markham et al. 1977) are hosts of *S. kunkelii* under controlled conditions. Infected *C. roseus* developed symptoms equivalent to those on maize: stunting, leaf chlorosis, loss of apical dominance, increase in flower number and reduction in flower size (Markham et al. 1977, Daniels 1983).

D. maidis adults have been collected in the field from several mono- and dicotyledoneous plants in addition to *Zea* and *Tripsacum* species, including *Triticum aestivum*, *Solanum* spp (Larsen et al. 1992, Summers et al. 2004), *Avena fatua*, *Sorghum halepense*, *Hordeum vulgare*, *Rottboelia* spp, *Callistephus chinensis*, *Apium graveolens*, *Medicago sativa* (Summers et al. 2004), *Avena sativa* and *Sorghum bicolor* (Virla et al. 2003). However, in an exhaustive study of the host range of *D. maidis* (Pitre 1967), this leafhopper could be reared only in species of the Andropogoneae tribe (Gramineae), and nymphs could reach adult stage only in corn and teosintes.

II-d - Insect-Pathogen interaction

D. maidis transmits *S. kunkelii* in a propagative manner, and individuals acquiring the pathogen remain infectious during their entire lifespan (Nault 1980). When leafhoppers feed on diseased plants, phytopathogenic spiroplasmas accumulate in the gut and reach the hemolymph by passing through cells of the midgut epithelium (Kwon et al. 1999, Ozbek et al. 2003). Spiroplasmas multiply in the hemolymph and reach the ducts of salivary glands by passing through the gland cells, to be deposited in other plants when the insect salivates during probing (Alivizatos and Markham 1986a, Wayadande and Fletcher 1995, Kwon et al. 1999). This movement and multiplication of the spiroplasmas

inside the body of the insects takes about 17-25 days, after which the insect is able to transmit the pathogen (Davis 1974, Nault 1980, Alivizatos and Markham 1986b).

Both acquisition and inoculation are dose-dependent events. Indeed, when the extent of both acquisition and inoculation access period increases from 0.5 h to seven days, transmission efficiency increases progressively, reaching values near to 100%. Moreover, longer acquisition periods increase the initial dose of spiroplasmas reaching the insect hemolymph, and consequently the incubation period is shorter (Alivizatos and Markham 1986b).

The effect of spiroplasma multiplication upon the lifespan of different leafhopper species also affects their transmission efficiency. For instance, the lifetime of leafhopper species other than *D. maidis* is reduced when they acquire the spiroplasmas (Madden et al. 1984). For this reason, while *D. maidis* individuals transmit *S. kunkelii* with efficiencies near to 100%, leafhoppers of other species die prematurely, often before the spiroplasmas could reach the salivary glands, and consequently have a low transmission rate, from 9 to 38% (Madden and Nault 1983, Madden et al. 1984).

II-e - Insect-Host Interaction

The life cycle of *D. maidis* is synchronized to maize ontogeny (Gamez and Saavedra 1986, Nault 1990). When maize is not present in the field during the winter, adults take refuge nearby, in not well-known microhabitats (Nault 1990, Virla et al. 2003). In spring, young maize plants are colonized almost as soon as they emerge by adults of *D. maidis*, mainly inoculative females (Larsen et al. 1992, Virla et al. 2003, Summers et al. 2004), responsible for the initial spread of the disease. As the maize grows, the spiroplasma concentration increases in the phloem (Gussie et al. 1995), and subsequent generations of *D. maidis* acquire and spread it within the field (secondary spread).

When the maize matures, it becomes a less-suitable host for insect feeding, probably due to reduction in nitrogen content in leaves. Therefore, the leafhoppers migrate to find younger, nitrogen-rich maize plants if any are present (Larsen et al. 1992). On the other hand, those that remain in the mature plant develop adaptations to survive winter until the next growing season, such as the synthesis of cryoprotectant compounds (Larsen et al.

1992, Larsen et al. 1993). As *D. maidis* has a high rate of population increase (Nault and Madden 1985, Madden et al. 1986), many insects are available to act as a primary inoculum source for late-seeded maize, accounting for the high disease incidence in such plantings (Hruska et al. 1996).

Both the number of inoculative leafhoppers entering a maize field and the rate of insect population increase (Power 1987) are factors influencing the number of plants that will be infected and the rate of disease spread during the season. According to Saxena (1969), both events can be described as a sequence of stages. The first step is leafhopper orientation toward the plant. If this stimulus is positive, the interaction will continue successively with feeding, assimilation of ingested food, weight gain, development, and oviposition. Consequently, the more suitable a plant is as a host to insects, the more extended in time will be this interaction (Saxena 1969, 1985).

Several plant characters can elicit an insect response at a distance; these include visual (shape, color), hygro (water, vapor) and olfactory (volatiles) stimuli (Saxena 1969, 1985). Attraction of migrating *D. maidis* adults to young maize plants is triggered by a positive interaction of visual and chemical cues, as the insects are attracted by the shape and color of young maize tissues (Todd et al. 1990a), and when they are near the plant, such attraction is reinforced by the detection of plant volatiles (Todd et al. 1990b). In addition, the high water content of young plants early in the season could also be involved in the attraction (Saxena 1969) because leafhoppers have essentially a liquid diet (DeLong 1971).

Probing begins almost immediately after the contact with maize seedlings, within about 2-3 minutes (Wayadande and Nault 1996), suggesting that gustatory and/or mechanical stimuli perceived during feeding reinforce leafhopper attraction to the host (Todd et al. 1990a). If the plant is acceptable as a food source, the insect will continue feeding; otherwise it will depart to find a more suitable host (Saxena 1969, 1985).

The corn leafhopper, *D. maidis*, feeds primarily on phloem sap (Wayadande and Nault 1996), like other cicadellids of the subfamily Deltocephalinae (Rapusas and Heinrichs 1990, Lett et al. 2001). To obtain nutrients, the insect must introduce its stylets into plant tissues, a process called probing (Backus 2000). Stylets pierce the leaves,

moving inside an insect-secreted salivary sheath that gels rapidly as the stylets penetrate, lubricating the contact surfaces between insect and plant (Tonkyn and Whitcomb 1987, Walker 2000). Once the stylets reach phloem sieve tubes, they release watery saliva to inhibit the wound response of the plant, which would otherwise reduce ingestion (Walker 2000, Lett et al. 2001).

Leafhoppers can probe not only in phloem, but also in other plant tissues (Saxena and Khan 1985, Rezaul Karim and Saxena 1991, Wayadande and Nault 1996), and the latter is increased when insects feed on an unsuitable host (Campbell et al. 1982, Rapusas and Heinrichs 1990). Unsuitable plant species or genotypes have resistance mechanisms that allow them to avoid insect infestation. This plant resistance to insects may be composed of two elements: antixenosis and/or antibiosis (Saxena 1969).

In antixenosis, the plant is a non-preferred host for insects, either for orientation (attraction), feeding or oviposition (Saxena 1969). In these non-preferred plants, feeding is limited or non-existent, and insects emigrate to find a more suitable host (Power 1988, Sauge et al. 1998a). Antixenosis is an interesting resistance mechanism because it decreases the contact time between plant and insects, what probably would reduce the efficiency of pathogen transmission. This mechanism would be particularly effective in reducing the number of insects bringing the primary inoculum.

Antibiosis occurs after the plant has been colonized by insects, and is characterized by reduced feeding, development, survival and reproduction (Saxena 1985, Sauge et al. 1998a). As a consequence of alimentary constraints, lifespan and offspring number are significantly reduced (Saxena 1969, Jimenez-Martinez et al. 2004). This would reduce the rate of population increase of insect vectors, in turn lessening the magnitude of secondary disease spread in the field and the number of adults migrating to other fields.

Both antixenosis and antibiosis may be caused by mechanisms differing in physiological origin and/or tissue localization. Some resistant plants synthesize toxins or deterrent components, or lack attractant molecules (Saxena 1969, Cole 1997). In other cases, these phenomena may be caused by the poor nutritional quality of the plant (Sauge et al. 1998b, Jimenez-Martinez et al. 2004). Also, resistance factors may be located in

different tissues transversed by the insect stylets: epidermis, mesophyll, or phloem (Helden and Tjallingii 2000).

The study of probing behavior using electrical penetration graph (EPG) technology can help to establish the mechanism and location, at the tissue level, of the traits conferring plant resistance. For example, using EPG it was found that leafhoppers exposed to resistant rice plants spend less time feeding from phloem than do those exposed to susceptible cultivars, decreasing the efficiency of transmission of the phloem-restricted rice tungro viruses (Dahal et al. 1990, Rapusas and Heinrichs 1990, Rezaul Karim and Saxena 1991). In addition, resistance factors can be located not only within phloem, but also at the tissues encountered successively, from the surface to the phloem sieve element, during probing (Walker 2000). Hence, EPG technology contributes to our understanding of the bases of plant resistance in pathosystems in which hemipteran vectors are involved (Helden and Tjallingii 2000), and can assist plant breeders to select and to characterize resistant genotypes (Wilkinson and Douglas 1998, Lett et al. 2001, Sauge et al. 2002).

The probing behavior of *D. maidis* was studied to analyze its efficiency as a vector of maize chlorotic dwarf virus (Wayadande and Nault 1993, 1996), but because of the low resolution limits of the EPG equipment available at that time and the short acquisition access periods provided, the results of that study do not completely explain the probing behavior of *D. maidis* as a vector of persistently-transmitted pathogens. Furthermore, no information is available about how this probing behavior differs in plants expressing different levels of disease resistance. Such information could provide a basis to identify the mechanism(s) and target(s) (either insect or pathogen) of disease resistance.

II-f - Pathogen-Host Interaction

Typical symptoms of *S. kunkelii* multiplication in maize are stunted plant growth due to upper internode shortening, chlorotic stripes extending from the base of the leaves to the tips (Kunkel 1946); and proliferation of ears in different nodes (Nault 1980). The symptoms are most severe when the plants are inoculated in early stages of ontogeny (Stoner 1964, Hao and Pitre 1970, Scott et al. 1977). However, both symptom

manifestation and severity may be noticeably modified by temperature, possible variants in the pathogen, and the host genotype (Nault 1980). For these reasons, correct identification of the etiological agent in the field may be difficult. Still more symptom confusion may occur due to the common existence of mixed infections with maize bushy stunt phytoplasma (MBSP) and maize rayado fino virus (MRFV), which sometimes cause symptoms resembling those caused by *S. kunkelii* infection (Bajet and Renfro 1989).

Some physiological mechanisms of spiroplasma pathogenicity have been proposed. Spiroplasma colonies may block phloem sieve tubes, impairing movement of metabolites from sources to sinks (Daniels 1983). As spiroplasmas lack many metabolic functions, they compete for –and deprive host cells of– some chemical substances (Bove et al. 2003). Indeed, plant stunting, chlorosis, and leaf size reduction could result in nutrient reduction caused by competition for sterols (Chang 1998) or fructose (Bove et al. 2003). Third, toxins or byproducts of spiroplasma metabolism could interfere with the host metabolism (Chang 1998). Finally, alterations in the plant host's hormonal balance such as an increase in abscisic acid concentration could lead to stunting, yellowing, stomatal closure and early leaf drop (Daniels 1983).

Maize genotypes showing resistance to corn stunt have been obtained (Scott and Rosenkranz 1974, 1975, 1977, Jeffers 2002). However, host resistance may be broken if the pathogen has a potential to generate new strains differing in antigenic properties or having enhanced pathogenicity (Burdon 1993). Indeed, field evidence suggests that some maize cultivars change dramatically their level of resistance in different geographic locations or different years, which suggests the possible presence of different pathogen strains (Silva et al. 2003). Nevertheless, the basis of the *S. kunkelii*-maize relationship is unknown, and it remains unclear whether the host is able to recognize the pathogen and to mount a defense from the pathogen attack (Fletcher et al. 2005).

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CHAPTER III

EVALUATION OF RESISTANCE TO *DALBULUS MAIDIS* IN MAIZE HYBRIDS

DIFFERING IN RESISTANCE TO CORN STUNT DISEASE IN THE FIELD

Abstract

Corn stunt is one of the most important diseases of maize (*Zea mays* L.) in the Americas. The causal agent, *Spiroplasma kunkelii*, is a mollicute transmitted by the corn leafhopper, *Dalbulus maidis*. Although resistant maize genotypes have been developed, no characterization has been done of this resistance. Antixenosis and antibiosis are two strategies of plants against insects that may serve as pathogen vectors. The aims of this study were to compare the levels, if any, of antixenosis and/or antibiosis to the vector *D. maidis* of two field-resistant and two susceptible maize hybrids, and to relate these phenomena to the vectors' efficiency of inoculation of the pathogen *S. kunkelii*. An antixenotic component, measured as *D. maidis* settling non-preference and oviposition rates, was not found. However, one resistant hybrid, X1297J, exhibited antibiosis as decreased nymphal survival and adult longevity. This hybrid could be useful in areas where maize is grown sequentially throughout the growing season, potentially reducing the number of vector insects migrating to late-seeded fields. In the resistant hybrid X1273A, however, no evidence for antibiosis or antixenosis was found. Inoculation efficiency was similar for all the genotypes evaluated, whether resistant or susceptible, indicating that resistance mechanisms would not prevent pathogen transmission once inoculative insects settled on the plant. However, our results cannot preclude the possibility that other mechanisms of host resistance to *D. maidis* could be expressed in field but not in controlled conditions.

Introduction

Corn stunt is one of the most important maize (Zea mays L.) diseases in America (Bradfute et al. 1981, Bajet and Renfro 1989, Nault 1990). Since it was first described (Alstatt 1945, Frazier 1945) its prevalence and economic impact have been considerable (Stoner 1964, Summers et al. 2004). Annual losses of up to $60 million have been reported in Florida (Bradfute et al. 1981), and in other countries disease outbreaks have caused loss of entire maize crops (Power 1987, Hruska et al. 1996).

The economic losses caused by corn stunt disease have increased in both geographical range and incidence during recent years. The main reasons for this increase are expansion of maize-producing areas, improper cultural practices, and lack of resistant maize germplasm (Hruska et al. 1996, Summers et al. 2004). As an example, in the United States, Nicaragua and Brazil continuous maize production provides a consistent, year-round food source for vector leafhoppers, supporting increased population numbers and consequently higher incidence of disease (Hruska et al. 1996, Summers et al. 2004).

Corn stunt disease is caused by the mollicute *Spiroplasma kunkelii* (Davis et al. 1972). This spiroplasma is transmitted by leafhoppers (Hemiptera: Cicadellidae) in a persistent-propagative manner, and individuals acquiring the pathogen remain infectious during their entire lifespan (Nault 1980). *Dalbulus maidis* is the most important vector of the pathogen due to its ample geographical distribution, from the southern United States to Argentina (Nault 1990, Summers et al. 2004), and to its high efficiency of spiroplasma transmission (Alivizatos and Markham 1986).

The obtention of genotypes resistant to corn stunt has been pursued as a mechanism to reduce the negative impact of the disease (Scott and Rosenkranz 1974, 1975, 1977). Plant host resistance is an environmentally friendly alternative for disease control, is compatible with other integrated pest management components, and minimizes negative impact on non-target organisms (Saxena and Khan 1989). In addition, because genes for different mechanisms of resistance can be introduced into the same genotype, this method can be highly stable (Saxena and Khan 1989). For instance, insect-resistant rice cultivars have been adopted to reduce damage of tungro disease (Saxena and Khan 1989).

Host resistance can be directed either to the insect, to the pathogen, or both. In the case of other leafhopper-transmitted pathogens, insect-resistant cultivars had lower disease incidence than did susceptible ones (Heinrichs and Rapusas 1983a, Costamagna et al. 2005). However, in the case of corn stunt, no information is available regarding the mechanisms of resistance, nor their effects upon pathogen transmission. Such knowledge would facilitate identification and characterization of existing and future maize genotypes with resistance to corn stunt.

Plant resistance against leafhoppers is composed of two elements: antixenosis and antibiosis (Saxena 1969). In antixenosis the plant is a non-preferred host for the insects, for orientation (attraction), feeding and/or oviposition (Saxena 1969, Saxena and Khan 1989). The insects emigrate from these non-preferred plants to find a more suitable host (Cheng and Pathak 1972, Heinrichs and Rapusas 1983a). As the efficiency of both acquisition and inoculation of spiroplasmas increases with time (Alivizatos and Markham 1986), the shorter period of contact between plant and insects caused by antixenosis would reduce transmission efficiency. Hence this mechanism would reduce the amount of primary inoculum by migrating leafhoppers.

Antibiosis, when present, occurs after the insects have settled on the plant, and may consist of reducing metabolism of ingested food or producing compounds toxic to the insects (Saxena 1985). These alimentary constraints significantly reduce the rate of population growth of the insect vectors (Saxena 1969, Kawabe 1985), probably lessening both the magnitude of secondary disease spread in the field and the number of adults migrating to other fields.

The hypothesis tested in this work was that antixenosis and/or antibiosis in maize hybrids resistant to corn stunt in field reduce the efficiency of *S. kunkelii* inoculation by *D. maidis*. The objectives were to assess whether specific maize cultivars found corn stunt-resistant in field showed resistance to the vector leafhopper, and, if so, to identify the nature of that resistance, and to relate the resistance to the efficiency of inoculation of the pathogen *S. kunkelii*.

Materials and Methods

I - Plants, insects, spiroplasmas and test conditions

Four maize (*Zea mays*, L) hybrids, developed in geographical areas of Brazil where corn stunt disease occurs, were tested. The hybrids, designated X1273A (7), X1E73 (1), X1286B (3) and X1297J (6), were provided by Pioneer Hi-Bred International, Inc. For each hybrid, the number in parentheses indicates the level of corn stunt field resistance, estimated on a scale of 1 to 9, with 1 being highly susceptible and 9 immune (highly resistant). In for some tests, hybrids were compared in pairs (X1273A-X1E73 and X1297J-X1286B), which included hybrids with a common ancestor. Sweet corn var. Golden Bantam served as a susceptible control. In all the experiments, plants were used at the two-leaf stage, since inoculation of *S. kunkelii* at this time results in high reductions of yield (Hao and Pitre 1970).

A colony of healthy *D. maidis* DeLong and Wolcott was initiated at Oklahoma State University in 1997 from insects obtained from L. R. Nault, Ohio Agricultural Research Development Center (Wooster, Ohio). The Ohio State colony was initiated from insects collected originally from Mexico, and was maintained on plants of sweet corn var. Golden Bantam. Insect maintenance was carried out in aluminum-framed cages (Wayadande and Fletcher 1995), placed in a growth room at a constant temperature of 26 °C, with a 16:8 h (light:dark) photoperiod (Nault 1980).

Spiroplasma kunkelii Whitcomb strain CR2-3X was used for inoculation tests. This strain was collected in Costa Rica (Castro et al. 1992), and cultured in LD8A3 medium (Lee and Davis 1989). A passage 9 from the original isolation was used.

Plants were seeded in 15 cm-diameter pots. When plants were used either singly or in pairs, the insects were confined in tubular lexan cages 20 cm in height and 12 cm in diameter, covered by a nylon mesh in the upper part. In the settling preference test, one plant of every genotype (five genotypes total) were seeded together in a 20-cm diameter pot, and the insects were confined in tubular cages 30 cm in height and 20 cm in diameter.

II - Variables measured

II-a - Settling Preference

Five, two-week old adult *D. maidis* mated females were caged on a single pot containing five maize plants, one of each test genotypes (four hybrids plus Golden Bantam). The number of insects settled on each plant was recorded at 1, 3, 8 or 24 hours after their release into the cage and expressed as a percentage of the total number of insects. The treatments were replicated ten times per time of recording, each pot being considered as a replication.

II-b - Ovipositional Response

In a free-choice test, groups of five gravid, two-week old *D. maidis* females were released into cages containing a pair of maize plants for a period of 5 days. Each pair (X1273A-X1E73 and X1297J-X1286B) included hybrids with a common ancestor, but being one resistant and the other susceptible. At the end of the test the insects were removed, the plants harvested, and the number of eggs deposited in the plant tissues counted with the aid of a dissecting microscope. Treatments were replicated ten times, each pot being considered as a replication. The results were expressed as number of eggs per female per day.

A no-choice test was also carried out. Experimental details were the same as in the free-choice test, except that each group of five insects had access to only one of the maize genotypes. Treatments were replicated fifteen times, each pot being considered a replication. The results were expressed as number of eggs per female per day.

II-c - Insect Survival

Survival of *D. maidis* nymphs was tested on each maize genotype by caging 20 second-instar nymphs on a single maize plant for a period of 20 days, when all the surviving insects had eclosed to the adult stage. At the end of the test, surviving insects were counted. Treatments were replicated ten times, each pot being considered a replication. The results were expressed as percentage of survival.

Survival of *D. maidis* adults was tested by caging groups of 10 two-week-old females or males with a single maize plant. Insect survival was recorded weekly for 7

weeks. Plants were replaced periodically to assure availability of fresh tissue and to eliminate plant deterioration as a potential cause of insect mortality. Treatments were replicated fifteen times, each pot being considered a replication. The percentage of surviving insects was plotted over time.

II-d - *S. kunkelii* Inoculation Efficiency

Nymphs of *D. maidis* at second- to third-stage acquired *S. kunkelli* by feeding in sachets made of two layers of Parafilm® membrane stretched over a 50 ml plastic cup. A solution (500 µl) of D10 medium (Alivizatos 1982) containing a suspension of *S. kunkelii* at a titer of about 10^8 cells/ml was placed between the membranes. The acquisition access period (AAP) was 24 hours. After 28 days of incubation on healthy maize plants (var. Golden Bantam), insects were sexed (insects eclosed to the adult stage) and caged singly, each on a single maize plant, for an inoculation access period (IAP) of either 1 or 5 days (Alivizatos and Markham 1986). At the end of the IAP the insects were removed, and the plants kept in a greenhouse for 60 days for symptom expression and spiroplasma detection by ELISA in the basal part of the uppermost leaf (Gussie et al. 1995). Plants testing negative in the ELISA test were retested by PCR using primers F2 and R6, which amplify a segment of the gene encoding spiralin (Barros et al. 2001), after extraction of nucleic acids extraction using CTAB (Doyle and Doyle 1994). Experiments were replicated three times using 30 plants in each replication. The results were expressed as percentage of test plants in which the pathogen *S. kunkelii* was detected.

III - Statistical Analyses

Results were analyzed with an analysis of variance (ANOVA) test, except for the free-choice ovipositional response, in which t-tests were performed for each pair of hybrids. Means were compared with Fisher's LSD test. Prior to ANOVA, percentages equal to zero were considered as 0.001, and the values of percentages were subjected to arcsine transformation.

Results

There was no difference (p=0.0686) in the settling preference of *D. maidis* females on genotypes differing in field resistance to corn stunt disease (Table III-1). There also

was a significant (p=0.0015) effect of time from the beginning of the test, but no interaction between genotype and time (p=0.7055). Not all the insects settled on plants at 1 and 3 hours after caging, but all of them were found on the plants at both 10 and 24 hours. High variability in settling was found among test replications, preventing a strong statistical distinction among the genotypes.

Table III-1: Settling preference[#] of *D. maidis* females on maize plants of different genotypes.

Insect location	Time (hours)			
	1	3	10	24
X1273A (7)	8 ± 4.4 bcd	8 ± 3.3 cd	16 ± 4.0 bcd	18 ± 6.3 bcd
X1E73 (1)	10 ± 4.5 bcd	10 ± 4.5 bcd	26 ± 6.0 abc	30 ± 4.5 ab
X1297J (6)	12 ± 4.4 bcd	8 ± 3.3 cd	10 ± 3.3 bcd	18 ± 7.0 bcd
X1286B (3)	10 ± 3.3 bcd	12 ± 6.1 bcd	20 ± 7.9 bcd	14 ± 4.3 bcd
Golden Bantam	14 ± 5.2 bcd	18 ± 4.7 bcd	28 ± 5.3 abc	20 ± 5.2 bcd
Not on plants	46 ± 4.4 a	34 ± 4.5 a	0 ± 0.0 d	0 ± 0.0 d

(#): measured as percentage of insects settled on each plant.
Numbers between parentheses indicate the resistance rating in field (1: susceptible, 9: immune).
Different letters indicate significant differences at Fischer's LSD test (p<0.05).

Oviposition rates of *D. maidis* females did not vary among maize hybrids differing in field resistance to corn stunt (Table III-2). In the free-choice test, the number of eggs laid in the susceptible and resistant hybrids in each genotype pair were similar (p=0.7756 for X1273A-X1E73 and p=0.9833 for X1286B-X1297J). Furthermore, oviposition rates on all tested maize genotypes were indistinguishable in the no-choice test (p=0.6148), in which the susceptible cultivar Golden Bantam was also included.

Table III-2: Ovipositional rate[#] of *D. maidis* females caged on different maize genotypes.

Genotype	Free-Choice	No-Choice
X1273A (7)	4.4 ± 1.0 a	7.7 ± 0.3 a
X1E73 (1)	5.0 ± 1.3 a	6.9 ± 0.4 a
X1297J (6)	5.4 ± 1.0 a	6.9 ± 0.4 a
X1286B (3)	5.1 ± 0.9 a	7.2 ± 0.3 a
Golden Bantam		7.7 ± 0.4 a

(#): measured as number of eggs laid per female per day.
Numbers between parentheses indicate the resistance rating in field (1: susceptible, 9: immune).
Different letters indicate significant differences at Fischer's LSD test (p<0.05). In free-choice experiments, t-tests were performed per each pair of hybrids (X1273A-X1E73 and X1286B-X1297J).

Survival of *D. maidis* at both immature and mature stages varied when insects were exposed to different maize genotypes (Table III-3, Figure III-1). Percentage of nymphs becoming adults was lower on all the hybrids than that on the susceptible cultivar Golden Bantam (p<0.0001), particularly on hybrid X1297J. Adult survival was also affected by genotype (p<0.0001), sex (p=0.0210) and their interaction (p=0.0003). In this case, only the insects on hybrid X1297J had reduced survival when compared to control insects on Golden Bantam, and survival of males was lower than that of females.

Table III-3: Nymphal survival[#] of *D. maidis* on maize plants of different genotypes.

Genotype	Survival (%)	
X1273A (7)	46.5 ± 4.5	b
X1E73 (1)	49.0 ± 3.8	b
X1297J (6)	17.5 ± 3.4	c
X1286B (3)	54.0 ± 5.2	b
Golden Bantam	73.0 ± 6.9	a

(#): measured as percentage of surviving insects.
Numbers between parentheses indicate the resistance rating in field (1: susceptible, 9: immune).
Different letters indicate significant differences at Fischer's LSD test (p<0.05).
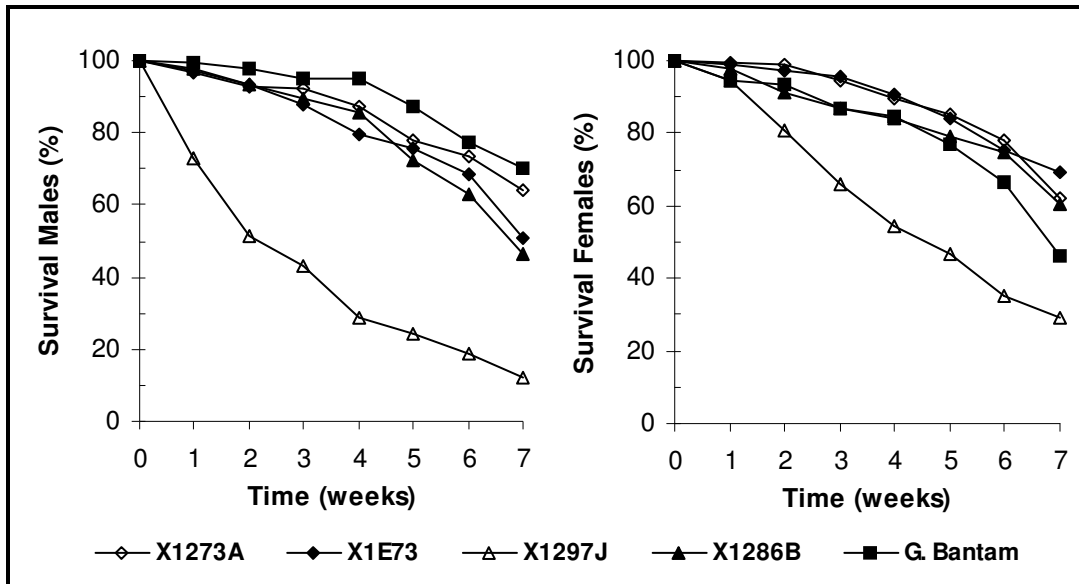
Figure III-1: Survival (%) of adult *D. maidis* on different genotypes of maize.

Adults of *D. maidis* were able to inoculate the pathogen *S. kunkelii* efficiently to plants of all the maize genotypes tested (Figure III-2). Although adults of both sexes were

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assayed, the results were pooled in Figure III-2 because efficiency of inoculation was not significantly different (p=0.2827) for males and females. Neither the effect of genotype (p=0.4672) or IAP length (p=0.7104), nor the IAP x genotype interaction (p=0.1835), were significant.
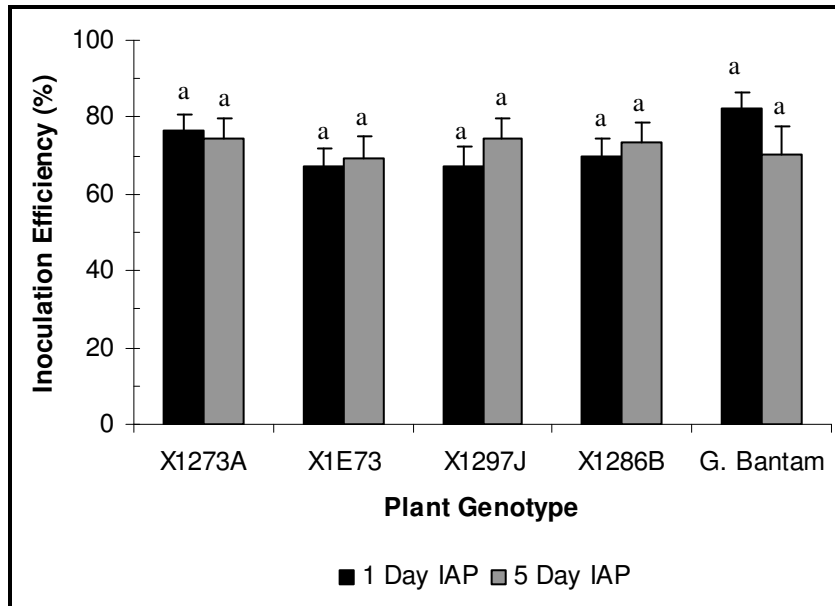
Figure III-2: Inoculation efficiency (%) of *D. maidis* on different genotypes of maize. Different letters indicate significant differences using Fisher's LSD test (p<0.05).

Discussion

Plant acceptance has an important effect upon fitness and, consequently, transmission capability of leafhoppers (Saxena and Khan 1989). In rice, damage caused by viruses was significantly reduced by the use of vector-resistant cultivars (Hibino et al. 1987, Dahal et al. 1990). In the case of corn stunt, resistant genotypes have been obtained, but no information on the nature of such resistance was available. For this reason, we sought to determine if the resistance of two maize hybrids to this disease was targeted to the insect or to the pathogen. Additional goals were to analyze the resistance with respect to antixenosis and antibiosis, and to determine the effect of these processes upon *S. kunkelii* inoculation by *D. maidis*.

The lack of settling preference of *D. maidis* females on maize genotypes differing in resistance to corn stunt disease is inconsistent with findings in other auchenorrynchan

species. Indeed, lower settling preference on resistant rice cultivars was observed for the leafhopper *Nephotettix virescens* (Cheng and Pathak 1972, Heinrichs and Rapusas 1983a), and for the planthoppers *Delphacodes kuscheli* (Costamagna et al. 2005), and *Sogatella furcifera* (Khan and Saxena 1985, Ye and Saxena 1990). In these species, a clear preference for susceptible genotypes was found between 6 and 10 hours. In contrast, we found no differences in settling preference of *D. maidis* over a period of up to 24 hours.

Our findings suggest that the disease resistant maize genotypes included in this study do not interfere with insect settling during the process of host finding and acceptance by the vector. On one hand, plant host finding and acceptance by *D. maidis* begins with their detection of visual and chemical cues (Todd et al. 1990), followed by ingestion probes within in less than five minutes of alighting on the plant. For this reason, although chemical cues determining resistance could have been masked by the close proximity among the experimental plants during the experiments, a time period of 1 to 3 hours would allow insects to test all the genotypes until they found a susceptible one. Alternatively, the high efficiency in inoculation of spiroplasmas by *D. maidis* (Figure III-2) shows that the insects are able to penetrate different plant tissues and reach phloem. Hence, this evidence suggests that the insects did not encounter any resistance factors in deeper tissues either.

The lack of oviposition preference of *D. maidis* females in our work resembles the behavior of several homopteran species on rice. Insect-resistant and susceptible rice cultivars were accepted equally as oviposition hosts by females of the leafhoppers *Nephotettix cincticeps* (Kawabe 1985) and *N. virescens* (Cheng and Pathak 1972); and of the planthoppers *Nilaparvata lugens* (Sogawa and Pathak 1970, Ye and Saxena 1990) and *S. furcifera* (Heinrichs and Rapusas 1983b, Khan and Saxena 1985). Hence, our results are consistent with those found in other species of auchenorrynchans, and suggest that antixenosis did not play a role during oviposition of *D. maidis* females with these maize genotypes under the conditions of our study.

The reduction in *D. maidis* survival rate on resistant maize hybrid X1297J is similar to that reported previously for homopterans on rice. After exposure to plants of resistant

cultivars, reduced survival of nymphs and adults was found for the leafhoppers *N. virescens* (Cheng and Pathak 1972, Heinrichs and Rapusas 1983a), and *N. cincticeps* (Kawabe 1985); and also of the planthoppers *N. lugens* (Sogawa and Pathak 1970, Ye and Saxena 1990), and *S. furcifera* (Heinrichs and Rapusas 1983b, Khan and Saxena 1985). However, survival of *D. maidis* at the adult stage on the maize hybrids we tested is quantitatively different from that of leafhoppers and planthoppers on rice. Indeed, while adult homopteran survival rates on some resistant rice cultivars was reduced to 20% after 5 days in *N. virescens* (Cheng and Pathak 1972, Ye and Saxena 1990) and *S. furcifera* (Heinrichs and Rapusas 1983b), average survival rate of *D. maidis* in corn stunt resistant maize hybrids X1273A and X1297J was 98% and 85% in the first week, respectively. This difference in survival rate may have significant implications with respect to inoculation efficiency of pathogens.

Survival on corn stunt resistant maize hybrids was affected more negatively at the nymphal than adult stage. Similar differences in susceptibility to disease resistant plants between life stages have been found in other leafhopper species (Saxena 1969), and were attributed to higher nutritional demand by immature forms, which require levels sufficient to support weight gain and molting. Higher levels of mortality for immature stages than adults may help in reducing the population numbers prior to eclosion to adult stage and migration to other plants.

The lack of maize antixenosis and the delayed effect of antibiosis on adult *D. maidis* were correlated with high inoculation efficiencies of the pathogen *S. kunkelii*. Initially, a relatively long (5 days) IAP was assayed to maximize the opportunity of *S. kunkelii* transmission (Alivizatos and Markham 1986), but no differences could be found in inoculation efficiency among the maize hybrids. It is likely that during a period as long as 5 days, the nutritional needs of those insects caged on resistant plants would overcome an initial resistance mechanism, resulting in probing and inoculation of the pathogen. However, when the IAP was only 1 day the levels of inoculation were also high, and there were no differences among maize genotypes. Consequently, our results differ notably from those reported in a study of the effect of breeding for insect resistance in rice. In the latter crop, the efficiency of inoculation of phloem-associated viruses was

lower when the leafhoppers fed on resistant cultivars, as compared to the efficiency on leafhopper-susceptible cultivars (Hibino et al. 1987, Dahal et al. 1990).

The hybrid X1297J has one or more traits that result in reduced nymphal survival rate and reduced adult longevity of *D. maidis*. However, this resistance mechanism would not protect the plants from inoculative adults, because the increase in mortality occurred long after the leafhopper's inoculation efficiency reached its maximum value of about 5 to 7 days (Alivizatos and Markham 1986). Yet, a decrease in survival of nymphs would likely limit the amount of secondary pathogen spread within a field, or between fields. As young plants are the most susceptible to disease (Hao and Pitre 1970), reductions in the number of inoculative *D. maidis* adults migrating to other fields could be an effective tool for corn stunt disease control in areas where corn crops are grown sucesively throughout the season or year.

Although the maize hybrid X1273A has been reported to carry field resistance to corn stunt disease, our results did not confirm this resistance under laboratory conditions. In rice, several genotypes resistant to viral pathogens were not resistant to the vectors (Heinrichs and Rapusas 1983b). In contrast, *D. maidis* inoculation efficiency and maize symptom severity (data not shown) were similar among all the hybrids assayed, regardless of the genotype resistance rating. This lack of difference suggests that once the spiroplasmas colonized the plant host, their ability to multiply and cause symptoms was similar in both susceptible and resistant genotypes.

It is possible that maize hybrid X1273A expresses other resistance mechanisms in the field that are not triggered under the controlled conditions in our experiments. For instance, as *D. maidis* is able to migrate long distances while searching for suitable hosts (Nault 1990), a deterrent that might operate only at long range would not be effective when the insects are caged in close contact with plants. Alternatively, it is possible that visual and/or olfactory stimuli of the host plants associated with host deterrence were not expressed by hybrid X1273A in the controlled conditions in our tests.

Resistance to insects is a promising tool that has the potential to reduce the negative impact caused by vector-transmitted pathogens in several important crops (Saxena and Khan 1989). Our work has shown that maize genotypes can be characterized for

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resistance using the parameters described here, and additional research could provide useful insight for this approach to disease management. At present additional studies are being performed using EPG (Electrical Penetration Graph) technology, aiming for a deeper characterization of these resistant genotypes. It is hoped that this information will be relevant to efforts to develop new maize genotypes with durable pathogen and/or leafhopper resistance, reducing the damage caused by corn stunt disease.

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CHAPTER IV

CHARACTERIZATION OF PROBING ACTIVITIES OF THE CORN

LEAFHOPPER *DALBULUS MAIDIS* (HEMIPTERA: CICADELLIDAE)

Abstract

The corn leafhopper, *Dalbulus maidis*, is a vector of three maize pathogens that have become limiting factors for maize production in some areas of the Americas. Insect probing behavior plays an important role in vector acquisition and inoculation of pathogens and hence the disease spread, and is relevant to the characterization of potential sources of insect-resistance in maize. Hence, we sought to analyze and to understand the different probing activities performed by this insect while feeding on corn plants. Using electrical penetration graph (EPG) technology, six distinct waveforms were characterized and correlated with major probing activities of *D. maidis* by monitoring transmission of spiroplasmas and excretion of honeydew. Major waveforms comprise stylet pathway (waveform 1), active ingestion (waveform 2), nonvascular probing (waveform 3), phloem conditioning (waveform 4), phloem ingestion (waveform 5) and oviposition (waveform 6). Our results support previous findings with this species, and also indicate that some waveforms (2, 4 and 5) are related to voltages generated during probing, as was found previously for other hemipteran species. This work provides basic information relevant to the understanding of probing behavior of *D. maidis* and to characterize potential sources of insect-resistant maize.

Introduction

The corn leafhopper, *Dalbulus maidis*, is a major pest of maize (*Zea mays* L.) in the Americas. Its epidemiological importance is due to its prevalence from the southern United States to central areas of Argentina (Nault 1990), and to its high efficiency in transmitting three important maize pathogens: the mollicutes *Spiroplasma kunkelii*, maize bushy stunt phytoplasma (MBSP) (Nault 1980), and the maize rayado fino virus (MRFV) (Gamez 1973). These three pathogens, alone or in combination, cause "corn stunt," a disease complex that has become a limiting factor for corn production in some areas of The United States and Latin America.

Changes in agricultural practices have played an important role increasing the amount of insect population sizes, and hence the amount of disease. Indeed, although the host range of *D. maidis* includes only corn and teosintes (Pitre 1967), the persistence of inoculative insects after maize crops (Larsen et al. 1992, Ebbert and Nault 1994), the ability of this leafhopper species to move long distances (Nault 1990), and the increase in acreages planted in maize monocultures (Hruska et al. 1996, Summers et al. 2004) have led to significant increases in the prevalence of diseases whose causal agents are transmitted by the corn leafhopper. Furthermore, insects harboring any of the three corn stunt pathogens, insects remain inoculative for the rest of their lifetime (Gamez 1973, Madden et al. 1984) because of pathogens' ability to multiply in the insect body.

The feeding behavior of *D. maidis* has a central role in the transmission efficiency of the corn stunt pathogens. Because the efficiencies of both acquisition and inoculation of these pathogens increase with the extent of insect-plant contact (Alivizatos and Markham 1986) and because such interaction lasts longer in plants suitable for insect feeding (Saxena 1969, 1985), these plants are exposed to a higher pathogen pressure than are plants that are less accepted by insects (Saxena 1969, 1985). For this reason, control of leafhopper-transmitted pathogens, such as the rice tungro viruses, has relied primarily on insect-resistant cultivars, which prevent successful feeding by insects (Rezaul Karim and Saxena 1991, Azzam and Chancellor 2002).

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Although insect life history parameters have been used extensively to identify sources of resistance (Saxena and Khan 1989), they provide little information about the mechanism of resistance in different tissues. EPG (electrical penetration graph) technology has been particularly helpful in efforts to overcome this difficulty and to discriminate among genotypes differing in resistance mechanisms. This approach has been effective in the characterization of activities performed by hemipteran insects while probing (insertion of the stylets into the plant tissues) (Backus 2000). For instance, extensive research characterized the probing behavior of several *Nephotettix* species (Cheng and Pathak 1972, Heinrichs and Rapusas 1984, Rapusas and Heinrichs 1990, Rezaul Karim and Saxena 1991) and *Nilaparvata lugens* (Khan and Saxena 1988, Kimmins 1989, Alam and Cohen 1998, Hattori 2001), important pests of rice. In turn, this information facilitated the detection and characterization of rice genotypes resistant to tungro disease (Azzam and Chancellor 2002).

The probing behavior of leafhoppers that serve as vectors of maize pathogens is not as well understood as that of other leafhoppers. Studies of the probing behavior of *Cicadulina* spp (Kimmins and Bosque-Perez 1996, Lett et al. 2001), and of *Graminella* spp and *D. maidis* (Wayadande and Nault 1996) have provided some information. The latter study identified the major activities of probing behavior related to inoculation of the maize chlorotic dwarf virus (MCDV), but because EPG equipment available at that time was less discriminatory than that used today the data lacked sufficient resolution to describe precisely the probing behavior of this important maize pest. Wayadande and Nault (1996) correlated inoculation of the phloem-restricted MCDV with production of x-waveforms, which were correlated with stylet position in phloem cells. As the three pathogens transmitted by *D. maidis* are all associated with phloem sieve tube elements, the activities performed by this insect in phloem tissue will determine the transmission efficiency of this leafhopper.

The objective of this work was to characterize the major biological activities performed by *D. maidis* during probing. This goal was reached by identifying discrete waveforms in the EPG output, and correlating each of them with stylet position and insect activity. Specific metrics used to characterize insect activity included efficiency of transmission of the pathogen *S. kunkelii* and characterization of excreted honeydew in

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each waveform. These data will contribute to our understanding of the behaviors related to transmission of persistently-transmitted pathogens and to the characterization of mechanisms of plant resistance to *D. maidis*. Ultimately, the findings will be applied to strategies for reducing the damage caused by this insect species and the pathogens that it transmits.

Material and Methods

I - Insects, plants and spiroplasmas

A colony of healthy *D. maidis* DeLong and Wolcott was initiated at Oklahoma State University in 1997 from insects obtained from L. R. Nault, Ohio Agricultural Research Development Center (Wooster, Ohio). The Ohio State colony was established initially from insects collected in Mexico, and was maintained on plants of sweet corn var. Golden Bantam. Insect and plant maintenance were carried out in aluminum-framed cages (Wayadande and Fletcher 1995), placed in a growth room at a constant temperature of 26 °C, with a 16:8 h (light:dark) photoperiod (Nault 1980). All the experiments were performed using maize plants var. Golden Bantam, at the three-leaf stage. The youngest fully expanded leaf of each plant was folded over a horizontally-placed plastic cylinder (10 cm diameter) to expose the abaxial surface, preferred by *D. maidis*. *Spiroplasma kunkelii* Whitcomb strain CR2-3X (passage 9) was used for transmission tests. This strain was collected originally in Costa Rica (Castro et al. 1992), and cultured in LD8A3 medium (Lee and Davis 1989).

II - EPG recordings

Ten to fourteen-day-old *D. maidis* females were anesthetized by confining them singly in glass tubes (1.5 X 15 cm), which were chilled on ice for 2-3 minutes. Immobilized insects were placed with the help of an "abdomen" type aspirator (sized to fit tightly around the abdomen), on a stage constructed under a stereomicroscope (Nikon 530 220, Melville, NY), and secured by application of a gentle vacuum. Insects were tethered to an 18.75 µm-diameter, 2-3 cm-long gold wire (Sigmund Cohn, Mount Vernon, NY) using silver conductive paint (Ladd Research Industries, Burlington, VT) to

attach the wire to the pronotum. After tethering, insects were starved for 1 hour before recordings were initiated.

EPG recordings were performed using a four-channel, Type 3 AC/DC Missouri monitor (Backus and Bennett 1992) for a period of 20 hours. Throughout the test, constant equipment settings consisted of: alternate current (AC) with 100 mV, 1000 Hz substrate voltage, input resistance of 10^8 Ω (Ohm), and amplification (gain) of 500 X. Substrate voltage was applied through an electrode inserted into the soil, and the gold wire was glued to the insect electrode (Figure IV-1). This insect electrode consisted of a copper wire (around 0.5 mm diameter) soldered to a brass nail that was inserted in the electric circuit. The insects and plants were placed inside a Faraday cage (2' X 2' X 4', constructed of an aluminum frame with a steel base), to reduce the interference of external electrical noise. Output waveforms were converted to digital format at 100 samples per second using a DI-720 analog-to-digital board, acquired with Windaq/Pro software (Dataq Instruments, Akron OH), and stored in a hard drive.
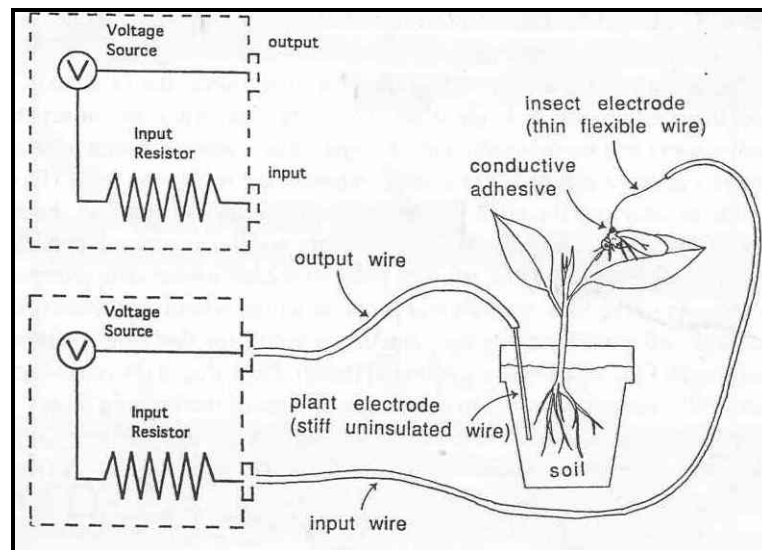
Figure IV-1: Schematic version of the EPG system used in our experiments. (Picture taken from Walker, 2000).

III - Correlation of EPG parameters with *S. kunkelii* transmission

Inoculative insects were obtained by feeding second- to third-stage *D. maidis* nymphs on D10 medium (Alivizatos 1982) containing *S. kunkelii* at a titer of approximately 10^8 cells/ml. Approximately 500 µl of this suspension was placed inside feeding sachets constructed of two layers of Parafilm® membranes stretched over a 50 ml

plastic dose cup, into which the test insects were introduced for an acquisition access period (AAP) of 24 hours. After 28 days of incubation on healthy maize plants (var. Golden Bantam), insects eclosed to the adult stage and were used for inoculation tests.

To correlate waveform type with pathogen inoculation, wire-tethered inoculative insects were allowed to probe on spiroplasma-free plants while being recorded (First inoculation access period, IAP). Probing was interrupted by removing the insect from the plant when the desired waveform or waveform combinations were observed (described below). After recording this first inoculation access period (IAP), the insects were chilled on ice (as described above) and gently detached from the gold wire. Insects where then placed singly on another maize plant for a second IAP of 3 days (Alivizatos and Markham 1986) to determine if they were inoculative. All the plants were kept for 60 days in a growth chamber (25 °C, with a light:dark photoperiod of 16:8 hours) for symptom expression and diagnosis. Presence of *S. kunkelii* was confirmed by PCR using primers F2 and R6, which amplify a segment of the gene encoding spiralin (Barros et al. 2001), after extraction of nucleic acids using the CTAB method (Doyle and Doyle 1994).

Diseased plants obtained in the inoculation experiments were used for acquisition tests. To correlate waveform type with acquisition, spiroplasma-free insects had access to *S. kunkelii* infected plants while being recorded, until the probing was interrupted as described for inoculation. The insects were then detached from the gold wire and caged singly for a period of 2 weeks, to allow multiplication of spiroplasmas. After this period, the insects were tested singly for *S. kunkelii* using PCR as described.

For both inoculation and acquisition tests, insects were sorted into one of three groups, depending on the types of waveforms that they had displayed by the time the probes were interrupted: Group 1, with waveform 1 and waveform 2 (no probing in phloem); Group 2: same as Group 1 plus waveform 4 (phloem probing includes x-waveform only); Group 3: same as Group 2 plus waveform 5 (includes phloem ingestion). Grouping was done in this way because of the sequential appearance of different waveforms; waveform 5 was preceded always by waveform 4, which in turn was always preceded by waveforms 1 and 2. The results were expressed as percentage of

test plants that became infected (inoculation test), or percentage of insects that acquired *S. kunkelii* (acquisition test).

IV - Honeydew excretion

Rate of excretion and pH of honeydew also were used to correlate waveforms with insect activities. The rate of honeydew excretion was obtained by observing the insect probing using a stereomicroscope, and counting for 20 minutes the number of honeydew droplets released after each particular waveform started, expressed as number of droplets per minute. The pH was determined by spotting pH indicator paper (range pH 4 to 8) with individual honeydew droplets deposited on the leaf surface. Buffers of known pH were used as standards, spotting 2 µl on the pH indicator tape.

V - Statistical analysis

Outputs of EPG recordings were inspected visually, and notes were made on the types of waveforms and the time of appearance of each waveform. These data were stored in a spreadsheet for the analysis and interpretation of results. Probing behavior and its association with either inoculation or acquisition of spiroplasmas were analyzed using chi-square contingency tests (Prado and Tjallingii 1994). For inoculation tests, only those insects that transmitted *S. kunkelii* either to the plant in which recording took place (first IAP), or to the test plant (second IAP) were considered inoculative and were included in the statistical analysis.

Results

Characterization of waveforms of *D. maidis*

To identify the most optimal conditions for EPG recording, several input resistances ranging from 10^6 Ω to 10^{13} Ω were screened. An input resistance of 10^8 Ω was chosen because it allowed identification of the same waveforms seen at higher input resistances, but also provided positional information of each waveform (relative to the baseline and the highest voltage detected, or relative amplitude), which was lost at higher input resistances. When females of *D. maidis* probed on maize plants, six different waveforms (Table IV-1, Figures IV-2 and IV-3) were identified in the EPG output; these were

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designated 1 through 6 according to their time of first detection. The recordings showed a flat baseline when insects were not probing, while the output voltage of all the probe-related waveforms was higher than that of the baseline.

Table IV-1: Absolute and relative amplitude (%),and time to first event (min) of waveforms recorded in probing of *D. maidis* females (n=10) on maize plants of the susceptible variety Golden Bantam.

Waveform	Relative Amplitude	Absolute Amplitude	Time to First Event
1	75.7 ± 10.1	76.2 ± 10.7	2.9 ± 2.1
2	42.7 ± 8.0	6.7 ± 3.4	45.9 ± 75.4
3	53.9 ± 14.6	10.4 ± 5.2	102.5 ± 141.3
4	54.9 ± 10.0	5.9 ± 5.2	83.7 ± 66.4
5	63.1 ± 14.9	4.5 ± 2.3	138.1 ± 94.7
6	52.1 ± 8.7	95.8 ± 6.8	217.6 ± 136.1

Waveforms 1 to 3 occurred early in the insect-plant interaction (Table IV-1). All the probes started with waveform 1, which is characterized by a sudden increase in voltage, a high absolute amplitude (distance between peaks and valleys in the same waveform), and variable frequency, making an overall irregular waveform of short duration (Figure IV-2). Shape of waveform 2 is regular, with low absolute and relative amplitude and a constant frequency of 5 Hz (Figure IV-2F). The average duration of this waveform can be highly variable, from short events lasting a few seconds, up to 20 minutes without interruption. Waveform 3 (Figure IV-2A) is similar to waveform 2 in relative and absolute amplitude, but its frequency is much lower and more variable. It was detected in few insects and for brief time periods, shifting from and to waveform 2 within the same probe.

In contrast to the short duration and early appearance of waveforms 1 to 3, waveforms 4 and 5 lasted longer and took place later in the insect-plant interaction (Table IV-1). The frequency of waveform 4 (Figure IV-2, insets A to C) increased from 0.2-0.3 Hz at the beginning to 5 Hz when it merged into waveform 5. There also was a gradual increase in relative amplitude toward the latter part of the waveform 4, reaching the same relative amplitude as in waveform 5. During early stages of waveform 4 there usually were 2-5 "spikes" (sudden increase in voltage with an irregular form), as shown in Figure IV-2A. However, many probes of *D. maidis* females were naturally interrupted before

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reaching the characteristic 5 Hz frequency when waveform 5 should begin. Waveform 5 (Figure IV-2E and G) had a high relative amplitude, a frequency of 5 Hz, and two different parts: a short one (0.4-2.0 seconds) with low absolute amplitude, and another with higher absolute amplitude lasting 1.5-4.0 seconds. Duration of both phases varied not only among insects, but also among probes and even in the same waveform event.
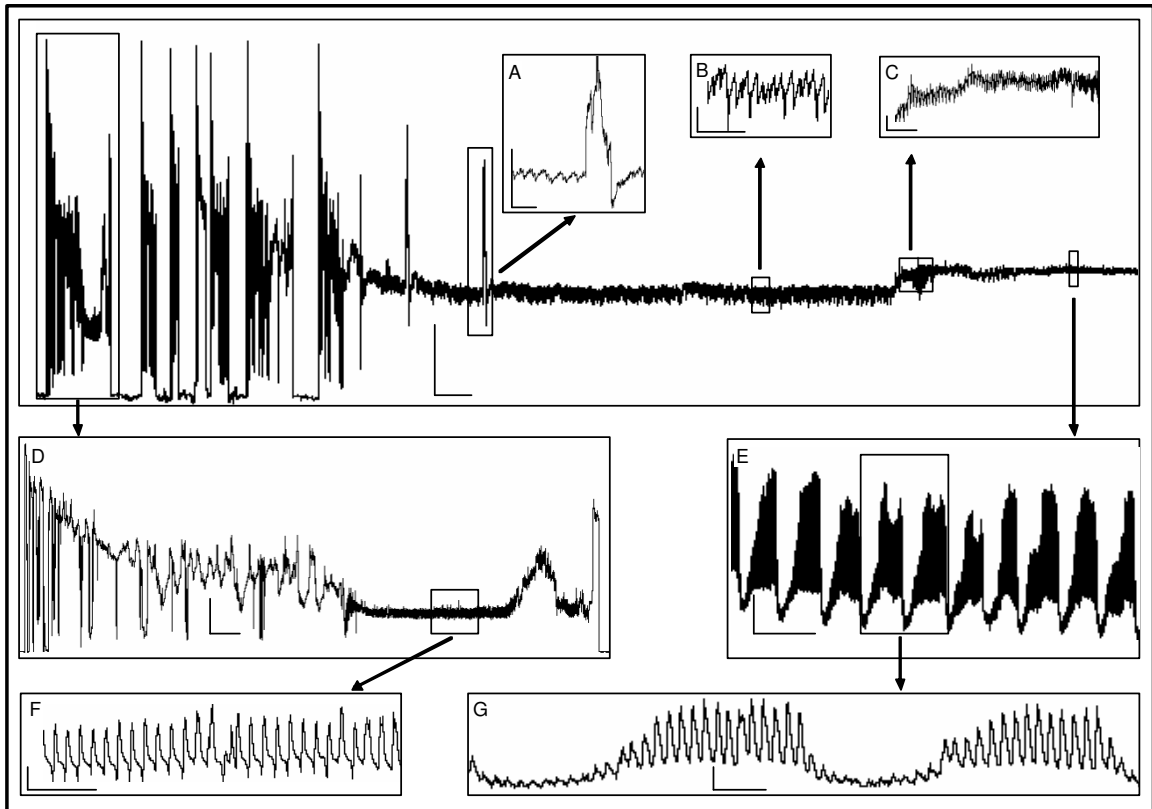
Figure IV-2: Sample of major waveforms displayed during probing of *D. maidis* females (horizontal bar: 100 s, vertical bar: 1000 mV). Inset A: spike during early stages of the waveform 4 (horizontal bar: 10 s, vertical bar: 1000 mV). Inset B: middle stage of waveform 4 (horizontal bar: 10 s, vertical bar: 100 mV). Inset C: transition from waveform 4 to waveform 5 (horizontal bar: 10 s, vertical bar: 100 mV). Inset D: expansion of first probe containing an event of waveform 2 (horizontal bar: 10 s, vertical bar: 1000 mV). Inset E: waveform 5 (horizontal bar: 10 s, vertical bar: 100 mV). Inset F: waveform 2 (horizontal bar: 1 s, vertical bar: 50 mV). Inset G: expansion of waveform 5 (horizontal bar: 1 s, vertical bar: 20 mV).

Waveform 6 (Figure IV-3B) corresponded to oviposition, and each single waveform was visually correlated to insertion of the ovipositor and deposition of a single egg. For this reason, it was correlated with either spiroplasma transmission or honeydew excretion. No histological examinations were carried out to determine the tissues in which the eggs were deposited.

Although waveforms 2 and 5 shared similar absolute amplitude and a 5 Hz frequency, they differed in some other characteristics: waveform 5 had a higher relative amplitude, a short period of low absolute amplitude followed by a longer period of high amplitude, and always followed waveform 4. On the other hand, waveform 2 had low relative amplitude, lacked a period of low absolute amplitude, and always followed waveform 1. In additional experiments to characterize these two waveforms, the recording conditions were adjusted to detect the emf component (voltages generated during the insect-plant interaction, such as muscle contraction of the insects) of the EPG output, using an input impedance of 10^{13} Ω, 0 V in the input voltage and a DC (direct current) output (Walker 2000). Using these settings, the frequency and shapes of waveforms 2, 4 and 5 remained unaltered (results not shown), indicating that they were caused by voltages generated in the insect-plant interaction, or emf component (Walker 2000).
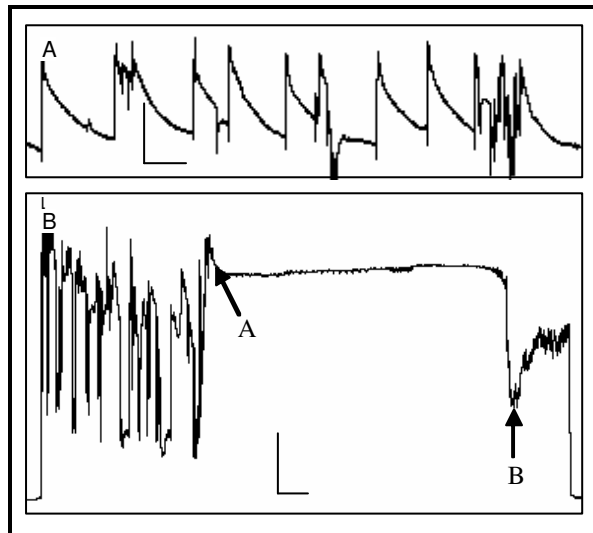
Figure IV-3: Sample of less common waveforms displayed during probing of *D. maidis* females. Inset A: waveform 3 (horizontal bar: 10 s, vertical bar: 100 mV). Inset B: waveform 6 (Horizontal bar: 10 s, vertical bar: 1000 mV). Arrows indicate the moment of ovipositor insertion (A) and removal (B).

Correlation of EPG waveforms with biological activities

Table IV-2 shows the efficiency of inoculation and acquisition of the pathogen *S. kunkelii* by insects whose probes were interrupted at different stages. No spiroplasmas were inoculated when probing was interrupted after both waveforms 1 and 2 were displayed. The Chi Square analysis indicated that inoculation of spiroplasmas always

correlated to waveform 4, with no further increase in inoculation efficiency when probing continued to include waveform 5 (p=0.418).

Table IV-2: Inoculation and acquisition efficiency of the pathogen *S. kunkelii* by insects which probing was interrupted in different waveforms.

		Inoculation		
Waveforms	**# Total plants**	**# Infected plants**	**% Infected plants**	
1 + 2	15	0	0.0	a
1 + 2 + 4	31	26	83.9	b
1 + 2 + 4 + 5	26	22	84.6	b
		Acquisition		
Waveforms	**# Total insects**	**# Infected insects**	**% Infected insects**	
1 + 2	18	2	11.1	a
1 + 2 + 4	47	26	55.3	b
1 + 2 + 4 + 5	40	37	92.5	c

Treatments followed by different letters are significantly different according to Chi square test (p<0.05).

Although inoculation correlated to only one waveform, insects acquired spiroplasmas regardless of the probing activities performed. A low acquisition rate occurred when insect probing was interrupted after only waveforms 1 + 2, but acquisition efficiency increased significantly when the probes included waveform 4, and still more insects acquired the spiroplasmas if they probed until they performed waveform 5.

Table IV-3: Characteristics of honeydew excreted by *D. maidis* females displaying ingestion waveforms (n=10).

Waveform[A]	pH	Rate[B]
2	4.96 ± 0.67 a	0.34 ± 0.13 a
5	7.12 ± 0.38 b	0.65 ± 0.15 b

[A]: Waveforms 1, 3, 4 and 6 not analyzed because honeydew excretion was rarely observed during these waveforms.
[B]: Excretion rate: number of droplets per minute.
Different letters indicate significant differences by Fisher's LSD test (p<0.05) for each parameter.

Characteristics of honeydew varied during different waveform events of *D. maidis* probing behavior (Table IV-3). *D. maidis* produced honeydew only infrequently when waveforms 1, 3, 4 or 6 were displayed. On the other hand, honeydew excretion correlated

consistently to waveforms 2 and 5. Honeydew was excreted at a lower rate and was of lower pH during waveform 2 than during waveform 5, although measurement of the excretion rate during the former waveform was difficult due to its short average duration (around 7 minutes) and occasional shifting from and to waveform 3.

Discussion

Interpretation of the probing activities associated with different EPG waveforms is based on similarities of *D. maidis* waveforms with those of other leafhopper species, transmission of the phloem-associated pathogen *S. kunkelii* and characteristics of honeydew excretion. Electronic monitoring of probing behavior of *D. maidis* females reveals EPG waveforms similar to those previously described for other leafhoppers, including those reported in an earlier characterization by Wayadande and Nault (1996) for this same species. However, the information obtained in this work provides a higher level of detail than was possible to gather during most of the previous research in leafhoppers, and therefore can explain better the probing behaviors related to pathogen transmission.

Waveform 1 resembles the formerly described "salivation" waveform (now designated "stylet pathway") of several leafhopper species, including *D. maidis* (Wayadande and Nault 1996), *Perkinsiella saccharicida* (Chang 1978), *Nephotettix cincticeps* (Kawabe and McLean 1980), and *N. virescens* (Rapusas and Heinrichs 1990), and is similar also to waveform 1 of *Cicadulina mbila* (Lett et al. 2001) and waveform 1 of *Homalodisca coagulata* (Backus et al. 2005). Common characteristics include a short duration of the waveform event (about 1 minute), a sudden increase in voltage at the beginning of the waveform, an irregular waveform shape, and high absolute amplitude.

Waveform 2 is similar to waveforms related to either "non-sieve element ingestion" in *D. maidis* (Wayadande and Nault 1996), "xylem ingestion" in *N. cincticeps* (Kawabe and McLean 1980, Kawabe 1985) and *N. virescens* (Rapusas and Heinrichs 1990), and "active ingestion" either from xylem or mesophyll cells by *C. mbila* (Lett et al. 2001). Waveform 2 is similar to those described in *D. maidis* and *Nephotettix* spp, in its presence after a "salivation" (now "stylet pathway") waveform, its regular shape and its

low relative amplitude. Also, recent evidence on *C. mbila* (Lett et al. 2001) shows that its and our "waveform 2" share a 5 Hz frequency and a emf origin, in addition to the remaining characteristics.

Waveform 3 occurred rarely, and its characteristics include medium absolute and relative amplitude, a sudden increase followed by a gradual reduction in voltage, and low and variable frequency (Figure IV-3). Waveform 3 is associated with waveform 2, the insect moving back and forth between these two waveforms. In *N. virescens* (Rapusas and Heinrichs 1990) and *N. cincticeps* (Kawabe and McLean 1980, Kawabe 1985) a similar waveform was designated the "resting" waveform and in *D. maidis* (Wayadande and Nault 1996) it was called "nonvascular probing" because in both cases the stylet tips were located in mesophyll cells and no ingestion, as measured by excreted honeydew, was detected. A similar waveform (waveform "D") was associated with mesophyll ingestion in the xylem-feeder *Graphocephala atropunctata* (Almeida and Backus 2004), and in the mesophyll-feeders *Empoasca kraemeri* and *E. fabae* (Calderon and Backus 1992). However, this waveform was correlated visually in our tests with the insect moving actively while keeping the stylets inserted, so it could be related to the waveform 3 of *C. mbila*, (Lett et al. 2001), a similar waveform (although of inverted shape) that was interpreted as "stylet work".

Waveform 4 obtained in this study is identical to the x-waveform described previously in this species (Wayadande and Nault 1996), and resembles the so-called "x-waveform" (Kawabe and McLean 1980) or "TIp (trial ingestion from phloem) waveform" (Kawabe 1985, Rapusas and Heinrichs 1990) found in other leafhoppers. All these waveforms occurred immediately before the phloem ingestion waveform, and displayed low absolute amplitude and high relative amplitude, and the presence of 3-5 "spikes" (sudden increase in voltage) at their early stages. Our results confirm the previous findings in *D. maidis* (Wayadande and Nault 1996), in which this waveform occurred in approximately 80-120 cycles of 5-10 seconds each. However, the higher resolution level attained in this work shows that, instead of the flat line formerly described, the frequency of waveform 4 increases until reaching the 5 Hz of the next waveform. It was also determined that this waveform has an emf origin. The "waveform 4" in *C. mbila* (Lett et al. 2001) also has a frequency of 0.4-0.6 Hz, similar to that in the

early stages of our "waveform 4", that was attributed by the authors to activity of a salivary pump, based on former evidence in aphids (Tjallingii 1978). However, an increase in frequency similar to the one found in *D. maidis* was not described in *C. mbila*, and the overall shapes of the two waveforms differ (Lett et al. 2001). However, it is known that shape of this waveform changes significantly among leafhopper species (Wayadande and Nault 1996).

Waveform 5 shares some characteristics with "phloem ingestion" waveforms of other hemipteran species. However, although "phloem ingestion" waveforms of leafhoppers obtained using old AC-based EPG systems (Chang 1978, Kawabe and McLean 1980, Kawabe 1985, Rapusas and Heinrichs 1990, Wayadande and Nault 1996) appeared as flat lines, our results uncover a complex structure with a 5 Hz frequency. In this sense, our "waveform 5" is similar both in frequency and emf origin to the phloem ingestion waveform detected using DC-based EPG in *C. mbila* (Lett et al. 2001) and in the aphid *Rhopalosiphum padi* (Prado and Tjallingii 1994).

Waveform 6 was visually correlated with insertion of the ovipositor into plant tissues, so it was doubtless an oviposition waveform. This waveform resembles remarkably the same waveform in *N. lugens*, the only other auchenorrynchan species in which this waveform was characterized so far (Hattori and Sogawa 2002). In this planthopper species, the presence of stylet pathway waveform at the beginning and at the end of the waveform was correlated visually with stylet insertion and removal, establishing that oviposition occurs while the stylets are inserted in the plant tissues. Hence, the similarity of these two waveforms suggests that stylet insertion and removal could also be happening during oviposition in *D. maidis*.

Dynamics of pathogen transmission can reinforce the interpretation of waveforms with respect to tissue localization and insect activity during different stages of probing behavior (Walker 2000). However, research correlating waveforms with transmission of phloem-associated pathogens has been very limited, not only in leafhoppers but also in other hemipterans. The only information available relates the inoculation of MCDV (maize chlorotic dwarf virus) to maize by *Graminella nigrifrons* (Wayadande and Nault 1993), MSV (maize streak virus) to maize by *C. storey* (Kimmins and Bosque-Perez

1996), TYLCV (tomato yellow leaf curl virus) to tomato by *Bemisia tabaci* (Jiang et al. 2000), and BYDV (barley yellow dwarf virus) to wheat by *Sitobion avenae* (Scheller and Shukle 1986) and by *R. padi* (Prado and Tjallingii 1994). In each of these cases, waveforms similar to our "waveform 4" were performed while stylet tips were inserted in phloem sieve tube members, without significant excretion of honeydew. Our results with *D. maidis* agree with those findings, in that the phloem-located pathogen *S. kunkelii* is inoculated during waveform 4, with little excretion of honeydew.

Information about acquisition of phloem-associated pathogens was reported only in the cases of *R. padi* acquiring BYDV (Prado and Tjallingii 1994) and *Myzus persicae* acquiring CaMV (cauliflower mosaic virus) (Palacios et al. 2002). In the first case, pathogen acquisition was highly correlated to phloem ingestion, reminiscent of our waveform 5 in the frequency of 5 Hz, its emf origin, and the excretion of basic honeydew. However, our results differ with those of Prado and Tjallingii, in that *S. kunkelii* can be acquired also during other waveforms; and the acquisition of CaMV by *M. persicae* (Palacios et al. 2002) that do not ingest from phloem could be due to the presence of this virus not only in phloem, but in mesophyll cells as well.

Our results related to honeydew excretion also agree with those of Wayadande and Nault (1996). First, waveforms 1 and 3 are similar to their "salivation" and "nonvascular probing" waveforms, in which no significant excretion of honeydew was detected. Also, the findings of Wayadande and Nault (1996) that both the "x-waveform" and the "phloem ingestion" waveform occur in phloem sieve tube members, but that honeydew is excreted only during the latter waveform, are equivalent to our findings on waveforms 4 and 5, respectively. These observations suggest that waveforms 4 and 5 of *D. maidis* take place with stylet tips inserted into phloem cells, as do waveforms 4 (designated as phloem salivation) and 5 (related to phloem ingestion and similar to our waveform 5) of *C. mbila* (Lett et al. 2001).

Despite the good correlations we obtained for the waveforms described above, interpretation of the biological activity of waveform 2 remains inconclusive. Data on honeydew pH and excretion rate agreed with those of Wayadande and Nault (1996), who related this activity to "nonsieve element ingestion" because salivary sheath tips were

found mostly in mesophyll cells. Similar results in *C. mbila* (Lett et al. 2001), were interpreted as active ingestion, either from mesophyll, bundle sheath or xylem cells. However, in *N. cincticeps* (Kawabe and McLean 1980), a waveform similar to our waveform 2 correlated with position of salivary sheath termini in xylem cells and excretion of honeydew at a rate higher than that seen for phloem ingestion (about 7 drops per minute). Hence, our results of honeydew excretion could be due to probing in mesophyll cells or in xylem cells for the brief periods observed for *D. maidis* (Table IV-1), probably until insects gained water lost during pre-experiment handling.

The name proposed for waveform 1 is "stylet pathway," because the waveform resembles analogous waveforms in other leafhoppers (Kawabe and McLean 1980, Rapusas and Heinrichs 1990, Wayadande and Nault 1996, Lett et al. 2001), histological examination indicates that probing activity during this waveform is limited to parenchymatous cells (Wayadande and Nault 1996), and there is negligible honeydew excretion and *S. kunkelii* transmission. Although the name "salivation" was used for this waveform in previous literature (Kawabe 1985, Wayadande and Nault 1996), we feel that "stylet pathway" describes more precisely the activities related to this waveform, which include not only secretion of saliva to facilitate stylet movement through plant tissues, but also rupture of cells during probing, and probably also ingestion of cell contents (Helden and Tjallingii 2000, Lett et al. 2001, Backus et al. 2005).

We propose that waveform 2 represents active ingestion, either from xylem or parenchyma cells. Indeed, this waveform is similar to "waveform 2" of *C. mbila* (Lett et al. 2001) and "waveform G" of *R. padi* (Prado and Tjallingii 1994) including a waveform frequency of 5 Hz and emf origin; and the positional information characterizing this waveform as of low relative amplitude, like the xylem ingestion waveform in *N. cincticeps* (Kawabe and McLean 1980) and in *N. virescens* (Rapusas and Heinrichs 1990). The origin of these similar waveforms has been correlated only in aphids (Tjallingii 1978) with suction of the cibarial pump to ingest xylem sap.

The finding that some insects acquired the phloem-associated pathogen *S. kunkelii* during this stylet pathway and/or active ingestion waveform(s) was not initially expected, but previous research on other vectors found similar results (Prado and Tjallingii 1994).

These authors stated that the low level of BYDV acquisition by *R. padi* females that did not produce a phloem-related waveform could be due to the puncture of sieve tube members without continuing the probe with a phloem salivation waveform. Similarly, it is possible that *D. maidis* punctures phloem cells without continuing with waveform 4 behavior, but acquires the pathogens either by passive ingestion because of positive phloem pressure (Tjallingii 2006) or by active sampling of phloem sap (Kawabe and McLean 1980). Inoculation of spiroplasmas could also occur during an accidental puncture of sieve tube members. However, it is likely that wound healing reactions would seal off the damaged cell(s) in the absence of phloem salivation, preventing or reducing the likelihood of further movement of spiroplasmas (Tjallingii 2006, Will et al. 2007). In contrast, the phloem-associated pathogens entering the insect would not be constrained in their movement, but would be able to colonize the insect body.

Interpretation of biological activity related to waveforms similar to our "waveform 3" has been difficult for a number of leafhopper species (Kawabe and McLean 1980, Kawabe 1985, Rapusas and Heinrichs 1990, Wayadande and Nault 1996, Almeida and Backus 2004). Indeed, the lack of significant honeydew excretion found by us in *D. maidis* has been interpreted, in other leafhopper species, as resting with the stylets in place within the plant tissues (Kawabe 1985, Rapusas and Heinrichs 1990) or as mesophyll ingestion (Wayadande and Nault 1996, Almeida and Backus 2004). Since mesophyll lacks a strong positive pressure, ingestion of this tissue would require active suction of cell contents by the cibarial pump; hence, confirmation of the activity related to this waveform would require correlation between the EPG output and cibarial pump pulses, as was carried out in *R. padi* for waveform "G" (Tjallingii 1978).

The biological activity related to waveform 4 is responsible for the inoculation of *S. kunkelii* to maize plants, and also for a high proportion of acquisition by insects when they probe in infected plants. These results agree with previous findings of inoculation of phloem-associated viruses by *G. nigrifrons* (Wayadande and Nault 1993), *C. storey* (Kimmins and Bosque-Perez 1996) and *Sitobion avenae* (Scheller and Shukle 1986). Indeed, it is known that spiroplasmas traverse the gut wall, multiply in the insect body and reach the salivary ducts after crossing the cells of the salivary glands (Wayadande and Fletcher 1995), which strongly indicates that these pathogens are inoculated during

salivation into host tissues. Also, as spiroplasmas colonize sieve tube members and the termination points of salivary sheaths were also traced to these cells (Wayadande and Nault 1996) strongly suggest that the biological activity related to this waveform takes place in association with these cells.

The biological activity associated with waveforms similar to waveform 4 of *D. maidis*, defined usually as x-waveform, has been a topic of debate since its first detection in the pea aphid, *Acyrtosiphon pisum* (McLean and Kinsey 1967), and its subsequent detection in leafhoppers (Chang 1978, Kawabe and McLean 1980, Wayadande and Nault 1993). In the seminal work of McLean and Kinsey (1967) it was suggested that both salivation (to block the plant defense response) and ingestion of small quantities of phloem sap would occur during this activity. Our results for *D. maidis* agree with this conclusion, because spiroplasmas move from one organism to the other during this waveform.

Recent evidence (Tjallingii 2006, Will et al. 2007) seems to prove the early hypothesis of McLean and Kinsey (1967) that phloem salivation would prevent the accumulation and polymerization of phloem contents leading to blockage around stylet tips, impairing insect ingestion. Additionally, it is likely that phloem sap is ingested passively, regulated by timely opening and closing of the precibarial valve (Lett et al. 2001). In *D. maidis*, increasing the frequency of waveform 4 to the same frequency as that of the waveform related to phloem ingestion could indicate that salivation events are "conditioning" the phloem to allow continuous ingestion. The increasing flow of phloem sap into the insect would make it necessary to block incoming sap flow more frequently as its speed increases. For this reason, the term "phloem salivation" does not define completely the events taking place. Hence, we propose the term "phloem conditioning" for this waveform, because this term reflects both salivation (proposed to inhibit host defenses) and ingestion (probably as a consequence of passive influx of phloem contents) occurring in phloem cells, as demonstrated by spiroplasma movement in both directions.

The fact that waveforms 4 and 5 occur without changes in the voltage level indicate that events related to both waveforms take place at the same cell type, and that waveform 5 is always preceded by waveform 4 suggests that salivation into the sieve tubes is

necessary to allow the initiation of waveform 5 (Wayadande and Nault 1993, 1996). Waveform 5 is proposed to be related to phloem ingestion, because of the phloem location of salivary sheath tips during this waveform (Wayadande and Nault 1996), the acquisition of spiroplasmas that takes place during this waveform and the excretion of basic honeydew.

In this work, major biological activities related to the probing behavior of *D. maidis* were correlated to different electrical waveforms, using an EPG approach. These results reinforce the conclusions made previously by Wayadande and Nault (1996) for this species, but provide additional detail and understanding of the biological activities that accompany plant tissue probing. Therefore, as has been noted for other insect species (Walker 2000), this work provides a basis from which to understand the dynamics of pathogen transmission and the mechanisms of plant resistance, and will be a valuable tool in the management of diseases whose pathogens are transmitted by *D. maidis*.

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CHAPTER V

PROBING BEHAVIOR OF *DALBULUS MAIDIS* (HEMIPTERA: CICADELLIDAE) HAVING ACCESS TO CORN STUNT-RESISTANT AND SUSCEPTIBLE MAIZE PLANTS

Abstract

Plant resistance can be an effective means to limit the damage caused by corn stunt, an important disease of maize (*Zea mays* L.) in the Americas. As *Spiroplasma kunkelii*, the causal agent, is transmitted primarily by the leafhopper *Dalbulus maidis*, we sought to determine if genotypes rated corn-stunt resistant in the field are leafhopper resistant. To achieve this objective we used electrical penetration graph (EPG) technology to measure the probing activities of *D. maidis* on susceptible and resistant plant genotypes. The probing behavior of this leafhopper is more dynamic, and its phloem feeding is more prominent, than was previously reported for this or other leafhopper species. The probing behavior of *D. maidis* was somewhat altered by the resistance level of the plant genotype. Compared to insects probing on the susceptible variety Golden Bantam, on which they were reared, insects probing in resistant hybrids salivated for longer periods of time before ingesting from phloem, and displayed increased xylem ingestion. However, phloem ingestion was not prevented in resistant hybrids during an access period of 20 hours, even though previous reports indicate that inoculation of spiroplasmas to plants is highly efficient during a 20 hours access period. Hence, it is likely that traits conferring resistance at earlier stages of the insect-plant interaction, play a more important role in the tested maize genotypes than do factors related to the probing behavior of *D. maidis*.

Introduction

Corn stunt is one of the most important diseases of maize (*Zea mays* L.) in the Americas (Bradfute et al. 1981, Bajet and Renfro 1989, Nault 1990). This disease and associated economic losses have increased in both geographical range and incidence during recent years as a result of the expansion of maize-producing areas, lack of resistant maize germplasm and continuous maize production (Hruska et al. 1996, Summers et al. 2004).

The causal agent of corn stunt, *Spiroplasma kunkelii* (Davis et al. 1972), is a bacterium (Class Mollicutes) transmitted by leafhoppers (Hemiptera: Cicadellidae) in a persistent-propagative manner (Nault 1980). The corn leafhopper, *Dalbulus maidis*, is the most important vector of the pathogen because of its prevalence from the southern United States to Argentina (Nault 1990, Hruska et al. 1996, Summers et al. 2004) and of the high efficiency with which it transmits the spiroplasma (Alivizatos and Markham 1986). The host range of *D. maidis* is somewhat restricted; it can be reared only on species of the Andropogoneae tribe (Gramineae), and can complete its life cycle only on maize and teosintes (Pitre 1967).

S. kunkelii is transmitted only by the leafhoppers and not by mechanical contact or through seeds. Thus, the behavior of these insects has a major influence on the amount of disease in the field. As *D. maidis* has a high rate of population increase and insects prefer young plants (Nault and Madden 1985, Madden et al. 1986, Nault 1990), many leafhoppers migrate from early to late-seeded maize, contributing to the high disease incidence in such plantings (Hruska et al. 1996). In some countries continuous maize production over all seasons provides a consistent, year-round food source for vector leafhoppers, supporting increased insect population size and, consequently, disease incidence (Hruska et al. 1996, Summers et al. 2004). For this reason, control measures to reduce the damage caused by leafhopper-borne spiroplasmas are often aimed at reducing the number of inoculative leafhoppers entering a maize field and/or the rate of insect population increase.

Since phytopathogenic spiroplasmas are transmitted during leafhopper feeding, the extent of insect-plant contact has a central significance on acquisition and inoculation of these pathogens. Indeed, the efficiency of both events rises from about 20 to 80% if the access time is increased from 1 to 48 h (Alivizatos and Markham 1986). Even more precisely, the efficiency of transmission of spiroplasmas could be related to the time spent probing in phloem (Chapter 2), in which *S. kunkelii* resides (Davis et al. 1972).

The development of corn stunt-resistant genotypes has been pursued as a sound alternative to insect chemical control to reduce the negative impact of this disease (Scott and Rosenkranz 1974, 1975, 1977). However, the inability to identify the nature of the resistance hindered the development of strategies aimed at durable resistance, such as the deployment and pyramiding of resistance genes (Cook et al. 1987, Hogenboom 1993). For this reason, the availability of a method to identify and characterize maize genotypes with resistance to corn stunt would contribute to the design of effective strategies for disease control.

The study of probing behavior using electrical penetration graph (EPG) technology can help to establish the mechanism and location, at the tissue level, of the traits conferring plant resistance. For example, using EPG it was found that leafhoppers exposed to resistant rice plants spend less time probing from phloem than do their cohorts exposed to susceptible cultivars, decreasing the efficiency of transmission of the phloem-inhabiting rice tungro viruses (Dahal et al. 1990a, Rapusas and Heinrichs 1990, Rezaul Karim and Saxena 1991). In addition, resistance factors can be located not only within phloem, but also at the tissues encountered successively, from the surface to the phloem sieve element, during probing (Walker 2000). Hence, EPG technology contributes to our understanding of the bases of plant resistance in pathosystems in which hemipteran vectors are involved (Helden and Tjallingii 2000), and can assist plant breeders to select and to characterize resistant genotypes (Wilkinson and Douglas 1998, Lett et al. 2001, Sauge et al. 2002).

The probing behavior of *D. maidis* was studied to analyze its efficiency as a vector of maize chlorotic dwarf virus (Wayadande and Nault 1993, 1996), but because of the low resolution limits of the EPG equipment available at that time and the short

acquisition access periods provided, the results of that study do not completely explain the probing behavior of *D. maidis* as a vector of persistently-transmitted pathogens. Furthermore, no information is available about how this probing behavior differs in plants expressing different levels of disease resistance. Such information could provide a basis to identify the mechanism(s) and target(s) (either insect or pathogen) of disease resistance.

In this work, we tested the hypothesis that the time spent by *D. maidis* probing in phloem will be higher in maize plants of susceptible genotypes than in resistant ones. The objectives were to analyze the probing behavior of *D. maidis* adults at the tissue level and to characterize the resistance to *D. maidis* in maize hybrids exhibiting different levels of field resistance to corn stunt.

Materials and Methods

I - Insects and plants

A colony of healthy *D. maidis* DeLong and Wolcott was initiated at Oklahoma State University in 1997 from insects obtained from L. R. Nault, Ohio State University (Wooster, Ohio). This Ohio State University colony was started initially from insects collected in Mexico, and was maintained on plants of sweet corn (*Zea mays*, L) var. Golden Bantam, at a constant temperature of 26 °C, with a 16:8 h (light:dark) photoperiod (Nault 1980). Insect and plant maintenance were carried out in aluminum-framed cages (Wayadande and Fletcher 1995), placed in a growth room at a constant temperature of 26 °C, with a 16:8 h (light:dark) photoperiod (Nault 1980).

Four maize hybrids, developed in geographical areas of Brazil in which corn stunt disease occurs, were tested. The hybrids, designated X1273A (7), X1E73 (1), X1286B (3) and X1297J (6), were provided by Pioneer Hi-Bred International, Inc. For each hybrid, the number in parentheses above indicates the level of corn stunt field resistance, estimated on a scale of 1 to 9, 1 being highly susceptible and 9 immune (highly resistant). These four hybrids were grouped into two pairs, X1273A-X1E73 (with resistance levels 1 and 7), and X1297J-X1286B (with resistance levels 6 and 3). Each pair (X1273A-X1E73 and X1297J-X1286B) included hybrids with a common ancestor, but being one

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resistant and the other susceptible. Variety Golden Bantam served as a susceptible control. In all the experiments, plants were used at the three-leaf stage. The youngest fully expanded leaf of each plant was folded over a horizontally-placed plastic cylinder to expose the abaxial surface, preferred by *D. maidis*.

II - EPG recordings

Ten to fourteen-day-old *D. maidis* females were anesthetized by confining them singly in glass tubes (1.5 X 15 cm), which were incubated on ice for 2-3 minutes. Immobilized insects were placed with the help of an "abdomen" type aspirator (sized to fit tightly around the abdomen), on a stage constructed under a stereomicroscope (Nikon 530 220, Melville, NY), and secured by application of a gentle vacuum. Insects were tethered to an 18.75 µm-diameter, 2-3 cm-long gold wire (Sigmund Cohn, Mount Vernon, NY) using silver conductive paint (Ladd Research Industries, Burlington, VT) to attach the wire to the pronotum. After tethering, insects were starved for 1 hour before recordings were initiated.

EPG recordings were performed using a four-channel, Type 3 AC/DC Missouri monitor (Backus and Bennett 1992) for a period of 20 hours. Throughout the test, constant equipment settings consisted of: alternate current (AC) with 100 mV, 1000 Hz substrate voltage, input resistance of 10^8 Ω, and amplification (gain) of 500 X. Substrate voltage was applied through an electrode inserted into the soil, and the gold wire was connected to the electric circuit by gluing it to a copper wire (around 0.5 mm diameter) soldered to a brass nail that was inserted in the output of the electric circuit. The insects and plants were placed inside a Faraday cage (2' X 2' X 4', constructed of an aluminum frame with a steel base) to reduce the interference of external electrical noise. As five plant genotypes were tested and the monitor has only four channels (allowing four insect-plant combinations to be monitored simultaneously), both the single plant genotype being excluded and the channel assigned for the other genotypes each day were selected at random using a stochastic table. To avoid differences in probing behavior that could be due to variations in circadian cycle, the daily recordings always started at 5:00 pm.

Output waveforms were converted to digital format at 100 samples per second using a DI-720 analog-to-digital board, acquired with Windaq/Pro software (Dataq Instruments,

Akron, OH), and stored in a hard drive. Outputs of EPG recordings were inspected visually, and notes on the types of waveforms and the time of appearance of each waveform were recorded. Waveform activities were correlated with probing activities as described earlier (Chapter 2). Twenty-one insect-plant combinations were recorded per plant genotype, using new insects and plants for each replication.

III - Statistical analysis

Three types of parameters were considered for the statistical analysis: a descriptive statistic of non-sequential parameters (Backus et al. 2007), an analysis of conditional probabilities of different probing activities using the sequence of waveforms (Wayadande and Nault 1996, Backus et al. 2007), and a series of sequential parameters able to distinguish resistance located in phloem and non-phloem tissues (Helden and Tjallingii 2000).

In all the cases the numerical values for each parameter across the plant genotypes were compared using analysis of variance (ANOVA), and subsequent pairwise comparisons were made by Fisher's LSD test ($p<0.05$). Data were log-transformed previous to the ANOVA test.

Results

Table V-1 shows parameters of the probing behavior of *D. maidis* on the susceptible variety, Golden Bantam. All insects displayed all probing behaviors except that nonvascular probing occurred in only about 50% of the insects in each maize genotype (results not shown). Insects spent most of the time (95%) with their stylets inserted into plant tissues. The activities of longest duration were phloem ingestion (62% of the total time) and phloem contact (15%), phloem-related activities together comprising up to 15.2 hours (77%). The insects also spent 2.2 hours (9% of the total time) probing in other plant tissues, including active ingestion and nonvascular probing.

D. maidis probed actively into plant tissues of the susceptible variety, Golden Bantam. An average of 80.1 probes was recorded within a period of 20 hours, with the probes interrupted by short non-probing periods (averaging 1 min each). The average duration of the probes not including phloem phase was 1.5 minutes, and most of these

included only brief penetrations. On the other hand, although far fewer included probing in phloem (8.2 times for phloem contact, of which 4.2 included phloem ingestion), those that involved phloem contact lasted significantly longer than those that did not (21 minutes for x-waveform and 178 minutes for phloem ingestion).

Table V-1: Nonsequential parameters of probing behavior of *D. maidis* females having access to maize plants of the susceptible variety Golden Bantam.

Activity	WDI	NWEI	WDEI
Nonprobing	82.4 ± 30.8	80.1 ± 41.6	1.03 ± 0.08
Nonvascular Probing	35.6 ± 8.8	3.0 ± 2.1	11.9 ± 1.7
X-Waveform	178.1 ± 12.8	8.2 ± 4.7	21.7 ± 2.3
Phloem Ingestion	730.6 ± 31.8	4.2 ± 1.6	174.0 ± 27.0
Pathway	82.9 ± 7.8	81.0 ± 41.4	1.02 ± 0.08
Active Ingestion	90.6 ± 15.5	11.3 ± 5.8	8.1 ± 0.94

WDI: Waveform duration per insect (total time an insect spent in each activity, in minutes). NWEI: number of waveform events per insect (number of times that each activity was detected). WDEI: waveform duration per event per insect (average duration of each activity, in minutes).

The probing behavior of *D. maidis* females on the susceptible variety, Golden Bantam, can be explored from another perspective by an analysis of conditional probabilities (Wayadande and Nault 1996). This approach considers the statistical likelihood that each probing behavior will be followed by each of the remaining ones. Figure V-1 shows that nonprobing is always followed by the stylet pathway waveform, and in most (76%) of the cases the insects finish the probe without any other activity. Numerous short probes take place before the insect succeeds in ingesting from the target tissue, and most of these brief probes occur before phloem ingestion.

The right side of Figure V-1 shows the probing behavior associated with activities not related to phloem tissue. Insects ingested from xylem/mesophyll in 13% of the events after the stylet pathway, and such active ingestion was usually followed (63% of the instances) by stylet removal. However, in 28% of the cases active ingestion was followed by pathway waveform, and in 8% of the cases the insect changed to nonvascular probing. This nonvascular probing was followed sometimes by removal of stylets, but other times (43%) by active ingestion.

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The phloem phase of the probing behavior is shown on the left side of Figure V-1. In ten percent of the instances stylet pathway continued with phloem contact waveform, of which half (51%) then progressed to phloem ingestion. If phloem ingestion did not occur, the most common behavior was removal of the stylets (41% of the events leading to nonprobing), rather than continuation of probing (8% returning to stylet pathway). Phloem ingestion was the most common terminal pattern, because it was followed in 82% of the cases by removal of stylets, and in only 10% of the cases by stylet pathway. The remaining 8% returned to phloem contact waveform, consisting of short periods of salivation interspersed within long phloem ingestion phases.
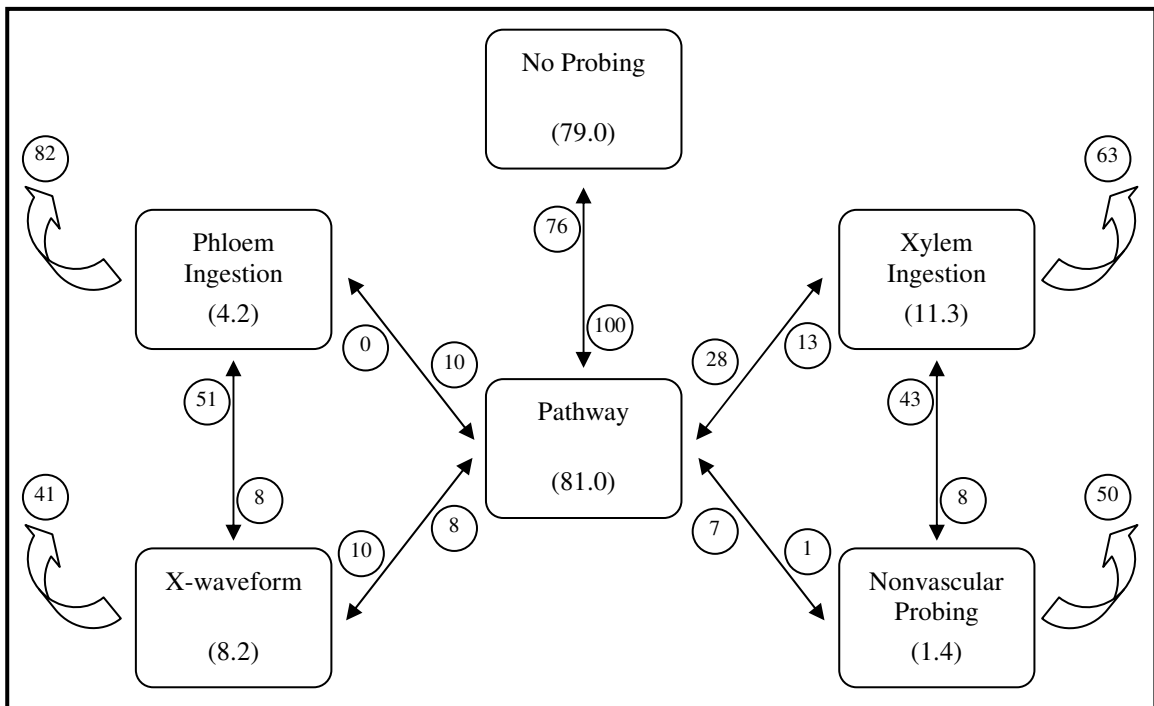
Figure V-1: Kinematic diagram of probing behavior of adult *D. maidis* females on the susceptible maize Var. Golden Bantam. The values inside the boxes indicate the average number of times that these events occurred per insect. Circled numbers near each arrowhead indicate the percentage of times that the preceding pattern is followed by the behavior next to this value. Thick arrows indicate the percentage of times that the precedent pathway was followed by no probing.

Table V-2 presents selected sequential parameters that reveal the dynamics of the probing behavior of *D. maidis*. Insects started probing quickly (within about 3 minutes) after settling on maize leaves of the susceptible variety, Golden Bantam. Most of the insects started active ingestion after 3 to 5 probes, within about 7 minutes. Although only one insect out of 21 ingested from phloem before doing so from xylem/mesophyll, the anomalous case resulted in the high average (45 minutes) "time to first active ingestion."

After an initial phase of active ingestion, the insects started another series of brief probes that lasted until they established phloem contact. These probes were similar in both duration and frequency (interval between two consecutive probes) to those occurring previous to active ingestion, but more probes were made before a phloem contact was reached (Table V-2). The phloem contact lasted about 35-45 minutes before phloem ingestion started, and the phloem ingestion period ranged from 10 minutes to 15 hours, taking place in late stages of the insect-plant interaction (138 minutes).

Table V-2: Sequential parameters of probing behavior of *D. maidis* females on maize plants of the susceptible variety, Golden Bantam.

Duration of specific behaviors (min)	
Stylet pathway before first phloem contact	1.4 ± 0.8
Stylet pathway before first phloem ingestion	1.4 ± 1.1
Phloem contact before first phloem ingestion	35.0 ± 11.5
Time between exposure and waveform initiation (min)	
First pathway	2.9 ± 2.1
First active ingestion	45.9 ± 85.4
First phloem contact	83.7 ± 66.4
First phloem ingestion	138.1 ± 94.7
Other sequential parameters	
Phloem ingestion index (%)[a]	69.6 ± 9.2
Phloem phase index (%)[b]	82.5 ± 7.8
Number of probes before first phloem contact	17.4 ± 13.5
Number of probes before first phloem ingestion	21.4 ± 14.8

[a] Phloem ingestion index: percentage of time the insects spend ingesting in phloem after their first established phloem contact. [b] Phloem phase index: percentage of time the insects spend probing in phloem after their first established phloem contact.

Table V-3 presents nonsequential and sequential parameters of probing behavior for which, in this study, significant differences were found during *D. maidis* probing on plants of different maize genotypes. The table does not show analysis of the conditional probabilities (Wayadande and Nault 1996), because no differences were found in any of those parameters. As the insect population used in our experiments had no previous contact with any of the four Brazilian maize hybrids used in this study, the variety Golden Bantam was included as a susceptible control. The use of this control treatment should have permitted the identification of traits potentially conferring resistance in

maize hybrids X1E73 and X1286B that could have been overcome by the pressure of local leafhopper populations, as was reported in the case of the rice leafhopper, *Nephotettix virescens*, and rice (Dahal et al. 1990b). For this reason, leafhopper probing behavior on each resistant maize hybrid was compared with that of both the susceptible maize hybrid counterpart and Golden Bantam.

TableV-3: Probing behavior of *D. maidis* on maize plants of different genotypes.

	Maize Genotype				
	G. Bantam	X1273A (7)	X1E73 (1)	X1297J (6)	X1286B (3)
Duration of waveform per event (WDE)					
Average	6.29 ± 0.53 b	5.07 ± 0.45 a	5.69 ± 0.51 a	5.02 ± 0.38 a	5.42 ± 0.47 a
Nonprobing	0.78 ± 0.04 c	0.78 ± 0.04 c	0.68 ± 0.04 a	0.73 ± 0.03 b	0.60 ± 0.02 a
Stylet Pathway	1.02 ± 0.04 a	1.07 ± 0.03 a	1.33 ± 0.28 c	1.14 ± 0.04 b	1.15 ± 0.03 b
Active Ingestion	7.99 ± 0.74 b	8.07 ± 0.75 b	7.66 ± 0.81 ab	6.03 ± 0.64 a	6.55 ± 0.56 a
Characteristics of waveforms per probe					
WDP (SP)	0.95 ± 0.04 a	1.03 ± 0.04 ab	1.23 ± 0.20 ab	1.00 ± 0.04 a	1.09 ± 0.04 b
WDP (AI)	6.86 ± 0.67 b	8.29 ± 0.93 b	6.54 ± 0.69 ab	5.52 ± 0.65 ab	6.81 ± 0.78 ab
NWEP (SP)	1.056 ± 0.007a	1.070 ± 0.008b	1.067 ± 0.007b	1.073 ± 0.007b	1.067 ± 0.007b
Characteristics of waveforms per insect					
NWEI (SP)	81.0 ± 41.4 a	101.0 ± 55.5 ab	87.0 ± 37.6 a	90.2 ± 52.1 ab	103.1 ± 48.0 b
NWEI (AI)	11.3 ± 5.8 a	16.3 ± 8.6 b	12.2 ± 7.6 ab	16.2 ± 9.1 ab	19.8 ± 9.6 b
WDEI (AI)	7.3 ± 0.9 ab	9.3 ± 1.6 b	8.2 ± 1.6 ab	6.1 ± 0.6 a	5.5 ± 0.7 ab
Sequential parameters					
Phloem phase index	82.5 ± 7.8 b	74.1 ± 13.6 a	80.4 ± 12.8 ab	77.5 ± 13.2 ab	77.6 ± 11.5 ab
# probes before PI	21.4 ± 14.8 a	44.6 ± 47.6 ab	36.1 ± 22.2 ab	29.8 ± 24.6 ab	36.8 ± 36.8 ab
Length SP before PC	1.5 ± 0.8 a	2.1 ± 1.1 bc	1.3 ± 0.7 a	2.6 ± 0.8 c	1.5 ± 1.2 ab
Length PC before PI	35.0 ± 11.5 a	49.1 ± 16.8 b	48.3 ± 18.4 b	48.5 ± 20.5 b	43.8 ± 16.2 ab

Numbers between parentheses indicate the field disease resistance rating in (1: susceptible, 9: immune). Different letters indicate significant differences by Fisher's LSD test (p<0.05) for each parameter. All durations are in minutes. WDE: waveform duration per event (average length of each waveform event). WDP: waveform duration per probe (average duration of each waveform type per probe). NWEP: number of waveform events per probe. NWEI and WDEI: described in Table V-1. PI: phloem ingestion. SP: stylet pathway. PC: phloem contact. AI: active ingestion.

Insects having access to the resistant maize hybrid X1273A had longer nonprobing periods than insects probing on the susceptible hybrid X1E73 (Table V-3). Moreover, average stylet pathway waveforms prior to phloem contact were shorter in the resistant maize hybrid X1273A, but the stylet pathway waveforms were longer than those of insect

probing on the susceptible hybrid X1E73. In addition to these parameters, the behaviors of insects probing on hybrid X1273A and those on Golden Bantam differed even more, because insects on the resistant hybrid X1273A had longer phloem contact before starting phloem ingestion. In addition, the phloem phase index indicates that insects on hybrid X1273A spent less time probing from phloem after they made their first phloem contact than did those probing on Golden Bantam, which implies a lower phloem acceptance on the hybrid (Helden and Tjallingii 1993, Helden and Tjallingii 2000). Also, the nonsequential parameters NWEP (number of waveform events per probe) and average duration per event indicate that the probes in hybrid X1273A are more complex, with more frequent changes in activities than those in Golden Bantam.

When compared to insects probing on the susceptible hybrid X1286B, those probing on the resistant hybrid X1297J produced shorter stylet pathway waveforms, and the no probing periods were longer. However, stylet pathways prior to phloem contact were longer in the resistant maize hybrid X1297J than in its susceptible counterpart. The comparison of the probing behavior between X1297J and Golden Bantam also indicated that insects probing on the former had longer phloem contact before starting phloem ingestion and a shorter average duration of waveforms. The same trend as in X1297J was found for X1273A, with probes including more events of shorter average duration per event, indicating more frequently changes in activity during the same probe in the resistant hybrid than in the susceptible variety, Golden Bantam.

Discussion

To characterize maize resistance to the leafhopper *D. maidis*, we began by reviewing the probing behavior of this insect because the 2-hour probing period assayed in previous work on this leafhopper (Wayadande and Nault 1996) might not be long enough to completely characterize a pathosystem in which highly efficient transmission of spiroplasmas requires long periods of insect-plant contact. Also, the lower sensitivity of the EPG equipment available at the time of the 1996 study was non-optimal to identify subtle differences in the probing behavior of *D. maidis* feeding on susceptible versus

resistant plants. For this reason, a more detailed characterization of the probing behavior of *D. maidis* was carried out.

The most noticeable difference between the results obtained in this study and in that of Wayadande and Nault (1996) is the proportion of insects achieving phloem contact and phloem ingestion. In this work, 80-90% of the insects made phloem contact, 40-80% (depending on the plant genotype) ingested from phloem within 3 hours, and all the insects ultimately reached phloem ingestion. In Wayadande and Nault's work (1996), in contrast, only 31% of the insects reached phloem, and 26% had ingested by the end of the 3-hour period. The differences between our results and those of Wayadande and Nault might relate in part to our use of intact plants to monitor the probing behavior. The excised leaves used in the previous study might have had altered chemical or physical properties of phloem sieve tube members, making this tissue less suitable for insect feeding. Also, more insects might have reached phloem ingestion in the earlier study if the recording had been extended for longer time periods.

Our results indicate that insects spend 78% of their probing time in phloem-related activities, 8% in active ingestion and 2% in nonvascular probing. In the work of Wayadande and Nault, phloem-related activities covered 36% of the time, while 26% of the time was spent in nonvascular probing and 30% in nonsieve element ingestion. Also, our results indicate that insects spend a high proportion (around 80%) of the time probing in phloem after they establish the first contact with that tissue, as described for other phloem-feeding hemipterans (Helden and Tjallingii 1993, Helden and Tjallingii 2000). These results indicate clearly the preference of *D. maidis* to probe in phloem over other tissues. However, like Wayadande and Nault (1996), we did observe early probing on non-phloem tissues, as demonstrated by the steady xylem ingestion at the beginning of the access period to plants, possibly to ameliorate dehydration (Saxena and Khan 1985, Kimmins 1989, Spiller et al. 1990).

Limiting insects' access period to plants could lead to underestimation of the importance of phloem probing by these insects. For instance, with *Nephotettix virescens* (Khan and Saxena 1985, Rapusas and Heinrichs 1990, Rezaul Karim and Saxena 1991), *N. malayanus* and *N. nigropinctus* (Rezaul Karim and Saxena 1991), *Graminella*

nigrifrons and *G. oquaka* (Wayadande and Nault 1996) the time spent in phloem feeding during a 3-hour period was also around 20 to 35 percent of the total accession time. However, it is possible that the phloem probing of these leafhopper species is also more prevalent at later stages of the insect-plant interaction, as we found for *D. maidis*.

The durations of several probing activities of *D. maidis* are similar to those of the leafhopper *Cicadulina mbila* (Lett et al. 2001). In the latter species, stylet pathway probing lasted an average of 1.5 minutes, active ingestion 2.4 minutes, phloem salivation 30 minutes, and phloem ingestion 85 minutes, similar to the durations measured for *D. maidis*. Considering the length of the salivation prior to phloem ingestion for both leafhoppers is 35-45 minutes, there is a big difference in length of phloem salivation between leafhoppers and other hemipteran species. For instance, in both aphids (Sauge et al. 1998, Tjallingii 2006) and whiteflies (Lei et al. 1999, Jiang et al. 2001), insects that access interior feeding sites by moving their stylets between cells, salivation in phloem typically occupies less than 5 minutes before phloem ingestion is attained.

Our conditional probabilities analysis of the probing behavior of *D. maidis* yielded results similar to those of Wayadande and Nault (1996). The minor differences between the two studies are probably due to the shorter recording period used in the 1996 study. For instance, it is likely that our finding of a lower proportion of probes leading to sites other than phloem is because such probing activities occur early in the in insect-plant interaction, and hence their relative importance decreases with time, in contrast to phloem-related activities. In addition, the lower proportion of phloem contacts ending in phloem ingestion, found in our assays, can be explained by the presence of some interrupted x-waveforms, which are not identifiable at the resolution level available in the earlier work. Finally, we detected some cases in which the phloem ingestions were interrupted by short x-waveforms. Such transitions were not identified by Wayadande and Nault, probably because of the low resolution of their system, in which the final stages of the phloem contact and the start of phloem ingestion are seen as a flat line.

Wayadande and Nault (1996) found that for *Graminella nigrifrons*, *G. oquaka* and *Ablysellus grex* the so-called nonsieve element ingestion (likely to be active ingestion) was preceded by x-waveforms, salivation (stylet pathway), or nonvascular probing. These

last two transitions were confirmed in our results with *D. maidis*, in the case of early probes ending in active ingestion, or the switch between active ingestion and nonvascular probing. However, the higher level of resolution available in this study allowed us to determine that whenever phloem contact is followed closely by active ingestion, a short stylet pathway waveform occurs between the two. This short pathway waveform suggests a change in cellular location of the stylet tips during the stylet pathway waveform, a phenomenon that might have been found in *Graminella* and *Ablysellus* by using a more sensitive system.

Probing behavior has been studied extensively in several different leafhopper species, including *Nephotettix* spp. (Saxena and Khan 1985, Dahal et al. 1990a, Rapusas and Heinrichs 1990, Rezaul Karim and Saxena 1991), *Cicadulina* spp (Kimmins and Bosque-Perez 1996, Lett et al. 2001), *Graminella* spp. and *D. maidis* (Wayadande and Nault 1996). However, only Lett and coworkers analyzed the moment of occurrence of the different activities during probing. *C. mbila* starts probing at around 0.7 minutes, reaches active ingestion in 6 minutes, phloem salivation in 85 minutes and phloem ingestion in 131 minutes (Lett et al. 2001). These results are similar to those we found for *D. maidis*, except that the latter insect required a longer time to first probe. However, this parameter is not a reliable indicator of probing behavior, because it is strongly influenced by the handling of the insects before the recording (Helden and Tjallingii 2000).

The use of EPG technology has demonstrated that the probing behavior of several leafhopper species differs depending on whether insects are given access to resistant or susceptible plants. Indeed, phloem ingestion decreased and xylem ingestion increased when several *Nephotettix* spp fed on insect-resistant plants (Kawabe 1985, Khan and Saxena 1985, Rapusas and Heinrichs 1990, Rezaul Karim and Saxena 1991). In contrast, the allocation of *D. maidis* probing time in different tissues was not largely influenced by the level of resistance or susceptibility of the maize genotypes tested in our experiments. However, investigation of the distribution of waveforms at different hierarchic levels of the probing behavior (Backus et al. 2007), and measurement of specific landmarks in the insect's probing behavior (Helden and Tjallingii 1993, Helden and Tjallingii 2000) allowed identification of several insect-resistance traits in both hybrids with field resistance to corn stunt.

A few probing behavior parameters were different in corn stunt resistance maize hybrids compared to their susceptible counterparts. In both resistant hybrids, longer stylet pathways durations were observed prior to phloem ingestions than was the case in the susceptible hybrids. There were also more waveform events in resistant plants (active ingestion per insect in X1273A and stylet pathway per probe in X1297J) than in susceptible ones. These data indicate that in the resistant hybrids there was greater probe complexity than displayed on the susceptible hybrids, encompassing a higher number of different probing activities in a single penetration into the plant tissue (Backus et al. 2007). According to previous research in different aphid species (Helden and Tjallingii 1993, Helden and Tjallingii 2000), these parameters indicate the presence of an insect resistance factor located in the epidermis or mesophyll. The shorter duration of stylet pathway waveform events, together with longer non-probing periods, support the presence of such a resistance factor in both hybrids. Both of these behaviors are consistent with insect stylet removal, in response to detection of the resistance factor.

Differences in the probing behavior of *D. maidis* on resistant hybrids, compared to that on var. Golden Bantam, suggest that insects raised on this susceptible variety encounter, in addition, a resistance factor located in the phloem. A longer x-waveform duration on the resistant cultivars was explained to be necessary to prevent clogging of phloem cells and allow ingestion (Will et al. 2007). Such phloem resistance may be stronger in X1273A than in X1297J, as reflected by the proportion of time spent in phloem probing after the first phloem contact (Helden and Tjallingii 2000). However, once this initial resistance was overcome, the time spent probing in phloem was equivalent in resistant and susceptible genotypes, and the leafhoppers were able to probe in phloem and hence transmit the spiroplasmas efficiently to all the genotypes analyzed (Chapter 1). This result is in contrast to that reported for the leafhopper *N. virescens* (Dahal et al. 1990a, Rapusas and Heinrichs 1990, Rezaul Karim and Saxena 1991), for which phloem probing duration was significantly reduced on resistant plants, lowering the efficiency of transmission of phloem-associated viruses.

EPG is a useful technique to characterize insect resistance in plants (Lei et al. 1999, Helden and Tjallingii 2000), detecting differences in the probing behavior that are typically not detected in other biological tests. However, despite such differences, both

the EPG results and the spiroplasma inoculation efficiency indicate that the insects overcame host resistance in less than 1 day of access to plants. For this reason, it is likely that other types of traits are associated with the field resistance to corn stunt of these hybrids. Our laboratory tests forced leafhoppers and plants into close contact, from which the insects were unable to escape. It is possible that resistance of these hybrids is detected by insects at a broader range, perhaps preventing insect settling on resistant plants. Our results emphasize the need for a detailed characterization of maize resistance to corn stunt, to minimize the damage caused by this disease.

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CHAPTER VI

GENOMIC VARIABILITY OF THE PHYTOPATHOGENIC MOLLICUTE

SPIROPLASMA KUNKELII

Abstract

Corn stunt disease has become a limiting factor in some areas of the Americas in recent years. Although resistant maize genotypes have been developed in the past, this resistance has been unstable in different geographical locations or through time. To better understand disease components that could affect the stability of host resistance, we assessed the genome variability of the etiologic agent, *Spiroplasma kunkelii*. Isolates were obtained from a number of geographical locations, and molecularly characterized by amplification of several regions of the spiroplasmal chromosome and sequencing of specific genes and amplification products. Polymorphism among isolates was related in part to viral insertions, but was also found in the nucleotide sequence of *Skarp*, a gene that encodes a membrane protein implicated in attachment to insect cells. The degree of polymorphism among isolates of different geographic origin was low, and the level of genomic variability was similar among isolates of different countries. The results suggest that the genome composition of this species is highly conserved among isolates of different geographical origin. Hence, it is unlikely that instability of maize resistance is due to generation of new pathotypes of *S. kunkelii*. Instead, other components of this complex pathosystem could account for the breakdown of resistance.

Introduction

The mollicute *Spiroplasma kunkelii* is one of three known phytopathogenic spiroplasmas, together with *S. citri* and *S. phoeniceum* (Fletcher et al. 2006). *S. kunkelii* is transmitted in a propagative manner by *Dalbulus maidis* and, less frequently, by several other leafhopper species, (Nault 1980), causing one of the most important maize diseases in America, corn stunt (Bradfute et al. 1981, Bajet and Renfro 1989, Nault 1990). The prevalence of this disease has been increasing in recent years due to the extension of maize cultivation to an almost year-long process (Hruska et al. 1996, Summers et al. 2004), and to the lack of consistently resistant maize germplasm (Jeffers 2002, Silva et al. 2003).

Although maize genotypes resistant to corn stunt have been obtained by selecting disease-free plants in the field (Scott and Rosenkranz 1974, 1975, Jeffers 2002), such genotypes have usually become susceptible after a period of time or in other geographical locations (Silva et al. 2003). At present, neither the target(s) nor the mechanism(s) of resistance have been characterized in maize, and it is not known whether the insect vector or the pathogen itself can overcome such resistance. For this reason, as part of a strategy to characterize the components of corn stunt disease, we sought to estimate genomic variability of *S. kunkelii*, and to determine its potential ability to generate new strains and to overcome host resistance.

Several mechanisms have been identified to cause variability in the genome of spiroplasmas. Changes in the DNA sequence can occur because of nucleotide substitutions during DNA replication or repair (Melcher and Fletcher 1999), deletion, rearrangement or duplication of DNA fragments (Robertson and Meyer 1992, Dybvig and Voelker 1996, Melcher and Fletcher 1999), integration of extrachromosomal DNA into the spiroplasmal chromosome (Nur et al. 1986a, Nur et al. 1987, Melcher and Fletcher 1999, Sha et al. 2000), or DNA exchange with other cells (Barroso and Labarere 1988). In particular, spiroplasma viruses can be an important source of genomic variability (Mouches and Bove 1983, Ye et al. 1996, Melcher and Fletcher 1999, Melcher et al. 1999), because viral DNAs are usually found integrated into the chromosome in one

or more copies, either intact or fragmented (Mouches and Bove 1983, Ye et al. 1996, Melcher et al. 1999, Sha et al. 2000, Bai and Hogenhout 2002).

Although a high level of genomic variability is characteristic of other members of the Class Mollicutes, such as *Mycoplasma* species (Dybvig and Voelker 1996, Citti et al. 2000, Rocha and Blanchard 2002), information available on the genus *Spiroplasma* is limited and unlikely to reflect a clear picture of its variability. For example, distantly related strains of *S. citri* were not consistently distinguished using a REP-PCR approach (Mutaqin 2005); but were discriminated by RAPD-PCR (Mello et al. in press) and by analysis of viral insertions located along the spiroplasmal chromosome (Bebear et al. 1996). Moreover, this species can undergo rearrangements of its DNA sequence over brief time intervals when subjected to different propagation histories (Fletcher et al. 1996).

In the characterization of *S. kunkelii* isolates performed so far (Gomes et al. 2004), the sequence of the gene encoding the membrane protein, spiralin, was almost identical across isolates collected in different areas of Brazil. However, the recent availability of the almost-complete genome sequence of *S. kunkelii* CR2-3X and the characterization of a set of isolates covering a broad geographical range should provide a better understanding of genome variability of this species. Features that might indicate active mechanisms of genomic variability in CR2, are the presence of DNA sequences of viral origin (Melcher et al. 1999, Bai and Hogenhout 2002) and of genes for horizontal movement of DNA (Bai et al. 2004, Davis et al. 2005, Wei et al. 2006), which can generate genetic diversity. Additionally, the genes of several membrane-associated proteins identified in the genomes of *Spiroplasma* species (Yu et al. 2000, Berg et al. 2001, Davis et al. 2005) are similar to those of highly variable proteins in *Mycoplasma* species (Citti et al. 2000, Rocha and Blanchard 2002, Comer et al. 2007), and are potentially variable in *Spiroplasma* spp as well.

The hypothesis tested in this work was that genome composition of *Spiroplasma kunkelii* varies in isolates of different geographical origin. The objectives were to characterize *S. kunkelii* isolates from diverse geographical and ecological habitats

through assessment of DNA sequence variability and to estimate the potential of this species to generate new genotypes.

Materials and Methods

I – Spiroplasma isolation and culture

Strains of *S. kunkelii* were obtained, through cooperation with researchers in Latin America and the United States, from a variety of different geographical locations and collection dates (Table VI-1). Spiroplasmas were isolated from symptomatic plants, which were previously tested by PCR using primers F2-R6 (Barros et al. 2001) to confirm the presence of *S. kunkelii*.

The spiroplasmas were isolated from leaf midribs, which were first surface sterilized by successive immersions in 70% ethanol, 10% commercial bleach, and 3 rinses in sterile water, for one minute each. The midribs were minced into pieces of about 2 mm^2 while immersed in 5 ml of LD8A3 broth (Lee and Davis 1989). After waiting five minutes to allow spiroplasma movement out of the plant tissues, the broth was filtered (0.22 µm) to remove walled bacteria. Immediately, 50 µl of the filtrate were added to 5 ml of fresh medium to initiate a "blind subculture", and the tubes were incubated at 31 °C.

The titer of spiroplasma cultures was estimated every other day by direct counts using dark-field microscopy at a magnification of 1250X. When the titer reached about 10^8 cells/ml, the cultures were aliquoted and stored (-80 °C), before the depletion of the medium nutrients affected the viability of cells, which was assessed as increased cell length and loss of helicity. For use in this study, 50 µl thawed culture was added to 25 ml fresh medium, which was used for DNA extraction when the titer reached 10^8 cells/ml.

II - DNA extraction

Total DNA was extracted using the CTAB method (Doyle and Doyle 1994). Briefly, 0.05 g of each plant leaf sample was ground with a mortar and pestle in the presence of liquid nitrogen. Ground material was incubated in a water bath for 60' at 60 °C in 5 volumes (V) of CTAB buffer to disrupt cell structures. Organic compounds were degraded by adding 1 V of chloroform:isoamyl alcohol (24/1) and vigorously stirring for 5'. Organic and DNA-containing phases were separated by precipitation for 10' at 12000

g in a bench microcentrifuge. The upper phase was recovered to a tube containing 1 V of cold isopropanol and incubated at -20 °C for 2 hours. DNA was precipitated by centrifugation for 10' at 12000 g, and the resulting pellet was washed with 70% ethanol, dried and resuspended in 100 µl of sterile water. The concentration of DNA was measured in a spectrophotometer (Nanodrop® ND-1000, Wilmington, DE), and an aliquot diluted to a final concentration of 4 ng/µl. To extract DNA from spiroplasma cultures, these were concentrated by centrifugation for 30' at 20000 g. The supernatant was discarded and 600 µl of CTAB buffer was added to the resulting pellet prior to incubation.

Table VI-1: Isolates of *S. kunkelii* used in this study.

Isolate	State	Country	Date Collected	# Pass
AR01	Tucumán	Argentina	7/28/2002	1
AR02-AR11	Chaco	Argentina	1/5/2006	1
AR12-AR14	Santiago del Estero	Argentina	1/5/2006	1
AR15	Santa Fe	Argentina	5/20/2006	1
AR16	Córdoba	Argentina	5/20/2006	1
AR17	Tucumán	Argentina	5/20/2006	1
AR18-AR23	Córdoba	Argentina	1/3/2007	1
BR01-BR03	Minas Gerais	Brazil	3/5/2007	1
CA01-CA31	California	United States	10/272006	1
CR2	Alajuela	Costa Rica	6/25/1987	8
FL01	Florida	United States	5/25/1987	Unknown
ME01	Unknown	Mexico	Unknown	Unknown
ME02-ME06	Jalisco	Mexico	11/28/2005	1
R8A2B (*S. citri*)	Unknown	Morocco	1985	Unknown
23-6 (*S. floricola*)	Maryland	United States	1978	Unknown
TS2 (*S. melliferum*)	Maryland	United States	1/6/1982	9
S. phoeniceum	Unknown	Unknown	1982	42

Pass: number of passage after isolation of tissues, at which DNA was extracted.

III - Detection of DNA polymorphisms

Several sets of primers were used to amplify different parts of the spiroplasma genome (Table VI-2). BOX, REP and ERIC primers (Louws et al. 1999) recognize repetitive elements present in Eubacteria. Primers recognizing the conserved ends of viral insertions (Fletcher et al. 2006) were designed to detect polymorphism in the distance between viral insertions: R8A2BR (5'-agatttgttgcacttcattttaca-3'), R8A2BL (5'-atttgttgttgcacttcgattaca-3') for virus SpV1 R8A2B; and C74R (5'-

tgtaatctttaaaatgtmtgtggt-3'), C74L1 (5'-aaaataaaataatgctttgttt-3') and C74L2 (5'-ttactttccaaaactgtaaaaacc-3') for virus SpV1 C74. Reverse complementary primers (with the same name plus the letter "i" at the end) were designed to detect polymorphism in the length of viral insertions. Finally, 10-mer primers (Operon Technologies, Alameda, CA) were chosen (Mello et al. in press) for RAPD (random amplification of polymorphic DNA).

Polymerase chain reactions (PCR) were performed using 20 ng of DNA template and 2 units of Taq DNA polymerase (Promega) in a reaction volume of 25 µl, with buffer provided by the supplier. The components of the final reaction included: 3 mM of $MgCl_2$, 1.25 mM of each dNTP, and 4 µM of each primer. Reactions were initially incubated for 10' at 95 °C, followed by 35 cycles of 1' at 94 °C, 1' at each corresponding temperature (for primers annealing, Table VI-2), and 5' at 72 °C. The reaction ended with an extension step for 10' at 72 °C. PCR products were electrophoresed in 3 % agarose gels at 10 V/cm for 90', stained with ethidium bromide and visualized under ultraviolet light (Sambrook et al. 1989).

IV - Sequencing of specific DNA segments

Selected isolates were sequenced in each DNA segment (see results). Amplification of several membrane associated protein genes was performed using primers F2-R6 (60 °C) for the gene encoding spiralin (Barros et al. 2001), 32F1-32R (66 °C) for the p32 gene (Killiny et al. 2005), SkB-SkABC (47 °C) for the gene ScA, coding for P58 (Comer et al. 2007), and P89L-P89R (60 °C) for SkARP1, the gene encoding P89 (P89L: 5'-ggcacaaaaatacacgagtgaag-3' and P89R: 5'-catcacaaccatacacaatccc-3'). Specific viral insertions were amplified using the primers R8A2BRi-R8A2BLi (62 °C), and C74Ri-C74L1i (60 °C). PCRs were performed as previously described, using for each primer combination the annealing temperature indicated between parentheses, and an extension time of 2'. A single band was obtained in all cases. Specific PCR products were isolated and cleaned with a GENECLEAN® SPIN Kit (BIO 101) to remove salts and enzymes required for the amplifications, and were used as template for sequencing.

V - Analysis of results

For REP-PCR and RAPDs, tests were performed three times, and only those bands present in all three replications were considered in the analysis. The presence-absence of bands in REP-PCR was assessed visually and converted into a binary form: 1: presence, 0: absence. Phylogenetic analysis were done with the software Mega4 (Tamura et al. 2007), generating a phylogenetic tree using the neighbor-joining (Saitou and Nei 1987) and maximum parsimony (Eck and Dayhoff 1966) algorithm following a bootstrap (1000 replicates with replacement) to determine robustness of the consensus tree (Felsenstein 1985). *S. melliferum* was considered the outgroup. Genetic diversity (π) was estimated by determining diversity for all the pairs of isolates for each geographical location, using a Jules-Cantor model in Mega4 (Tamura et al. 2007).

Sequence alignments, bootstrapping and construction of consensus trees by maximum parsimony (MP) were done with Mega4 (Tamura et al. 2007). The MP was obtained using the Close-Neighbor-Interchange algorithm, prior exclusion of uninformative characters.

Results

Amplification polymorphisms were detected in 7 out of 20 primer combinations used, yielding a total of 80 bands scored (Table VI-2). A single polymorphic amplification product was detected in most cases, except in the case of the primer pair C74R-C74L1, which identified two polymorphic DNA fragments. In total, only 9 out of 80 amplification products were polymorphic. Five polymorphic DNA fragments were identified by RAPD, while four polymorphic DNA segments were identified using primers recognizing conserved ends of viral insertions, 3 of them being polymorphic for distance between two consecutive insertions, and the fourth in the length of such insertions. No polymorphisms were detected using BOX, ERIC or REP primers.

Figure VI-1 shows the phylogenetic tree generated from the molecular analyses, which indicates a close relationship among all *S. kunkelii* isolates. The low bootstrap values at most of the branches indicate that even the small distinctions among isolates were not reliable. No positive correlation could be drawn between geographical origin of

isolates and structure in different clades of the tree, although most of the isolates from Argentina tended to be grouped together, along with a significant proportion of the California isolates. The six Mexican isolates seemed to be more dispersed. Hence, although spiroplasma species were outgrouped somewhat more reliably (bootstrap values about 40%), the distance among isolates was so small that bootstrap separation was weak.

Table VI-2: DNA polymorphism of *S. kunkelii* isolates using REP-PCR and RAPD.

Target DNA	Primers	TA (°C)	# Total bands	# Polymorphic bands
Repetitive elements	ERIC1-2	37	8	0
	REP1-2	50	4	0
	BOX	50	8	0
Distance between viral insertions	R8A2BR-L	42	5	1
	C74R-L1	42	4	2
	C74R-L2	42	2	0
Length of viral insertions	R8A2BRi-Li	42	5	0
	C74Ri-L1i	42	4	1
	C74Ri-L2i	42	4	0
Random	OPA09	37	3	1
	OPA13	37	6	0
	OPA15	37	3	0
	OPA18	37	5	1
	OPAW05	37	1	0
	OPB16	37	1	0
	OPB20	37	2	0
	OPC13	37	2	0
	OPH08	37	5	1
	OPN11	37	3	1
	OPQ06	37	5	0

TA: temperature of annealing.

To quantify the genetic diversity, average pairwise diversity (π) was estimated for each geographic location from which more than one isolate was collected. The fact that values for California (0.310 ± 0.303), Argentina (0.244 ± 0.225), Mexico (0.230 ± 0.159) and Brazil (0.084 ± 0.073) were similar to each other, indicates that the genome structure in all populations is equally diverse.

To further characterize genomic variability of *S. kunkelii*, DNA regions expected to be variable among specific isolates were sequenced and compared. Isolates ME01

through ME06; BR01 through BR03; AR01 through AR03; CA01, CA02 and CA24; CR2 and FL01 were selected for this analysis because they represent different geographical origins (from California, US to Argentina) and different collection dates (1982 for ME01 to 2006 in the other Mexican isolates), and were collected in areas expected to differ in pathogen variability (Mexico hypothesized to have the largest variability since it is the geographic origin of maize).
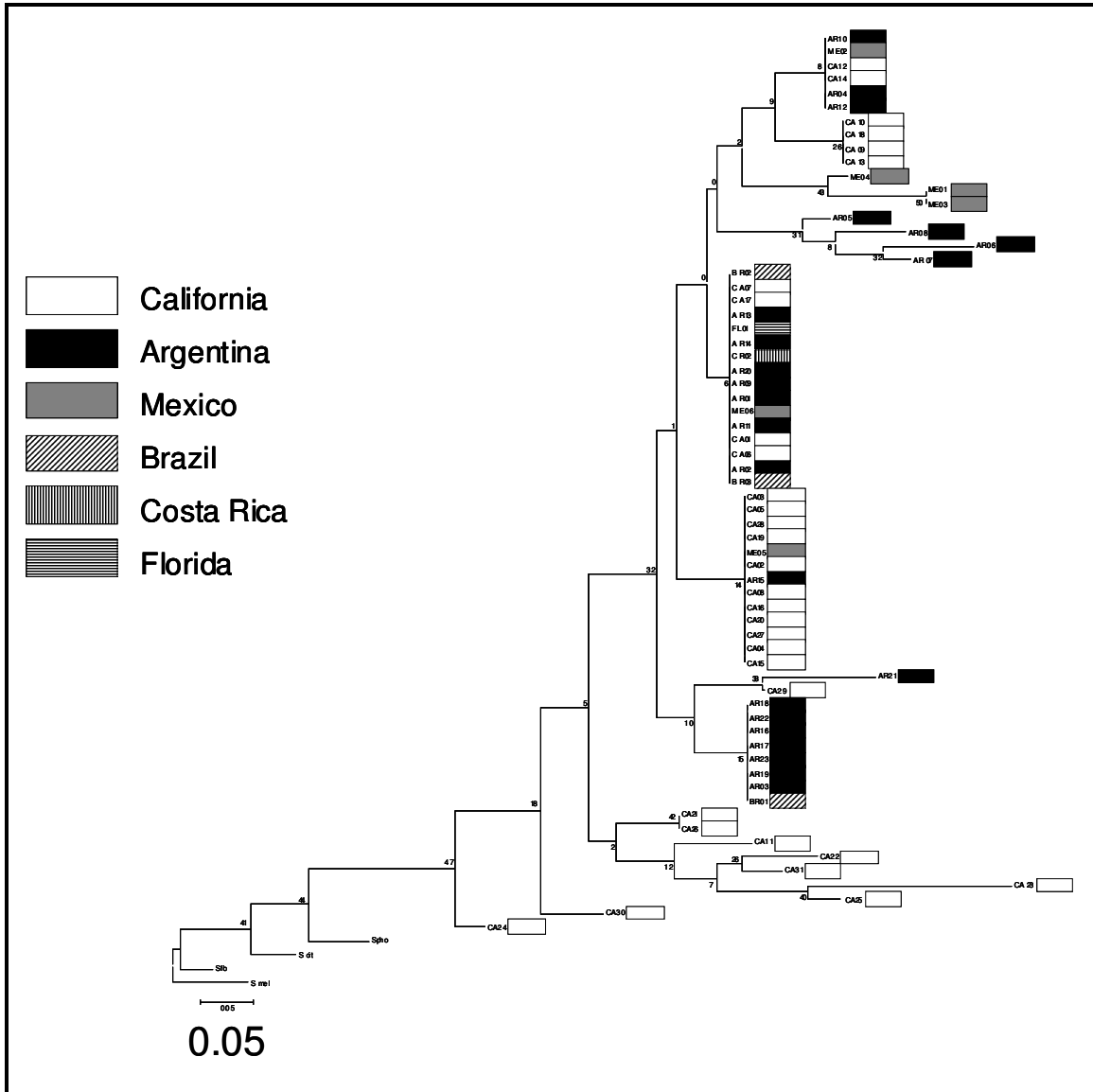

Figure VI-1: Phylogenetic tree of *S. kunkelii* isolates constructed by molecular data obtained by REP and RAPD analysis and inferred using the maximum parsimony method. The percentage of replicated trees in which the associated isolates clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. *S. melliferum* was used as outgroup.

Using primers designed to detect the length of viral insertions and PCR conditions of high stringency, single amplification products were obtained. The amplicon of the viral insertion R8A2B, which was 100% identical across all isolates sequenced, is part of the putative transposase gene (ORF 3) of the virus SpV1 R8A2B (gi:9626114). Results of the sequence of the SpV1 C74 viral insertion were unreliable, because more than one nucleotide residue per position was detected, possibly due to the presence of other(s) fragment(s) of similar size that differed in their internal sequences. Amplification products from the Sp1 gene (spiralin) and the ScA gene (P58) also were identical across isolates sequenced, coinciding also with the sequence in the *S. kunkelii* database (www.genome.ou.edu/spiro.html). No amplification was obtained from the p32 gene in the isolates of *S. kunkelii* included in this work, nor from *S. citri* isolates R8A2 or BR3-3X.
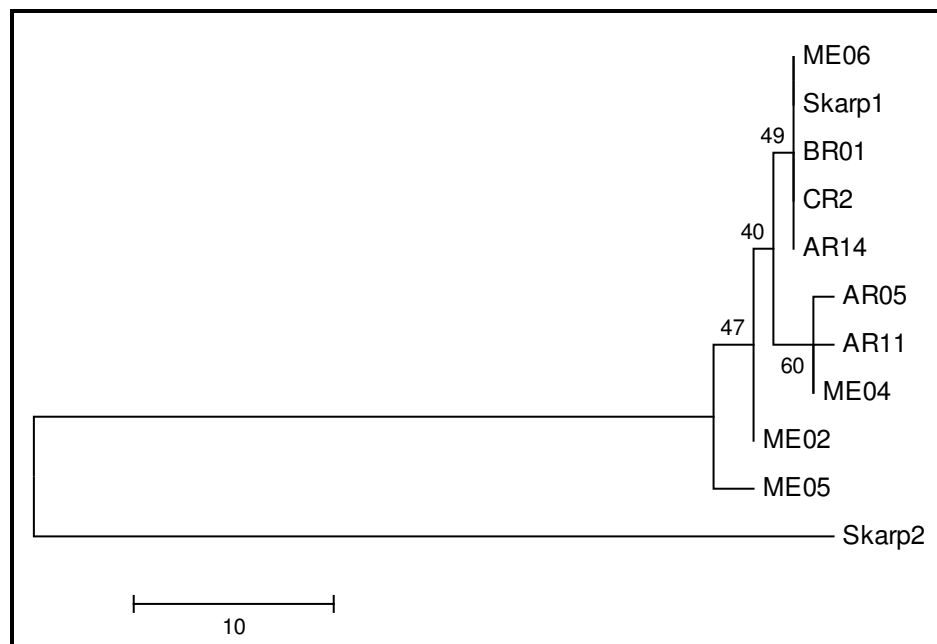

Figure VI-2: Phylogenetic tree of *S. kunkelii* isolates inferred using the maximum parsimony (MP) for the sequence of part of the Skarp1 gene. The MP tree was obtained using the close-neighbor interchange method. The tree is drawn to scale, with branch lengths in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 775 positions in the final dataset, out of which 7 were parsimony informative.

In relation to the Skarp1 gene (P89), as the sequences of the initial set of isolates were polymorphic, the whole set of isolates was submitted to sequence analysis. Indeed, a reliable sequence was obtained from a few isolates (Figure VI-2). Additionally, the

isolates AR01, AR03, AR04, AR06, AR07, AR09, AR10, AR12, AR17, CA15 and CA21 gave an amplification product whose sequences were identical to Skarp2, a similar gene found in *S. kunkelii* (gi:84663351). By using less stringent conditions during PCR (60 and 55°C of annealing temperature), amplification products were obtained from isolates AR02, AR08, AR22, ME01, ME03 and FL01, while no amplification products were obtained from the remaining isolates. However, because these sequences had many nucleotide residues per position they were not useful for comparison. Figure VI-2 shows that the isolates ME06, BR01 and CR2 have exactly the same sequence as that of the Skarp1 gene (gi: 55668082). Some variability was found among Mexican isolates, although it was supported by only seven parsimony informative sites, providing only weak support for the inference of relatedness among them.

Discussion

In this work, we used several approaches to assess genome variability among isolates of *S. kunkelii*, with the ultimate goal of identifying molecular signatures or profiles that could be correlated with geographic origin or date of isolation. In addition to RAPDs using primers that were effective in the identification of variation among *S. citri* isolates (Mello et al. in press), we also tested the primers ERIC, REP and BOX, commonly used to characterize bacterial species and isolates (Louws et al. 1999). The lack of strain discrimination, in our hands, of this method could be due to the high G+C content of these primers (over 65%), which could result in their recognition of conserved parts of the spiroplasmal genome. We expected that viral insertions could be useful for discriminating among *S. kunkelii* isolates, because the genome sequence of strain CR2 reveals that these insertions are abundant and of different lengths. However, only four polymorphic bands were obtained from virus-based primers in this study, although abundant polymorphisms were detected in other plant-pathogenic bacteria when repetitive elements were used as probes (Nelson et al. 1994, George et al. 1997).

Genome composition analyses by REP-PCR, RAPD and sequencing of several genes indicate that all of the *S. kunkelii* isolates included in this study are genetically highly similar, despite their origin from different geographical locations over a time span of 24

years. These results are in contrast to previous findings in *S. citri*, a closely related phytopathogenic spiroplasma species, in which abundant variability was characterized (Nur et al. 1986b, Nur et al. 1987, Ye et al. 1995, Ye et al. 1996, Melcher and Fletcher 1999). Many of the isolates included in the *S. citri* studies had been maintained for extended periods of time outside the insect and/or plant hosts, either by grafting (Fletcher et al. 1996, Ye et al. 1996), or by culturing (Nur et al. 1986b, Nur et al. 1987, Bebear et al. 1996, Ye et al. 1996). In the latter case, viral DNA can become progressively integrated into the spiroplasmal chromosome (Nur et al. 1987), accounting for some of the variability observed. Furthermore, as a consequence of culture or grafting maintenance, isolates can lose insect transmissibility (Wayadande and Fletcher 1995), and hence would not play an important role in natural variability observed in the field. In our study of *S. kunkelii* strains, we used low-passage cultures, passage 1 in most of the cases; the maximum passage number (9) was strain CR2, which remained highly transmissible in recent biological tests (Chapter 1). Hence, these strains should constitute a relatively accurate representation of existing *S. kunkelii* genome variability in nature. Our results, obtained using low-passage *S. kunkelii* isolates, resemble those reported for only low-passage strains of *S. citri* (Mello et al. in press). In this latter species, low-passage isolates yielded 17 out 159 (10.7%) polymorphic amplification products, compared to 9 out of 80 (11.2%) in *S. kunkelii*. Furthermore, Mello and coworkers found no correlation between molecular phenotype and geographical location or collection date, similar to our finding in *S. kunkelii*.

After integration of extrachromosomal DNA into bacterial chromosomes, gradual changes in the nucleotide sequence may occur over time if the integrated DNA provides no selective advantage to the host organism (Hacker and Carniel 2001). In our work, the finding that sequence of part of the gene coding for a putative transposase of viral origin is 100% identical across isolates could indicate that this sequence plays an important role in the metabolism of *S. kunkelii*, or that mechanisms of DNA replication and repair are highly efficient. The fact that the integrated sequence is different from that of the virus (40 out of 720 nucleotide residues changed) may indicate that this sequence was first modified after its insertion, and was stably maintained thereafter. Alternatively, this gene

could be variable in the SpV1 R8A2B virus, with the one inserted differing from that sequenced (gi:1260860).

Sequences of *S. kunkelii* genes encoding for the membrane proteins spiralin and P58 are identical across isolates. In contrast, the gene for spiralin in *S. citri* (Foissac et al. 1996) showed positive selection for amino acid changes, consistent with the evidence for low variability (98% identical) among isolates of *S. kunkelii* collected from different regions of Brazil (Gomes et al. 2004). Unfortunately those sequences are not available to determine if those nucleotide substitutions lead to positive selection for the gene encoding spiralin in *S. kunkelii*.

Using the primers for the plasmid-located Skarp1 gene (gi:45385178), the amplicon sequence obtained usually corresponded to the target gene. However, in some other strains the product amplified was from the Skarp2 gene, a closely related gene located in the spiroplasma chromosome (Wei et al. 2006). In those isolates the amplicon was identical (100% identity) to that of isolate CR2, obtained from Costa Rica in 1992. In contrast, there was some variability in the Skarp1 sequence among Mexican isolates. Since maize is thought to have originated in Mexico (Doebley 2004), and since isolates obtained from other geographical locations were identical to each other, this variability could reflect longer-term evolution in association with plant and insect hosts. However, the number of informative sites for this gene was too low to draw definitive conclusions about *S. kunkelii* phylogeny. The Skarp1 gene product has been associated with attachment to insect cells (Yu et al. 2000), and because of its location in a plasmid-located operon also containing genes similar to those of a type IV secretion system (Bai et al. 2004, Davis et al. 2005), it also may function in conjugation. The failure to detect this gene in some *S. kunkelii* isolates, despite using a variety of PCR conditions, could be due to mutation in the sites complementary to the primers, or to loss of the plasmid during isolation and culture. It would be interesting to test the insect transmissibility of these isolates.

Regarding the gene encoding the membrane-associated protein P32 (Killiny et al. 2006), no amplification product was obtained from the *S. citri* isolate used as outgroup, nor from any isolate of *S. kunkelii* including CR2, which retains insect transmissibility

(Chapter 1). Furthermore, the *S. kunkelii* database contains no significant match to the p32 gene as annotated in *S. citri* (gi:3678468). Hence, it is likely that this gene is present either in the part of the chromosome remaining to be sequenced (5% as of October 2007), or in the extrachromosomal DNA, as in *S. citri* (Killiny et al. 2006), and hence potentially lost during isolation and culture of these isolates. For this reason, it could be useful to confirm these results with Southern hybridization, to determine if *S. kunkelii* indeed has a copy of this or a similar gene.

The high conservation of genes encoding membrane proteins of *S. kunkelii* was unexpected. Based in the similarity of some of these proteins to highly variable mycoplasma adhesins (Citti et al. 2000, Lysnyansky et al. 2001, Rocha and Blanchard 2002), we anticipated finding similar variability among spiroplasma proteins having similar functions. However, that proteins spiralin (Killiny et al. 2005), P58 (Ye et al. 1997) and P89 (Yu et al. 2000) are associated with insect transmission could indicate a selection pressure to avoid changes in sequence. Furthermore, by changing the amino acid sequence of their proteins, mycoplasmas may avoid being recognized by the immune system of mammals (Rocha and Blanchard 2002). On the other hand, although there is no direct evidence for *S. kunkelii* and the leafhopper *D. maidis*, it has been found that *S. poulsonii* is not recognized by the immune system of its insect host, *Drosophila melanogaster* (Hurst et al. 2003). If this were the case for *D. maidis*, then the resulting selection pressure would be directed to maintain the sequences of these proteins, as seen in this study.

The high level of similarity between *S. kunkelii* isolates limits the potential to make inferences about the spread of this pathogen in both space and time. Our analyses suggest that *S. kunkelii* avoids high rates of mutation, resulting in a relatively modest rate of genomic change. Hence, it is unlikely that the observed re-emergence of corn stunt in formerly-resistant maize cultivars was caused by spiroplasma genomic variation leading to the overcoming of host resistance. Furthermore, these results suggest that maize resistance to *S. kunkelii* could be stable in both time and geographical locations, as implied by the high similarity in the genome of this pathogen.

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ADDENDUM

Table A-1: Paramenters of probing behavior of *D. maidis* on maize plants of different genotypes for which no differences were found among the genotypes tested.

Parameter	Maize Genotype				
	G. Bantam	X1273A (7)	X1E73 (1)	X1297J (6)	X1286B (3)
Duration of nonprobing after first SP	0.7 ± 1.2	0.5 ± 0.4	0.4 ± 0.2	0.4 ± 0.3	0.6 ± 0.4
Duration of nonprobing after first PC	0.7 ± 0.5	0.5 ± 0.2	1.0 ± 1.4	0.9 ± 1.0	0.5 ± 0.2
Duration of nonprobing after first PI	0.6 ± 0.3	1.0 ± 1.6	0.9 ± 1.1	0.9 ± 0.6	1.1 ± 2.5
Duration of SP before first PI	1.4 ± 1.1	2.1 ± 2.1	1.2 ± 0.7	2.6 ± 2.8	1.5 ± 1.7
Duration of SP before all PC	1.5 ± 0.6	1.8 ± 0.8	1.8 ± 1.6	2.2 ± 1.3	1.6 ± 1.0
Duration of SP before all PI	1.5 ± 0.5	2.2 ± 1.4	2.0 ± 2.7	2.7 ± 2.7	1.7 ± 1.1
Duration of PC before all PI	36.9 ± 12.5	48.6 ± 16.1	47.5 ± 17.7	48.5 ± 20.5	43.8 ± 16.2
Appearance of first NVP	102.5 ± 181.0	68.6 ± 108.6	162.2 ± 264.5	250.0 ± 252.9	98.3 ± 205.9
Appearance of first PC	83.7 ± 66.4	100.5 ± 98.8	100.8 ± 86.6	91.8 ± 74.0	114.1 ± 82.2
Appearance of first PI	138.1 ± 94.7	247.7 ± 229.7	217.7 ± 132.5	172.7 ± 105.2	208.5 ± 162.9
Appearance of first SP	2.9 ± 7.1	3.4 ± 5.9	4.5 ± 8.0	3.1 ± 4.7	1.5 ± 2.6
Appearance of first AI	45.9 ± 125.4	48.1 ± 187.2	16.1 ± 34.2	31.2 ± 100.7	16.2 ± 58.2
Duration of First SP	1.4 ± 1.5	1.4 ± 0.9	1.2 ± 0.7	1.5 ± 0.9	1.2 ± 1.0
Duration of First PC	27.7 ± 21.9	23.7 ± 26.1	31.5 ± 27.4	21.5 ± 23.6	23.6 ± 17.7
Duration of First PI	169.9 ± 135.0	223.4 ± 237.1	183.7 ± 205.9	220.8 ± 194.5	181.9 ± 103.1
Duration of First AI	6.1 ± 8.9	11.9 ± 19.4	12.3 ± 21.2	11.3 ± 23.4	11.0 ± 7.0
Duration of AI before first PC	36.8 ± 39.2	40.0 ± 50.3	48.5 ± 62.1	29.3 ± 31.6	53.9 ± 53.7
Duration of AI before first PI	44.0 ± 52.5	62.5 ± 81.3	62.0 ± 77.0	37.5 ± 39.8	59.7 ± 62.4
Percentage of AI before first PC	42.3 ± 31.1	33.1 ± 27.2	49.9 ± 32.7	40.4 ± 29.4	48.1 ± 30.1
Percentage of AI before first PI	46.1 ± 31.3	45.4 ± 33.6	62.8 ± 29.2	46.7 ± 31.8	49.5 ± 31.7
Number of probes before first AI	4.0 ± 6.4	2.2 ± 2.2	2.9 ± 3.3	2.6 ± 2.9	2.6 ± 6.3
Number of probes before first PC	17.4 ± 13.5	27.5 ± 29.3	24.5 ± 19.4	22.7 ± 23.9	29.7 ± 35.4
Number of probes before first PI	21.4 ± 14.8	44.6 ± 47.6	36.1 ± 22.2	29.8 ± 24.6	36.8 ± 36.8
Percentage of probes before first PC	25.0 ± 17.3	27.5 ± 17.8	31.3 ± 23.1	26.7 ± 21.8	30.4 ± 21.1
Percentage of probes before first PI	29.4 ± 17.3	43.8 ± 26.7	45.9 ± 25.0	37.2 ± 27.6	39.4 ± 26.8
Percentage of probes with PC	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
Percentage of probes with PI	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
Percentage of PC-PI-PC/PC-PI	5.3 ± 14.5	9.1 ± 23.6	14.3 ± 32.3	8.9 ± 15.4	8.7 ± 25.6

In none of these parameters statistically differences were found in the Analysis of Variance test. Numbers between parentheses for maize genotype indicate the field disease resistance rating in (1: susceptible, 9: immune). All durations are in minutes. Appearances of waveforms are measured in minutes after beginning of insect-plant contact. SP: stylet pathway. PC: phloem contact. PI: phloem ingestion. NVP: Nonvascular probing. AI: active ingestion. Potential Index: percentage of time spent in PC (or PI) after the initial contact with phloem is made (Helden and Tjallingii 2000, Reference in Chapter IV).

Table A-1 (cont): Paramenters of probing behavior of *D. maidis* on maize plants of different genotypes for which no differences were found among the genotypes tested.

Parameter	Maize Genotype				
	G. Bantam	X1273A (7)	X1E73 (1)	X1297J (6)	X1286B (3)
Number of sequences PC-PI	4.2 ± 1.6	3.1 ± 1.4	3.4 ± 1.2	3.2 ± 1.5	3.4 ± 1.3
Number of sequences PC-PI-PC	0.3 ± 1.0	0.2 ± 0.5	0.5 ± 1.1	0.4 ± 0.7	0.2 ± 0.5
Percentage transitions NVP-NP	19.4 ± 31.8	35.7 ± 41.7	38.1 ± 49.8	48.0 ± 40.8	28.1 ± 37.5
Percentage transitions NVP-SP	7.1 ± 23.9	2.7 ± 7.1	14.3 ± 35.9	14.2 ± 28.2	1.6 ± 7.2
Percentage transitions NVP-AI	21.1 ± 35.3	14.0 ± 27.3	0.0 ± 0.0	9.2 ± 19.5	17.9 ± 29.5
Percentage transitions PC-NP	34.2 ± 19.4	33.6 ± 22.8	30.0 ± 25.6	42.0 ± 22.9	37.6 ± 19.1
Percentage transitions PC-NVP	0.0 ± 0.0	1.6 ± 7.2	1.1 ± 3.6	0.5 ± 2.2	0.0 ± 0.0
Percentage transitions PC-PI	60.6 ± 22.0	51.0 ± 27.1	55.0 ± 30.1	50.3 ± 24.2	50.4 ± 22.5
Percentage transitions PC-SP	5.4 ± 8.0	13.9 ± 17.4	14.0 ± 16.6	7.3 ± 10.3	12.1 ± 11.6
Percentage transitions PI-NP	83.1 ± 21.3	82.1 ± 31.5	77.9 ± 33.9	84.0 ± 22.0	82.6 ± 27.5
Percentage transitions PI-PC	5.2 ± 14.4	9.1 ± 23.5	14.3 ± 32.3	8.9 ± 15.3	8.8 ± 25.7
Percentage transitions PI-SP	10.4 ± 16.0	7.5 ± 17.5	7.9 ± 16.0	7.1 ± 16.1	8.7 ± 15.2
Percentage transitions SP-NP	73.8 ± 9.5	73.8 ± 10.2	73.0 ± 15.9	70.8 ± 7.7	71.0 ± 11.2
Percentage transitions SP-NVP	0.7 ± 1.1	0.7 ± 1.0	2.2 ± 6.3	0.9 ± 0.9	1.0 ± 1.8
Percentage transitions SP-PC	10.6 ± 5.4	8.4 ± 4.9	10.4 ± 8.7	9.9 ± 6.4	9.2 ± 6.3
Percentage transitions SP-AI	14.8 ± 7.1	16.8 ± 7.2	14.4 ± 5.5	18.4 ± 5.5	18.7 ± 6.6
Percentage transitions AI-NP	63.8 ± 19.1	62.8 ± 16.7	70.4 ± 21.7	59.3 ± 17.8	60.2 ± 20.2
Percentage transitions AI-NVP	7.4 ± 13.2	5.0 ± 7.2	1.7 ± 4.8	6.3 ± 8.2	2.5 ± 3.9
Percentage transitions AI-SP	28.5 ± 17.9	32.2 ± 17.5	28.0 ± 21.6	34.0 ± 18.8	37.0 ± 19.8
Average duration of NVP	5.8 ± 7.2	7.2 ± 8.8	12.5 ± 27.2	16.1 ± 18.6	5.6 ± 7.6
Average duration of PC	26.3 ± 10.5	32.0 ± 15.8	32.7 ± 16.7	29.8 ± 12.9	29.4 ± 11.5
Average duration of PI	208.2 ± 123.7	228.6 ± 107.9	201.5 ± 122.6	247.9 ± 171.3	199.8 ± 74.1
Number of occurrences of NP	80.1 ± 41.6	98.2 ± 57.2	84.6 ± 37.1	86.8 ± 52.7	98.6 ± 48.6
Number of occurrences of NVP	1.4 ± 2.1	2.0 ± 2.6	1.0 ± 1.6	2.2 ± 3.5	1.6 ± 2.5
Number of occurrences of PC	8.2 ± 4.7	7.5 ± 4.8	7.8 ± 3.8	7.5 ± 4.2	7.8 ± 3.8
Number of occurrences of PI	4.2 ± 1.6	3.1 ± 1.4	3.4 ± 1.2	3.2 ± 1.5	3.4 ± 1.3
Number of occurrences of SP	81.0 ± 41.4	101.0 ± 55.5	87.0 ± 37.6	90.2 ± 52.1	103.1 ± 48.0
Number of occurrences of AI	11.3 ± 5.8	16.3 ± 8.6	12.6 ± 7.4	16.2 ± 9.1	19.0 ± 9.9
Percentage of time in NP	5.3 ± 2.8	6.8 ± 4.5	5.1 ± 2.5	5.5 ± 3.1	4.9 ± 1.9
Percentage of time in NVP	1.4 ± 2.1	2.3 ± 3.1	1.7 ± 3.1	3.5 ± 4.1	1.7 ± 2.8
Percentage of time in PC	15.1 ± 4.8	16.3 ± 5.7	20.2 ± 10.5	17.0 ± 7.0	18.8 ± 11.8
Percentage of time in PI	61.6 ± 10.0	51.8 ± 16.6	53.2 ± 16.4	54.4 ± 16.9	51.6 ± 15.0
Percentage of time in SP	7.0 ± 2.8	9.2 ± 4.8	9.9 ± 7.6	9.0 ± 3.7	10.1 ± 4.3
Percentage of time in AI	7.4 ± 5.5	11.2 ± 7.5	8.2 ± 8.0	8.3 ± 6.0	10.2 ± 7.0
Percentage of time Probing	94.7 ± 2.8	92.5 ± 5.3	94.9 ± 2.5	94.0 ± 4.0	95.1 ± 1.9

Numbers between parentheses for maize genotype indicate the field disease resistance rating in (1: susceptible, 9: immune). All durations are in minutes. Appearances of waveforms are measured in minutes after beginning of insect-plant contact. SP: stylet pathway. PC: phloem contact. PI: phloem ingestion. NVP: Nonvascular probing. AI: active ingestion. Potential Index: percentage of time spent in PC (or PI) after the initial contact with phloem is made (Helden and Tjallingii 2000, Reference in Chapter IV).

VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: HOST RESISTANCE AND DIVERSITY OF *SPIROPLASMA KUNKELII* AS COMPONENTS OF CORN STUNT DISEASE

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Institution: Oklahoma State University   Location: Stillwater, Oklahoma

Title of Study: HOST RESISTANCE AND DIVERSITY OF *SPIROPLASMA KUNKELII* AS COMPONENTS OF CORN STUNT DISEASE

Pages in Study: 110   Candidate for the Degree of Doctor of Philosophy

Major Field: Plant Pathology

Scope and Method of Study:

Corn stunt is an important plant disease for which successful control using resistant maize germplasm has been unsatisfactory in the past. It is unknown at present if the pathogen *Spiroplasma kunkelii* has the ability to generate a variety of pathotypes able to breakdown maize resistance, or whether the insect vector *Dalbulus maidis* plays a role in detecting components of such resistance. In this study, we sought to characterize these important components in this pathosystem, providing information useful to the design of strategies of disease control.

Findings and Conclusions:

Field-resistance to corn stunt was not completely correlated to parameters of resistance to insects, in tests performed in controlled conditions. An antixenotic response against *D. maidis* was not found, although one resistant hybrid exhibited antibiosis as decreased nymphal survival and adult longevity. Inoculation efficiency was similar for all the genotypes evaluated, whether resistant or susceptible, indicating that resistance mechanisms would not prevent pathogen transmission once inoculative insects settled on the plant.

The electrical penetration graph (EPG) technology was also used to characterize maize resistance. Six distinct waveforms were characterized and correlated with major probing activities of *D. maidis* by monitoring transmission of spiroplasmas and excretion of honeydew. The probing behavior of *D. maidis* was somewhat altered by the resistance level of the plant genotype. Insects probing in resistant hybrids salivated for longer periods of time before ingesting from phloem, and displayed increased xylem ingestion. However, phloem ingestion was not prevented in resistant hybrids.

S. kunkelii has minimal polymorphism, and a similar level of variability in different geographical locations. Strain variation was in part due to viral insertions, and in part to differences in a gene encoding a membrane protein implicated in attachment to insect cells. The results suggest that it is unlikely that instability of maize resistance is due to generation of new pathotypes of *S. kunkelii*. Instead, other components of this complex pathosystem could account for the breakdown of resistance.

ADVISER'S APPROVAL: Jacqueline Fletcher