Exitianus obscurinervis (Hemiptera: Cicadellidae), a New Experimental Vector of *Spiroplasma kunkelii*

E. CARLONI,[1,2] E. VIRLA,[3] S. PARADELL,[4] P. CARPANE,[1,5] C. NOME,[1] I. LAGUNA,[1] AND M. P. GIMÉNEZ PECCI[1]

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ABSTRACT "Corn stunt" caused by the mollicute *Spiroplasma kunkelii* (Whitcomb) is potentially one of the most severe diseases affecting the corn (*Zea mays* L.) crop in the Americas, and the leafhopper *Dalbulus maidis* (DeLong & Wolcott) is considered its most important vector. However, other insects seen quite frequently in corn crops might well be its vectors in Argentina. To identify any leafhoppers species other than *D. maidis* that can transmit *S. kunkelii*, transmission assays were conducted, using individuals of *Exitianus obscurinervis* (Stål) collected in field and reared under controlled conditions. *S. kunkelii* was transmitted to corn plants by *E. obscurinervis*. The pathogen was transmitted to seven of the 11 plants, which showed characteristic corn stunt symptoms, and the presence of the pathogen was confirmed by DAS-ELISA. The presence of *S. kunkelii* in the *E. obscurinervis* individuals used in transmission experiments was confirmed by polymerase chain reaction and electron microscopy. The current study shows the existence of a new experimental vector of *S. kunkelii*, the leafhopper *E. obscurinervis*, which acquired spiroplasmas from infected plants and inoculated it to healthy plants.

KEY WORDS *Dalbulus maidis*, corn leafhopper, corn stunt, spiroplasmas

Spiroplasma kunkelii (Whitcomb) (Whitcomb et al. 1986) is one of three pathogens causing the "corn stunt" complex, one of the most serious diseases affecting corn (*Zea mays* L.) crops in tropical and subtropical regions of the Americas (Bradfute et al. 1981, Hruska et al. 1996, Oliveira et al. 1998). Corn stunt was first reported in Argentina in the 1990/91 crop season in a subtropical corn production area in the provinces of Santiago del Estero and Tucumán (Lenardon et al. 1992). Since then, the disease has progressively detected in to other areas and in successive crop seasons in Santiago del Estero, Tucumán, Salta, Catamarca, and Chaco provinces (Giménez Pecci et al. 1997). It occurs in most of the subtropical provinces, and even in temperate ones, such as Córdoba, Santa Fe, and Buenos Aires (Giménez Pecci et al. 2002, 2003, 2005).

S. kunkelii resides in the phloem of infected plants (Davis et al. 1972, Nome et al. 2009) and it is transmitted in a circulative-propagative manner (Nault 1980) by some leafhopper (Hemiptera: Cicadellidae) species. Spiroplasmas enter the insect, multiply and circulate until they reach the accessory salivary glands (Kwon et al. 1999, Özbek et al. 2003, Ammar and Hogenhout 2005). The timespan of this process is the latency period, which can range from 17 to 23 d (Nault 1980). The insect then remains inoculative for the rest of its life (Nault 1980, Alivizatos and Markham 1986). Both spiroplasma acquisition and inoculation to healthy plants can occur with insect feeding periods as short as one hour, and vector efficiency increases with time to reach nearly 100% (Alivizatos and Markham 1986).

The transmission of *S. kunkelii* has been performed under controlled conditions with several leafhopper species, including *Dalbulus maidis* (DeLong & Wolcott), *D. guevarai* DeLong, *D. gelbus* DeLong, *D. quinquenotatus* DeLong & Nault, *D. tripsacoides* DeLong & Nault, *D. elimatus* Ball, *Baldulus tripsaci* Kramer & Whitcomb (Madden and Nault 1983), *D. longulus* DeLong (Nault et al. 1984), *Exitianus exitiosus* (Uhler), *Graminella nigrifrons* Forbes (Nault 1980, Bradfute et al. 1981), *Stirellus bicolor* Van Duzee (Nault 1980), and *Euscelidius variegatus* Kirschbaum (Markham et al. 1977). Of the known vector species of *S. kunkelii, D. maidis* and *S. bicolor* are the only ones occurring in Argentina (Virla et al. 1991, Paradell 1995, Paradell et al. 2001, Luft Albarracin et al. 2008). *D. maidis,* the corn leafhopper, is considered the main vector species for its high transmission efficiency (Nault 1980, Madden and Nault 1983), ≈100% in controlled conditions (Alivizatos and Markham 1986, Carpane et al. 2011), and wide geographic distribution (Triplehorn and Nault 1985, Oliveira et al. 2004, Paradell et al. 2001).

[1] INTA-IFFIVE, Camino 60 Cuadras Km 5 1/2 (X5020ICA), Córdoba, Argentina.
[2] Corresponding author, e-mail: edgardocarloni@gmail.com.
[3] CONICET, PROIMI-Biotecnología Division Control Biológico, Av. Belgrano y Pje. Caseros (T4001MVB), Tucumán, Argentina.
[4] División Entomología, Facultad de Ciencias Naturales y Museo, UNLP, Paseo del Bosque S/N, (B1900FWA) La Plata, Buenos Aires, Argentina.
[5] Current address: Monsanto Argentina SAIC, Ruta 188 Km 77 (2700), Pergamino, Buenos Aires, Argentina.

Previous studies reported that *D. maidis* is abundant and frequent in several provinces of northern Argentina (Paradell et al. 2001; Giménez Pecci et al. 2002, 2003; Virla et al. 2003; Luft Albarracin et al. 2008), typically to the north of parallel 30° SL. However, on some occasions the disease has been detected in temperate regions (Giménez Pecci et al. 2002) (to the south of parallel 30° SL), where the presence of *D. maidis* is occasional (Paradell et al. 2008a), suggesting that other insect species might be vectors of *S. kunkelii* in that region. Conversely, 31 leafhopper species have been reported in corn crops in Argentina (Paradell 1995; Paradell et al. 2001, 2005), some of which could be *S. kunkelii* vectors because they are taxonomically related to proven vector species (Paradell 1995). The most frequent of these in the temperate region is *Exitianus obscurinervis* (Stål, 1859) (Oman et al. 1990, Paradell et al. 2008b), which is widely distributed in Argentina, mainly in maize and associated spontaneous vegetation (Paradell et al. 2001, 2005; Remes Lenicov et al. 2006). The aim of the present work was to determine the role of *E. obscurinervis* as vectors of *S. kunkelii.*

Materials and Methods

Obtaining and Maintaining *S. kunkelii* Strain and Vectors. The *S. kunkelii* strain used in transmission assays was obtained from *D. maidis* individuals collected from corn plots in El Manantial (Dpto. Lules, Tucumán Province, Argentina) (26° 49′ 50.2″ S, 65° 16′ 59.4″ W), and later maintained in sweet corn (Carpane et al. 2006). The strain was maintained by insect transmission under greenhouse conditions, following Nault (1980), using *D. maidis* adults from healthy colonies as vectors at the Institute of Phytopathology and Plant Physiology (IFFIVE), Córdoba, Argentina. *S. kunkelii* presence in corn plants was confirmed by polymerase chain reaction (PCR) (Barros et al. 2001) and DAS-ELISA (AGDIA Inc., Elkhart, IN), as described in the section Symptom evaluation and pathogen detection in plants and insects.

D. maidis and *E. obscurinervis* individuals were collected from corn crops in El Manantial (Tucumán Province, Argentina), a subtropical area where *S. kunkelii* and *D. maidis* are endemic (Paradell et al. 2001; Giménez Pecci et al. 2002, 2003). Insect rearing and transmission assays were performed under greenhouse conditions at the IFFIVE. The insects were maintained in cages (80 × 50 × 25 cm) with fine mesh walls (type voile), exposed to controlled temperature conditions (24–27°C), photoperiod (16 h of natural light supplemented with artificial light, and 8 h of dark) and relative humidity (70–80%). Insect species were identified at the Entomology Division of the Facultad de Ciencias Naturales y Museo de La Plata (UNLP), Argentina. Insect individuals used were identified to species using both external and male and female genitalia's characters (Linnavuori 1959, Ross 1968). Voucher specimens of *E. obscurinervis* are deposited in the collections of the Museo de Ciencias Naturales de La Plata, Buenos Aires (MLP). The materials were studied and compared with the holotype deposited in the collection of the MLP.

Transmission Assays With Field-Collected *E. obscurinervis* Individuals. Insects were collected using sweep nets from corn plots in February (summer) of the 2004/05 and 2005/06 crop seasons. They were transported to IFFIVE in plastic cages containing corn leaves and moist blotting paper to avoid dehydration. The collected insects were individually placed on plants of pisingallo maize cultivar Pop Zélia at one-expanded leaf stage for 7 d. Experimental plants were then maintained under greenhouse conditions and tested weekly for the presence of disease symptoms.

Transmission Assays With *E. obscurinervis* Reared Under Controlled Conditions. *E. obscurinervis* were reared using the method described by Virla (1990a, 1990b), with modifications. To obtain a healthy insect colony, adults were collected during the 2005/06 season, placed in cages containing healthy plants for 7 d, and removed at the end of that period. Newly hatched nymphs were put in rearing cages until they reached adult stage to initiate a healthy colony. As a food source and oviposition site, a combination of the following plants was used: corn (*Zea mays* L. cultivar Pop Zélia), Bermuda grass (*Cynodon dactylon* (L.) Persoon), Johnsongrass (*Sorghum halepense* L. Persoon), oats (*Avena sativa* L.), rescuegrass (*Bromus unioloides* Kunth), and crabgrass (*Digitaria* sp.). The plants in contact with the insects were periodically replaced and maintained under greenhouse conditions to confirm the absence of *S. kunkelii* by symptoms observation and serological tests. The colony was maintained in these conditions for 90 d, and then, in the absence of diseased plants, was considered healthy.

To acquire *S. kunkelii*, ≈150 healthy *E. obscurinervis* adults were placed in contact with symptomatic corn plants during a 4-d oviposition period. They were then removed and the plants placed in cages under greenhouse conditions. Nymphs hatched from the eggs laid were kept on the infected plants for 10–14 d, which was considered the acquisition period. Then, the nymphs were moved to cages containing the combination of healthy plants mentioned above, where they were maintained for a 21-d incubation period. Surviving individuals were used for inoculation of corn plants cultivar Pop Zélia at the 1–3 leaf stage (three insects per plant) during an inoculation period of 7 d. As a negative control, the same number of insects obtained from healthy colonies was placed on five corn plants of the same corn cultivar. At the end of the inoculation period, the insects were removed from the plants and kept at −20°C until determination of the presence of *S. kunkelii.* The plants were maintained under greenhouse conditions for further diagnosis, being sprayed with insecticide once a week.

Symptom Evaluation and Pathogen Detection in Plants and Insects. The presence of the pathogen in plants was determined by evaluating the characteristic *S. kunkelii* symptoms (Nault 1980) and by the DAS-ELISA serological test, using locally developed antisera (Giménez Pecci et al. 2009). Ninety days after the end of the inoculation period (plants at reproductive stage), samples of the penultimate expanded leaf were taken and kept at −20°C until pathogen presence was determined by serology. Some plants were tested before day 90 because they showed severe damage after reaching the reproductive stage.

The presence of *S. kunkelii* in insects was confirmed by PCR. The three insects used for transmission of each plant were placed in a 1.5 ml microtube for extraction of nucleic acids (Doyle and Doyle 1994, with modifications). Each sample was macerated with 500 μl of CTAB buffer (0.1 M Tris HCl pH 8.0, 1.4 M NaCl, 0.02 M EDTA pH 8.0, 2 g/100 ml CTAB) and 1 μl of β-mercaptoethanol. Samples were incubated at 65°C for 30 min, and homogenized every 5–10 min. After cooling at room temperature for 1 min, 500 μl of chloroform/isoamyl alcohol (24/1 vol:vol) was added and the mixture was agitated in a vortex for 30 s. It was then centrifuged at 10,000 rpm for 5 min and the supernatant (300 μl) was placed in another tube containing 180 μl isopropanol for incubation at room temperature for 30 min. This was then centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The precipitate was washed with 70% ethanol (vol:vol), dried and resuspended in 30 μl of TE buffer (0.01 M Tris-HCl, 0.01 M EDTA pH 8.0).

The spiralin gene of *S. kunkelii* was amplified from DNA extracted from the insects, using the primers CSSF2/CSSR6 (Barros et al. 2001). Amplification reaction was performed in a final volume of 25 μl, containing 1 μl of DNA (20–200 ng), 1X of buffer (6 mM Tris-HCl pH 8.8, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, and 1 U of *Taq*DNA polymerase (Invitrogen, São Paulo, Brazil). The amplification was performed under the following conditions: 94°C for 2 min, 40 cycles of 94°C for 1 min, 60°C for 2 min, 72°C for 2 min, followed by a final extension period of 72°C for 10 min. Amplification products were run in 1.4% agarose gel along with a molecular weight marker (100 pb DNA Ladder, Promega Corp., Madison, WI) and stained with ethidium bromide for visualization under UV light.

In addition, six individuals of the species *E. obscurinervis* (samples 10 and 11, Table 1) were processed for observation under transmission electron microscope (TEM) (JEOL JEM EXII) (Joel Ltd., Tokyo, Japan), following protocols of Kitajima (1997) and Maunsbach (1998), with modifications. The insects were submerged in 2.5% (vol:vol) glutaraldehyde/paraformaldehyde fixative in 0.5 M cacodylate buffer for 6 mo. To determine the presence of *S. kunkelii* in salivary glands, the heads of the insects were dissected and washed in distilled water three times for 5 min. Sections were placed in a 0.5% (vol:vol) uranyl acetate solution overnight, and then rinsed and dehydrated in a graded ethanol series (50, 70, 90, and 100% vol:vol, respectively) for 10 min each. Tissues were infiltrated with a solution containing equal amounts of Spurr's resin and 100% (vol:vol) ethanol for 12 h. Finally, they were embedded in pure resin and polymerized with UV light at 4°C for 72 h. Ultrathin sections (70 nm) obtained with an ultramicrotome (RMCMT6000-XL) (Leica Microsystems Inc., Buffalo Grove, IL) were mounted on 50-mesh copper grids. The grids were treated with uranyl acetate and lead citrate and examined under TEM at 80 kV.

Table 1. Detection of *S. kunkelii* in corn (*Z. mays* cultivar Pop Zélia) plants and in leafhoppers, inoculated under controlled conditions using *E. obscurinervis* as vector (three individuals per plant)

Sample	Detection of *S. kunkelii*			
	Plant		Insects	
	Symptoms	Serology	PCR	TEM
1	−	−	+	
2	+	+	+	
3	−	−	(*)	NT
4	−	−	+	
5	−	−	+	
6	+	+	+	
7	+	+	(*)	NT
8	+	+	+	
9	+	+	+	
10	+	+	(*)	+
11	+	+	(*)	+
Control	−	−	−	

Positive assay (+) and negative assay (−) sample. No symbol indicates that no assay was performed. The presence of the *S. kunkelii* in the plants was confirmed by symptoms and DAS-ELISA. Control: five healthy plants. The presence of the pathogen in the insect was confirmed by PCR and TEM. *Insects not tested in PCR, but embedded in fixative for electron microscopy observation. NT (not tested).

To further identify the presence of *S. kunkelii* in the salivary glands of insects, ultrathin sections were immunolabeled with 10 nm gold particles (Sigma-Aldrich, St. Louis, MO). Grids were blocked at room temperature using PBS (1 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.13 M NaCl, 3 mM KCl) + BSA 1% for 30 min, followed by incubation for 2 h on drops of PBS and polyclonal antiserum (diluted 1/10 vol:vol) (Giménez Pecci et al. 2009). For TEM observations, sections were previously washed with PBS five times and then incubated again on drops of conjugated anti-IgG (goat anti-rabbit) serum (Sigma-Aldrich) with colloidal gold diluted 1/10 vol:vol in PBS.

Results

Transmission Assays Using Field-Collected Leafhoppers. None of the 24 field-collected insects used in transmission assays inoculated the pathogen, because none of the plants developed disease symptoms. In addition, serological tests did not detect the presence of *S. kunkelii* in any of the 24 plants used by these insects for feeding.

Transmission Tests Using *E. obscurinervis* Reared Under Controlled Conditions. The *S. kunkelii* strain was inoculated under controlled conditions by individuals of the species *E. obscurinervis* to seven (63%) of a total of 11 corn cultivar Pop Zélia plants that were in contact with insects fed on infected plants (three individuals per plant) (Table 1). The presence of the pathogen in all the plants with *S. kunkelii* symptoms was confirmed using DAS-ELISA. Conversely, no symptoms were observed in the other four plants used in the transmission or in the five control plants inoc-

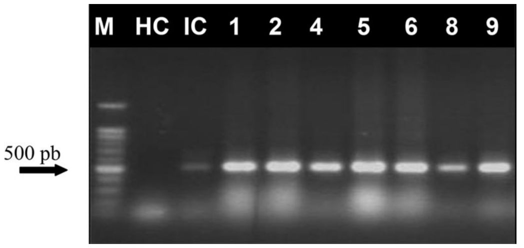

Fig. 1. Detection of *S. kunkelii* in *E. obscurinervis* used as experimental vectors. Electrophoresis in 1.4% agarose gel of the PCR product (500 bp) obtained with specific primers (CSSF2/CSSR6) for *S. kunkelii*. M: Molecular weight marker (100 bp DNA Ladder, Promega Corp.). HS: Healthy control (DNA of healthy *E. obscurinervis*). HC: Infected control (corn plant experimentally inoculated with the T1 isolate). Lanes 1–7: DNA of seven samples of *E. obscurinervis* (three insects/sample) experimentally inoculated with *S. kunkelii*.

ulated with insects from healthy colonies, neither was the pathogen detected by serological tests.

The plants that tested positive for *S. kunkelii* showed typical disease symptoms, as previously described (Nault 1980, Carpane et al. 2006). The first symptoms were marginal chlorotic striping at the base of newly developing leaves, appearing ≈60 d after the end of the inoculation period. They then spread until entire leaves were covered, affecting all the leaves that developed subsequently. At 90 d (end of assay), all the ELISA positive plants exhibited typical disease symptoms. Some plants presented stunted growth because of shortening of upper internodes. In addition, upper leaves showed cut margins. Reddening of leaf margins in experimental transmissions with *D. maidis*, was not seen in any of the plants, as observed by Carpane et al. (2006).

The presence of *S. kunkelii* in the insects used in inoculation assays was confirmed by PCR (Fig. 1). A fragment of ≈500 bp was amplified from the DNA samples taken from insects (the three individuals per plant pooled together) that were in contact with inoculated plants (samples 2, 6–11, Table 1). Additionally, the insects that had access to plants 1, 4, and 5 were positive by PCR, even though those plants were not inoculated with *S. kunkelii*, as demonstrated by the lack of symptoms and DAS-ELISA detection.

S. kunkelii was identified with TEM in *E. obscurinervis* individuals that transmitted the pathogen, based on size, morphology, and immunolabeling in salivary gland tissues (Fig. 2). The pleomorphic bodies found in salivary glands of inoculative insects (Fig. 2) were similar in shape and size to those previously described for *S. kunkelii.*

Discussion

During the 2004/05 and 2005/06 seasons, field-collected individuals of *E. obscurinervis* were used in inoculation assays to determine whether they are vectors of *S. kunkelii,* but none of these was found to be

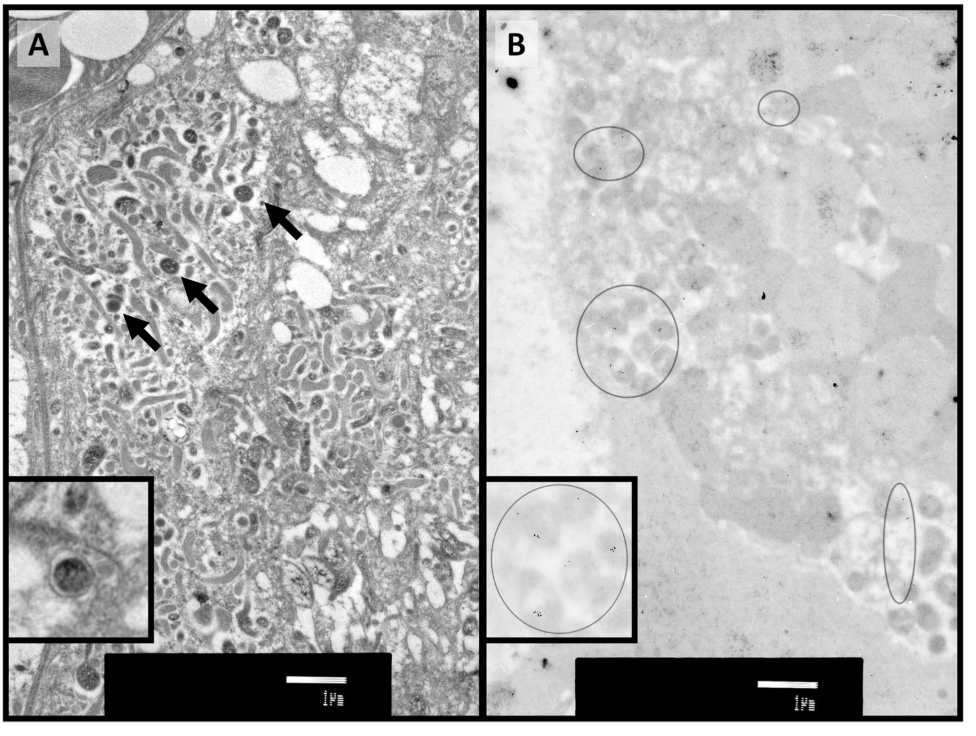

Fig. 2. Detection of *S. kunkelii* in salivary glands of *E. obscurinervis* in ultrathin sections. (A) Pleomorphic, single-membrane bodies inside vesicles (seen with more detail in the inset). (B) Immunolabeling with 10 nm gold particles using an antibody against *S. kunkelii*. Inset shows gold particles over pleomorphic bodies resembling spiroplasmas.

inoculative. Considering that the percentage of inoculative leafhoppers is low, such as 0.90 and 2.36% for *D. maidis* individuals (Ebbert et al. 2001, Carloni et al. 2006) and that in this assay only 24 insects were evaluated, it is still likely that inoculative individuals of this species are present in the field at very low levels, similar to those of *D. maidis.*

Experimental transmissions showed that *E. obscurinervis* transmits the pathogen *S. kunkelii.* Indeed, insects in contact with diseased plants were positive by PCR 21 d after the end of the acquisition period; TEM revealed corpuscles similar to *S. citri* and *S. kunkelii* (Kwon et al. 1999, Özbek et al. 2003) in the salivary glands of *S. kunkelii*-positive insects, which reacted in immunolabeling with *S. kunkelii*-specific antisera. The presence of *S. kunkelii* in salivary glands of *E. obscurinervis* suggests that the pathogen-vector transmission mechanism is circulative, similar to that of *D. maidis* (Ammar and Hogenhout 2005) and of *S. citri* in *Circulifer tenellus* (Wayadande and Fletcher 1995, Kwon et al. 1999), although whether *S. kunkelii* propagates in *E. obscurinervis* individuals still remains to be proved. In addition, plants accessed by inoculative *E. obscurinervis* showed the characteristic symptoms of corn stunt, and DAS-ELISA confirms the presence of *S. kunkelii* on them. *E. obscurinervis* is thus seen to be a vector of *S. kunkelii* under experimental conditions, suggesting that it can also be a natural vector in the field.

Seven of the 11 groups of insects in the transmission assays inoculated the pathogen *S. kunkelii* to plants, while the remaining ones had not inoculated the pathogen to plants after a latency period of 21 d despite harboring the pathogen. This could suggest that either the inoculation efficiency of *E. obscurinervis* is lower than that of *D. maidis* (Alivizatos and Markham 1986), or that *E. obscurinervis* requires a longer latency period than the average of *D. maidis,* which is close to 19 ± 2 d (Nault 1980). Because the latency and inoculation periods lasted 21 and 7 d, respectively, it could be also possible that the latency period of *S. kunkelii* in *E. obscurinervis* could last longer than the one tested. Further assays are needed to characterize in detail the behavior of *E. obscurinervis* as a vector of *S. kunkelii,* as well as of other pathogens that are transmitted by *D. maidis,* such as Maize rayado fino virus (MRFV) and Maize bushy stunt phytoplasma (MBSP) (Nault 1980, Nault et al. 1980).

This is the first contribution showing the capacity of *E. obscurinervis* to transmit *S. kunkelii* under experimental conditions, which suggests that *E. obscurinervis* may also be a field vector of this pathogen, with implications for the pathosystem of corn stunt in Argentina. Inoculative insects of this species could therefore be responsible for the occurrence of the disease in geographical areas, such as the corn belt of Argentina (Remes Lenicov et al. 2006), where the presence of *D. maidis* is occasional (Paradell et al. 2008a) and where *E. obscurinervis* is the most frequent species (Paradell et al. 2001). *E. obscurinervis* would also have a wider host range than *D. maidis,* because it has been successfully reared in sorghum (*Sorghum vulgare* L.), bromegrass (*Bromus* sp.) (Virla 1990a), and wheat (*Triticum aestivum* L.) (Virla 1990b), and has been found in the field on a wide range of wild and cultivated plants (Paradell 1995, Paradell et al. 2005, Remes Lenicov et al. 2006). The pathosystem of corn stunt in temperate areas of Argentina would thus be more complex than originally assumed, involving more than just the insect *D. maidis* and the corn crop as hosts of *S. kunkelii,* with other species serving as a pathogen reservoir and facilitating its survival.

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