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EXPERIMENTAL TRANSMISSION OF CORN STUNT SPIROPLASMA PRESENT IN DIFFERENT REGIONS OF ARGENTINA

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ABSTRACT - The aim was to transmit Corn stunt spiroplasma (*Spiroplasma kunkelii* Whitcomb) using field-collected *Dalbulus maidis* insects (Hemiptera - Cicadellidae), and diseased plants from places in the subtropical and temperate regions of Argentina. Field-collected *D. maidis* populations transmitted the spiroplasma in 39% of the cases when a density of five insects per plant was used. Transmissions were obtained from diseased plants collected in two subtropical locations. In all the symptomatic plants, the presence of spiroplasma was detected by serology and confirmed with PCR. The development of the symptoms and the pathogen titer varied between plants infected with insects from the same location, leading to the identification of two plant groups: one with severe symptoms and high pathogen titer, and the other with mild symptoms and lower titer, showing a direct relation between pathogen titer and the severity of symptoms.

KEY WORDS: *Zea mays* L.; Spiroplasma; Infectivity; Symptom diversity.

INTRODUCTION

Corn stunt is a disease produced by *Spiroplasma kunkelii* Whitcomb, a widely-distributed pathogen in neotropical areas of the American continent (Nault, 1980; Oliveira *et al*., 1998), where the disease is considered one of the most significant threats to maize production (Bradfute *et al*., 1981; Bajet and Renfro, 1989; Tsai and Falk, 1996; Massola Junior *et al*., 1999a). In Argentina, it was first detected in the 1990/91 crop year in the north-west (Lenardón *et al*., 1992), and since then it has become more and more important due to the steady growth in its incidence in the subtropical areas of the country, and to a slow advance into the temperate areas of high production, approaching the core maize area (Giménez Pecci *et al*., 2002a,b).

Typical symptoms of the disease are chlorotic stripes running from the base of the leaf laminas and extending slowly towards the apex; stunted growth of the plant due to the progressive shortening of the upper internodes, and the proliferation of ears in different nodes (Nault, 1980), so that grain yield is markedly reduced, even to zero if the plants are severely attacked (Virla *et al*., 2004). Nevertheless, both the manifestation and the severity of the symptoms can be considerably modified by temperature, the time of infection, variants in the pathogen and in the host genotype (Nault, 1980; Oliveira *et al*., 1998; Bedendo, 1999; Massola Junior *et al*., 1999b).

The pathogen is transmitted by hemipterous insects of the Cicadellidae family, and is not transmitted mechanically or through seeds (Tsai and Falk, 1996). The principal vector is *Dalbulus maidis* (DeLong and Wolcott, 1923), while other related species, *D. gelbus* DeLong, *D. elimatus* Ball, *Exitianus exitiosus* Uhler, *Graminella nigrifrons* Forbes and *Stirellus bicolor* Van Duzee, are less efficient vectors in the field (Nault, 1980). The manner of transmission is persistent propagative, and individuals acquiring the pathogen remain infectious during their lifespan (Nault, 1980). The only plant species affected by this pathogen in natural conditions belong to the genus *Zea*, including maize (*Z. mays* L.) and teosintes: *Z. diploperenis* Iltis, Doebley and Guzman, *Z. perennis* (Hitchc.) Reeves and Mangelsdorf, *Z. mays* x *Tripsacum floridanum* Porter ex Vasey L. and *Z. luxurians* (Durieu and Ascherson) Bird (Tsai and Falk, 1996). Out of these cicadellids, the only one found in Argentina is *D. maidis* (Paradell *et al*., 2001) and the only species of the genus *Zea* present is cultivated maize (*Z. mays*). None of these gramineae occur either wild or culti-

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vated, except in isolated cases in some maize phyto-improvement research institutions in the province of Buenos Aires.

Even though the distribution of this disease and of its vector *D. maidis* in Argentina have been studied in the last few years (GIMÉNEZ PECCI *et al*., 2002a,b, 2003), there is up to now no information available about its transmission with *D. maidis* individuals collected in Argentina, or about the behavior of isolates of the pathogen found in different regions of the country. Such studies can provide useful information for the understanding of the epidemiology of this disease and its subsequent management.

The aims of this work were: 1) to test the ability of populations of *D. maidis* from different parts of Argentina to transmit Corn stunt spiroplasma (CSS, *Spiroplasma kunkelii*), using infected insects originating in the field and populations of healthy insects kept in the laboratory. 2) to transmit different isolates of the pathogen, to study the symptoms shown under controlled conditions.

MATERIALS AND METHODS

Obtaining and maintenance of Dalbulus maidis *colonies*

The vector colony was started from insects collected in maize crops in the province of Tucumán. To begin the colony, adult insects were placed to lay their eggs on healthy maize plants at four-leaf stage for seven days, and then removed. Once the nymphs hatched, they were placed on healthy four-leafed maize plants until reaching adulthood. This procedure continued for about one year, from which time the colony was considered "healthy". The plants that were in contact with the insects were kept in greenhouses to test their state of health through the observation of symptoms and serology.

The "healthy" *D. maidis* colonies were kept in the greenhouse at an average temperature of 26°C, similar to that indicated for the experimental maintenance of the species by REMES LENICOV and VIRLA (1993). Aluminum-framed cages were built, 60 X 60 X 20 cm, covered with a fine "voile" type nylon mesh. Every week, one of the plants that were in contact with the insects was replaced with a new plant at the same stage. Periodically, adult individuals were chosen at random and their taxonomic identity and the purity of the colony were tested.

Experimental host

The maintenance of the vector colony and the transmission trials were made on *Zea mays* cv Pop Zélia (Popcorn), which is highly susceptible to the pathogen (OLIVEIRA *et al*., 2002).

Sources of inoculum and geographical origin

The initial inocula were obtained from symptomatic maize plants collected in the field or based on vector insects collected with an entomological net dragged through maize fields with symptoms of the disease.

These initial inocula were collected in different geographical regions (LORENZINI *et al*., 1995); of **subtropical** climate: Las Breñas (Chaco), Salta (Salta), Villa Trinidad (Santa Fe), Santiago del Estero (Santiago del Estero) and San Miguel de Tucumán (Tucumán); and of **temperate** climate: Salto (Buenos Aires), Villa del Rosario and Colonia Caroya (Córdoba) (Fig. 1). This distinction was made bearing in mind that the disease is found every year, with strong symptoms, in the subtropical region, and to a lesser extent in temperate areas (GIMÉNEZ PECCI *et al*., 2002a,b; GIMÉNEZ PECCI and LAGUNA, 2004); and matches its vector distribution (*D. maidis*), which is found only to the north of latitude 30° S (PARADELL *et al*., 2001).

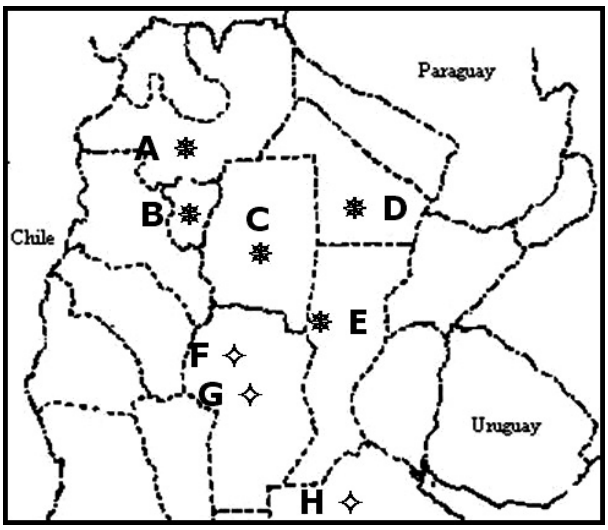

FIGURE 1 - Localities where the inocula were collected to begin CSS transmission under controlled conditions: ✳: Subtropical Climate: A: Salta (Salta); B: San Miguel de Tucumán (Tucumán); C: Santiago del Estero; D: Las Breñas (Chaco); E: Villa Trinidad (Santa Fe). ◇: Temperate Climate: F: Colonia Caroya (Córdoba); G: Villa del Rosario (Córdoba); H: Salto (Buenos Aires).

Transmission tests

The transmission attempts made and the total of inoculated and symptomatic plants are shown in Tables 1 and 2. The acquisition access (7 days), incubation (21 days) and inoculation access (7 days) periods were used following NAULT (1980).

To transmit the disease from symptomatic plants from the field, these were taken to the greenhouse and approximately 40 healthy 3rd - 4th stage nymphs were placed on them and allowed to feed for seven days. At the end of this period, the insects were moved to healthy maize plants at the four to six-leaf stage for 21 days, for incubation in the vector. Then the insects were placed on maize plants at the one-leaf stage, and allowed to feed for seven days, using a density of five insects per plant. Then the same insects were allowed to feed consecutively on different groups of plants for seven days.

When transmission was originated from field-collected insects, these were placed on maize plants at the one leaf stage for seven days to permit inoculation, at a density of five insects per plant. Just as in the previous case, the surviving insects were placed consecutively on different groups of plants. To estimate the probability of transmission (P) of single individuals, we used the equation of SWALLOW (1985):

$P = 1 - (1 - I)^{1/K}$

Where I is the proportion of diseased plants and K is the number of insects caged per plant.

Symptom development and pathogen detection

Once the inoculation access period ended, the plants in each group were taken to the greenhouse, where symptom development was monitored and they were sprayed weekly with insecticide to prevent possible contamination (Buprofezin, 50 g/100 liters). During the tests the average daytime temperature was 27.6°C and at night 18.5°C.

When all the plants exposed to the insects reached the reproductive stage, the penultimate leaf to appear on each plant was removed and kept in a freezer until the time for serological analysis. The presence of *Spiroplasma kunkelii* was determined by DAS-ELISA (Clark and Adams, 1977), using commercial reagents conjugated with alkaline phosphatase (AGDIA Inc, USA). The results were read at a wavelength of 405 nm and expressed as relative absorbance (RA), determined as the quotient between the absolute absorbance of each sample and the average value plus three standard deviations of six healthy controls, following Sutula *et al.* (1986).

To relate the symptomatology observed with the pathogen titer, serological analysis was made as from 40 days after infection. The RA of each sample was considered an indicator of pathogen titer. Diseased plants were identified as those with a RA greater than 1, and the higher the RA value, the greater the pathogen titer in the plant tissues was considered to be.

The serological results were corroborated through the PCR (Polymerase chain reaction) test. The nucleic acid extraction was carried out following Doyle and Doyle (1994), and the PCR reaction following Barros *et al.* (2001), using F2 and R6 initiators, specific for *S. kunkelii*, which amplify a 500 bp fragment of the spiralin gene.

Both the plants used as a source of inoculum and those transmitted in controlled conditions gave negative reaction both to Maize Bushy Stunt Phytoplasma (MBSP) and Maize rayado fino virus (MRFV), pathogens that are also transmitted by *D. maidis* and cause similar symptoms on maize (Tsai and Falk, 1996). PCR were performed to detect general phytoplasmas using primers R16F2 and R16R2 (Lee *et al.*, 1993), and DAS-ELISA test were performed to detect MRFV (data not shown).

RESULTS

1. Experimental transmission of CSS

Tables 1 and 2 present the results of the CSS transmissions under controlled conditions, using inocula from different regions of Argentina. Out of 12 attempts at transmission, six were successful. Infected plants with inoculum were obtained from three areas of subtropical climate. Las Breñas, Villa Trinidad and San Miguel de Tucumán. The source of inoculum in the first two places was diseased plants, and in that of S. de Tucumán were insects in the field.

A) Plant transmissions

Of the five places from which transmissions were attempted, two were successful: Las Breñas (Chaco) from the typically subtropical region and Villa Trinidad (Santa Fe) from the ecotone of the subtropical region towards the temperate area (Table 1).

From a total of 15 plants inoculated from the plant collected in Las Breñas, three registered a positive reaction. The inoculum source plant showed slight shortening of the upper internodes (stunting) and leaves with reddish margins, with average relative absorbance of 2.1. The three plants transmitted in controlled conditions showed severe stunting and chlorotic stripes starting from the base of the leaves, characteristic symptoms of CSS described by Nault (1980), without reddening of the margins of lower leaves. This evidence of typical symptoms in the transmitted plants was related to high relative absorbance in the ELISA test, with a mean value of 23.6.

TABLE 1 - *CSS transmissions under controlled conditions, using symptomatic plants collected in different areas of Argentina as inoculum source,* D. maidis *as vector and pisingallo maize cv. Pop Zelia as host.*

Geographic Region	Province	Locality	Collection Date	Number of Inoculated Plants	Number of Symptomatic Plants #
Subtropical	Chaco	Las Breñas	06/05/03	15	3
	Salta	Salta	02/05/03	3	0
Temperate	Santa Fe	Villa Trinidad	08/04/03	9	1
	Buenos Aires	Salto	26/11/03	3	0
	Córdoba	Colonia Caroya 1	16/04/03	18	0

(#) The presence of the pathogen was confirmed with DAS-ELISA and PCR in the inoculated plants that showed the symptoms described. Transmissions were performed with a vector density of five insects per plant.

In the transmission with inoculum from Villa Trinidad, only one plant of the nine transmitted showed symptoms of the disease. In this case, the original plant presented reddening of the leaf margins and normal height, with relative absorbance of 2.4. The transmitted plant showed very mild leaf chlorosis and relative absorbance near 2.0.

CSS transmission was not achieved from plants collected in Colonia Caroya (Córdoba), Salta (Salta), and Salto (Buenos Aires). The inoculum source plants from Colonia Caroya and from Salta showed slight chlorotic stripes and normal plant growth, with relative absorbances of 1.8 and 2.5 respectively. In the case of the inoculum from Salto (Buenos Aires) the symptoms shown were even slighter: chlorotic margins in the upper leaves and normal plant growth; the relative absorbance was 2.5.

B) Insect transmissions

Out of seven field collections of insects, five in the subtropical region and two in the temperate region, four transmissions were achieved (Table 2), all originating from the area of San Miguel de Tucumán (Tucumán).

From a total of 75 plants that were in contact with insects from San Miguel de Tucumán (in groups of five insects per plant) the disease was transmitted to 29, which represents 39% of the plants, or 9.4% of transmission probability by a single insect (SWALLOW, 1985). This is near to the proportion of inoculative *D. maidis* found by EBBERT *et al.* (2001), between 4 and 9%.

In all the transmissions, the diseased plants showed typical CSS infection symptoms, but its severity varied notably between plants. The plants with mild CSS symptoms showed normal growth in height, and only small chlorotic stripes starting from the base of the leaves; the severely affected plants however had a stunted appearance, and leaf chlorosis spread to cover the laminas completely. In the same way, relative absorbance was very variable, ranging from 1.3 to 26.6, with an average value of 16.0.

2 - Pathogen titer and plant symptoms transmitted with insects

The plants infected with the insects from Tucumán were used to establish the relationship between symptom severity and the pathogen titer estimated in the DAS-ELISA test. Great variability was a feature both of symptom severity and of the pathogen titer of infected plants. Following this, it was possible to separate the plants in two groups, one with **severe symptoms**, which included stunted plants, with marked shortening of internodes, and high relative absorbance, near 20 when the serological analysis was made as from 100 days after infection (Group 1), and another with **mild symptoms**, which included plants with normal growth in height, with relative absorbance near 10 (Group 2).

Disease symptoms in Group 1 (Table 3, Fig. 2) were marked by their rapid appearance and homogeneity between plants. After a 40 day post inoculation period in which no symptoms were observed, the plants began to display twisted, deformed leaves,

TABLE 2 - *CSS transmissions under controlled conditions, using field collected* D. maidis *as vectors and pisingallo maize cv Pop Zelia as host.*

Geographic Region	Province	Locality	Collection Date	Number of Inoculated Plants	Number of Symptomatic Plants #
Subtropical	Sgo del Estero	Sgo del Estero	02/05/03	1	0
	Tucumán	S.M. de Tucumán 1	10/02/03	20	11
		S.M. de Tucumán 2	17/02/03	19	9
		S.M. de Tucumán 3	01/05/03	13	3
		S.M. de Tucumán 4	11-12/03	23	6
Temperate	Córdoba	Colonia Caroya 2	16/04/03	4	0
		Villa del Rosario	27/02/03	2	0

(#) The presence of the pathogen was confirmed with DAS-ELISA and PCR in the inoculated plants that showed the symptoms described. Transmissions were performed with a vector density of five insects per plant.

TABLE 3 - *Symptom development and relative absorbance (RA) for CSS in the "Tucumán 1" isolate. Group 1: plants with severe symptoms and high pathogen titer. Group 2: plants with mild symptoms and lower pathogen titer.*

Days since infection	Group 1			Group 2		
	Symptoms	RA	n	Symptoms	RA	n
0 – 40	Without symptoms	N/D		Without symptoms	N/D	
40 – 70	Leaf chlorosis. Twisting of leaf tip and leaf deformation	7.8 ± 5.9	3	Mostly without symptoms. Slight leaf chlorosis in some plants	1.7 ± 0.8	8
70 – 100	Typical chlorosis and stunting (height less than 1 meter)	14.7 ± 4.2	7	Leaves with slight chlorotic stripes. Height greater than 1 meter	4.3 ± 2.0	3
100 – 150	Plant death	23.2 ± 2.7	4	Leaves with typical chlorotic stripes, axillary bud activation, multiple ears, phyllody	11.5 ± 2.1	3

N/D: not determined. n: number of plants in which RA was measured.

with cut edges, together with the characteristic symptomatology of the disease: chlorotic markings beginning in the base of the leaf laminas and extending to the tips. In subsequent leaves, chlorosis increased gradually until leaf laminas were completely white and necrosing rapidly. The plants were very stunted, with a height less than 1 meter. In no case did ears or tassels form. All the plants died quickly, a few days after reaching typical symptom stage.

This rapid development of symptoms was accompanied by high initial pathogen titers (Table 3, Fig. 3), from RA values of 7.8 to twisted, deformed leaf stage (40-70 days). Titers increased considerably during this time, with a slope value of 0.22 (Fig. 3), reaching a relative absorbance of 23.2 at the end of the ontogeny, when the severity of the symptoms was most marked, ending with the rapid death of the plants.

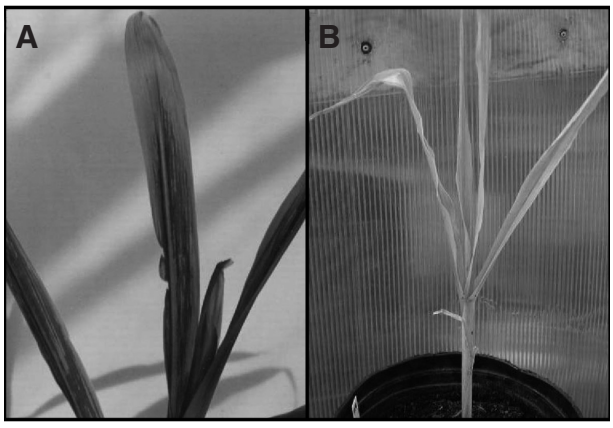

FIGURE 2 - Severe symptoms of CSS in corn, Group 1. A) leaves with strong chlorosis, cut edges and twisted tip. B) Stunted plant with little growth, and early death.

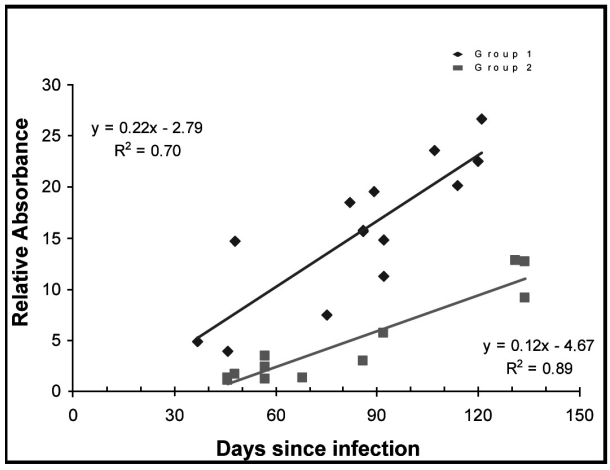

FIGURE 3 - Correlation between titer of *S. kunkelii* (determined as relative absorbance) and time since infection. Group 1: plants with severe symptoms. Group 2: plants with mild symptoms.

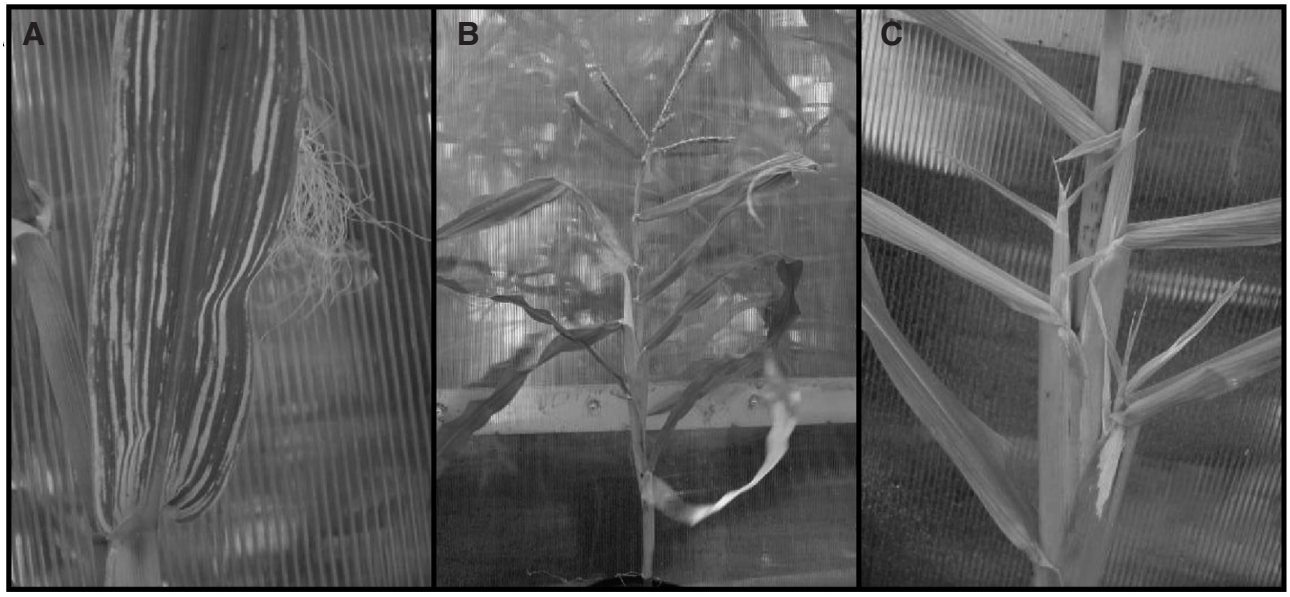

FIGURE 4 - Maize plants diseased with CSS, Group 2. A) Typical chlorotic markings beginning in the base of the leaves and extending towards the tips. B) Normal growth in height, formation of ears and tassels. C) Appearance of multiple ears, and of phyllody in some cases.

In the plants in Group 2 the symptoms manifested in a milder and slower form than in Group 1 (Table 3). The upper leaves showed a slight lightening, which turned into typical chlorotic stripes in the apical leaves (Fig. 4). In general, the height of the plants was normal, and they always formed male and female inflorescences. The development of symptoms was less homogeneous than in Group 1. 20% of the plants presented leaf margins with a reddish color in the lower leaves, at times followed by their necrosis; 30% of the plants had multiple ears.

The slower and milder expression of symptoms was related to lower pathogen titers, from a RA of 1.7 when presenting mild leaf chlorosis (40-70 days), up to 11.5 at the end of the cycle, when the plants showed normal growth and the apical leaves the characteristic chlorotic striping (Table 3, Fig. 4). Analyzing the correlation between the RA and the time since infection, the slope value was 0.12, which indicates a lower rate of spiroplasma multiplication than in the plants of Group 1, which presented a slope of 0.22.

DISCUSSION AND CONCLUSIONS

This paper reports the transmission of *Spiroplasma kunkelii* to maize plants, from diseased plants and from field-collected insects, using local populations of the vector *Dalbulus maidis*. Transmission was achieved from areas located in the subtropical region, while none were obtained from inocula collected in the temperate region.

In attempts to obtain isolates from diseased plants collected in field, only four diseased plants out of 48 (8%) were found. This indicate that the insects were unable to acquire and/or transmit the pathogen following acquisition, incubation and inoculation periods described previously (NAULT, 1980). These results seem to disagree with the high efficiency of *D. maidis* to transmit *S. kunkelii*, near to 100% (ALIVIZATOS and MARKHAM, 1986). This might be caused by the lack of viability of the spiroplasmas in the source plants. In our study we collected the plants in field and moved them onto greenhouse, and acquisition started usually three days after the collection. It is possible that during this time dehydration would kill the spiroplasmas, which are sensible to osmotic changes (CHENG and CHANG, 1994). Both PCR and DAS-ELISA recognize respectively DNA and proteins specific to the pathogen, but do not provide information about if the spiroplasmas are living or not. Nevertheless, we were not able to rule out the possibility that local isolates of *D. maidis* would be less efficient to transmit the spiroplasmas, as was found by EBBERT *et al.* (2001); nor lower transmissibility of the variants of *S. kunkelii* present in Argentina.

Based on insects collected in field, disease transmission was achieved only from vectors collected in Tucumán. According to the proportion of diseased plants obtained, we estimate that 9.4% of the insects in field were inoculative (SHALLOW, 1985). This agrees with earlier reports made by other authors, indicating that not all field-collected insects transmit the disease (GORDON *et al*., 1985; EBBERT *et al*., 2001; OLIVEIRA *et al*., 2002).

Symptoms began to manifest in diseased plants approximately 40 days after transmission. Even though other authors (BRADFUTE *et al*., 1981; BAJET and RENFRO, 1989) cited a shorter period for symptom expression (between 15 and 30 days), could be due to their having carried out the trials at higher temperatures. In this respect, NAULT (1980) indicated that symptom expression is earlier the higher the temperature to which the diseased plants are exposed. Bearing in mind this distinction, our results coincide totally with those reported by NAULT (1980) for a temperature regime similar to that of our trials (average 27-18°C by day and night respectively).

Prior studies of disease incidence in the field have indicated that serology or PCR may not always show a relationship between the presence of symptoms and the detection of CSS pathogen. Previous works (OLIVEIRA *et al*., 1998; MASSOLA JUNIOR *et al*., 1999a; GIMÉNEZ PECCI *et al*., 2002; GONÇALVES SILVA *et al*., 2002) found symptomatic plants with a negative reaction and symptom-free plants with a positive reaction. In the present work, made under controlled conditions, pathogen presence was closely related to the manifestation of symptoms, since in the cases in which symptoms were observed, the pathogen was detected by serology, and its presence confirmed by PCR. Likewise, no positive reaction for the spiroplasma was detected in any of the asymptomatic plants throughout the trial.

This disparity between the results obtained in this work and those of the authors mentioned could be due to the criterion used in determining the symptoms: while the previous authors included reddening of the leaf margins as a disease symptom, in this work only those plants whose leaves contained the chlorotic stripes described by NAULT (1980) were considered symptomatic, since in the few plants showing reddened leaf margins in lower leaves, chlorotic striping was always seen in upper leaves. Our results thus agree with those of BAJET and RENFRO (1989) and MASSOLA JUNIOR *et al*. (1999a), who detected the pathogen in all the plants in the field which had typical stripes in the leaves.

On the other hand, it was observed that the symptoms of the greenhouse-infected plants do not fully correspond with those on the field-collected plant source of the inoculum. This is the case in the transmissions from Villa Trinidad and from Las Breñas, in which the inoculum-source plants showed reddening of the leaf margins, while the infected plants did not show such reddening but rather chlorotic stripes at the base of the leaves, either very marked (Las Breñas) or slight (Villa Trinidad). This may be due to other factors affecting symptom expression, such as different host genotypes and environmental conditions (BEDENDO, 1999; MASSOLA JUNIOR *et al*., 1999a,b; TAVARES FERNANDEZ *et al*., 2002).

The direct relationship obtained between symptom manifestation and pathogen titer agrees with that detected by GUSSIE *et al*. (1995), who also found great variations in the pathogen titer and symptoms between plants infected by the same isolate in controlled conditions after 50 days. In our trials, it was possible to separate this variation into two plant groups: one group presenting high pathogen titer (RA near 20.0) and severe symptoms (Group 1); and another group with lower pathogen titer (RA near 10.0), and milder symptoms (Group 2). Therefore, it may be inferred that pathogen titer affects symptom severity, since high early titers are related with the appearance of typical symptoms in the plants in an early, severe form, and their rapid death. On the other hand, when the multiplication of the spiroplasma is lower, the symptoms are milder and later in time, so that the plants reach normal growth in height and form reproductive structures.

Based on the results obtained in this study, it can be asserted that symptom severity is directly related with the pathogen titer. Great variation in symptom severity and pathogen titer has been previously found (GUSSIE *et al*., 1995), and its possible causes have yet to be determined; among these may be mentioned: differences in the amount of initial inoculum, probably due to not all the insects collected in the field being diseased; possible variants of the pathogen differing in aggressiveness; and genetic variation in the host. For this reason, further studies will be necessary to establish its significance on the severity of symptoms, and thus on the yield of the affected plants.

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