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SHORT COMMUNICATION

Occurrence and distribution of single or mixed infection of phytoplasma and spiroplasma causing corn stunting in Brazil

Sarah R. Galvão[1] · Elizabeth O. Sabato[2] · Ivan P. Bedendo[1]

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Abstract

Maize bushy stunt and corn stunt have emerged among the most important diseases of maize in Brazil. To evaluate the single or dual presence of the phytoplasma and spiroplasma associated with corn stunting diseases, maize samples were collected across several locations in four Brazilian states. Multiplex PCR was performed for simultaneous detection of the bacteria. Eighty-nine out of 100 samples were positive with percentage values of 40%, 35%, and 25% for phytoplasma, spiroplasma, and mixed infections, respectively. Temperature may be an important driver of the prevalence of these mollicutes as phytoplasma prevailed in areas with mild temperatures and spiroplasma prevailed in warmer areas. These results extend knowledge of factors associated with corn stunting diseases, such as the potential role of temperature shaping the composition of the regional plant pathogenic populations.

Keywords Maize bushy stunt · Corn stunt · Mollicutes · *Zea mays*

Phytoplasmas and spiroplasmas are wall-less plant bacterial pathogens belonging to Mollicutes. They were discovered during the late 1960s and the early 1970s, respectively (Doi et al., 1967; Davis et al., 1972). Although spiroplasmas are best known to cause diseases in maize (Oliveira and Oliveira 2017) and citrus (Bové et al., 2003), phytoplasmas are associated with hundreds of diseases reported around the world in a large diversity of crops (Bertaccini and Duduk 2009). In maize, these bacteria are responsible for two major diseases known as corn stunt, caused by *Spiroplasma kunkelii* (Davis et al., 1972) and maize bushy stunt, associated with '*Candidatus* Phytoplasma asteris' (Lee et al., 1998), enclosed in the 16SrI-B group. Both stunting types may occur alone or simultaneously due to the presence of the leafhoppers *Dalbulus maidis* and *D. elimatus* that act as vectors (Nault, 1980).

In Brazil, these diseases were reported for the first time during the 1970s when they were considered of secondary importance to maize crops (Costa et al., 1971). Currently, these diseases cause severe damage to the grain production in all maize-producing regions and, non-rarely, epidemics reach 100% incidence that result in total yield loss (Sabato, 2017). Increase of incidence and severity has been likely driven by changes in the production system. In the last three decades, a new practice was introduced and areas commonly cultivated with maize have been used for soybean planting (Coelho et al., 2017). In these areas, the sowing time of corn is delayed till the end of soybean harvest. This kind of system, known as "second crop season" ("safrinha", in Portuguese), which now represents 60% of the Brazilian production, proved advantageous and became widely adopted in areas traditionally used for corn production (Sabato, 2017; Coelho et al., 2017). Even in areas where maize has been cultivated during the normal season, the second crop extends the time maize is present in the field during the year. In addition, in recent years, growers are adopting more intensively irrigation resources, allowing to obtain two to three grain harvests per year and, consequently, the maintenance of plants in the area. This superposition of plants in the field is highly favorable for the survival of these pathogens as well as their insect vectors. Therefore, infected leafhoppers can easily move from older to younger crops, spreading the diseases to other maize areas (Oliveira et al., 2002, 2015). The longer maintenance of corn in the field has led to an increase of the vector population, which found favorable conditions of temperature and host

✉ Ivan P. Bedendo
ibedendo@usp.br

[1] Departamento de Fitopatologia e Nematologia, ESALQ, Universidade de São Paulo, Piracicaba, SP 13418-900, Brazil

[2] Embrapa Milho e Sorgo, Sete Lagoas, MG 35701-970, Brazil

Fig. 1 Map of Brazil showing the geographic locations of the areas where the symptomatic corn plants were sampled for detection of spiroplasmas and phytoplasmas. Sample 1: state of Bahia (BA); samples 2, 3, and 4: state of Goiás (GO); samples 5, 6, and 7: state of Minas Gerais (MG); samples 8, 9, and 10: state of São Paulo (SP)

plant density throughout the year. These factors contribute to increase the risk of corn stunting diseases. This scenario explains recent major outbreaks from 2015 to 2017, which caused severe yield losses and the difficulty to manage these diseases (Sabato, 2017, 2018). Since the "second crop season" is routinely practiced and economically relevant, it is urgent to extend the knowledge about factors affecting epidemics by corn stunt complex diseases, such as the presence and distribution of the pathogen, which was the main objective of this work.

Surveys were conducted during 2017, and total of 110 samples were collected from plants exhibiting symptoms of stunting present in corn crops grown at distinct geographic areas of the Brazilian territory (Fig. 1; Table 1). The DNA was extracted from the leaves, according to a CTAB protocol (Doyle and Doyle, 1990) and used as template in multiplex PCR reaction mixture. The primers pair CSSF2/CSSR6 (Barros et al., 2001) was employed to detect spiroplasma and the primers R16F2n/R16R2 (Gundersen and Lee, 1996; Lee et al., 1993) for phytoplasma detection. The reaction was performed in a final volume of 25 μL containing 1 μL diluted DNA (50 ng); 18.7 μL sterilized water; 0.25 μL each primer (0.4–1.0 μM); 2 μL solution 2.5 mM each dNTP; 2.5 μL buffer 10X PCR and 0.17 μL Amplitaq 5 U μL^{-1}. Multiplex PCR was conducted by 35 cycles following the program: 15 s (30 s for the first cycle) at 94 °C, 15 s at 50 °C, and 15 s at 72 °C (5 min for final cycle). Independent analyses of negative samples using multiplex were performed with direct simple PCR for each of the two pathogens. The PCR products were analyzed by electrophoresis through a 1% agarose gel followed by staining in Sybr safe (Thermo Fisher Scientific) and visualized using a UV transilluminator. DNA from plants

Table 1 Number of plant samples positive for the presence of phytoplasmas and spiroplasmas, alone or in mixture, associated with maize bushy stunt and corn stunt, respectively. In parenthesis, the total number of plants

Sampling locations	Phytoplasma-infected	Spiroplasma-infected	Mixed-infected
Rio Verde (Goiás)	4 (14)	3 (14)	4 (14)
Campo Alegre (Goiás)	1 (7)	2 (7)	1 (7)
Goiatuba (Goiás)	2 (10)	4 (10)	4 (10)
Patrocínio (Minas Gerais)	3 (10)	2 (10)	2 (10)
Sete Lagoas (Minas Gerais)	13 (17)	0 (17)	0 (17)
Janaúba (Minas Gerais)	3 (19)	8 (19)	6 (19)
Mococa (São Paulo)	5 (11)	1 (11)	3 (11)
Casa Branca (São Paulo)	2 (6)	1 (6)	1 (6)
Paranapanema (São Paulo)	2 (5)	1 (5)	1 (5)
Luis E. Magalhães (Bahia)	1 (11)	9 (11)	0 (11)

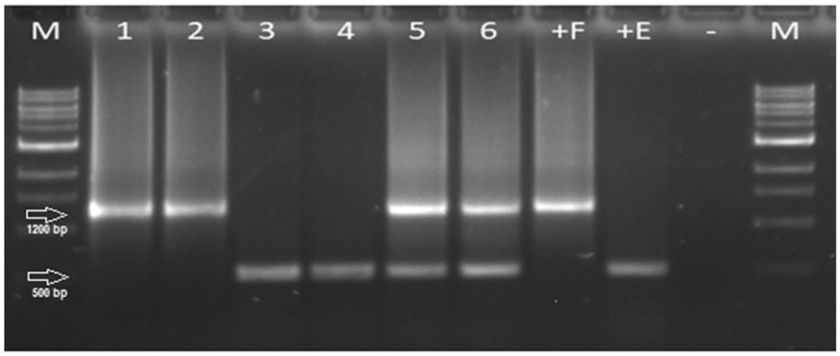

Fig. 2 DNA fragments amplified with multiplex PCR. Columns 1 and 2 represent samples infected by phytoplasmas; Columns 3 and 4 represent samples infected by spiroplasmas. Columns 5 and 6 represent samples with mixed infection. Letters +F and +E represent positive controls for phytoplasma and spiroplasma, respectively. Signal (-) and letter M represent negative control (water) and 1Kb Plus DNA Ladder

experimentally inoculated by the insect vector *D. maidis* with maize bushy stunt phytoplasma and corn stunt spiroplasma were used as positive control, and sterilized water served as negative control of reaction.

The multiplex PCR generated amplicons indicating the presence of phytoplasma and/or spiroplasma in 89 samples (Table 1). Phytoplasma and spiroplasma were identified in agarose by bands corresponding to the expected fragments of approximately 1200 bp and 500 bp, respectively (Fig. 2). Identical results were obtained for the positive controls, but no amplification occurred for the negative control (Fig. 2). The samples were tested negatives even after direct PCR for each pathogen.

Single infection by phytoplasma was found in 40% of the positive samples, while 35% of the symptomatic plants were infected only by spiroplasma. Both pathogens were simultaneously detected in 25% of the plants that exhibited symptoms. They were absent in 21 symptomatic samples.

The presence of single or mixed infection of phytoplasma and spiroplasma was variable across the sampled areas (Table 1; Fig. 3). In particular, the prevalence of phytoplasma was greater than spiroplasma in most of the sampled areas. However, spiroplasma prevailed in fields located in Campo Alegre, Goiatuba, Janaúba, and Luís Eduardo Magalhães. The occurrence of mixed infection was detected in most of the sampled areas, except those located in Sete Lagoas and Luís Eduardo Magalhães (Table 1; Fig. 3).

The multiplex PCR showed to be a simple and rapid tool to confirm the identity of the pathogens, since the symptoms induced by phytoplasma and spiroplasma may vary according to the corn genotype, the climatic condition, the period of sowing, and the infected vector population (Sabato, 2017). The tool allowed detecting the presence of the pathogens in 80% of the symptomatic samples. The failure to detect these prokaryotes in 20% of symptomatic plants can be mainly due to uneven distribution and age of the host during the infection (Oliveira et al., 2002).

Our results confirm the influence of the temperature on the prevalence of spiroplasma or phytoplasma reported previously (Oliveira et al.; 2007, 2015; Sabato et al., 2013). Spiroplasma was prevalent in warmer (25 to 30 °C) areas, such as those of Luís Eduardo Magalhães and Janaúba where the mean average temperature in the last 30 years was above 24 °C during the corn crop season. In contrast, phytoplasma was predominant in areas of mild temperatures, ranging from 18 to 22 °C. Among

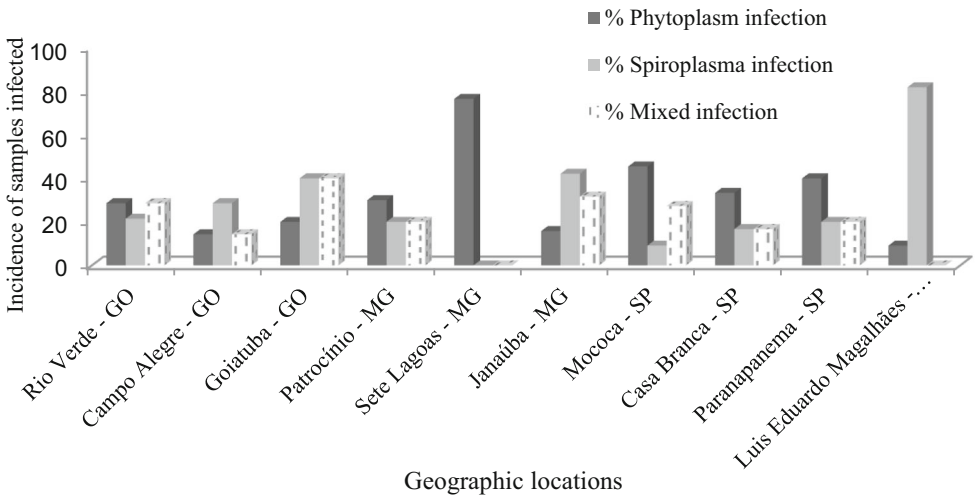

Fig. 3 Incidence level (%) of samples infected with phytoplasma, spiroplasma, and both pathogens, sampled in distinct geographic locations

these areas, the highest prevalence of phytoplasma was found in Sete Lagoas where mild temperatures are prevalent. The findings of the present study are in agreement with those reported in Mexico where phytoplasmas were more frequently found in areas of high altitude with milder temperature, located in the southeast region (Pérez-López et al., 2016). In contrast, results from another survey showed that spiroplasmas were present in leafhopper adults collected in low-elevation sites where normally occur higher temperatures (Moya-Raygoza et al., 2007). Accordingly, Nault (1980) showed a predominance of plants with symptoms induced by the spiroplasma at low altitudes, while plants displaying symptoms associated with the phytoplasma were found mainly at high-elevation areas. These statements reinforce the findings of the present study that evidenced the effects of lower and higher temperatures on the geographic distribution of both bacteria in areas cultivated with maize.

Interestingly, the prevalence of mixed infection increased when compared with data from almost two decades ago in Brazil. In 2002, surveys showed that only 5.8% of sampled plants were infected with both pathogens (Oliveira et al., 2002), while the results here presented showed that 25% of the symptomatic plants were infected. This notable increase is likely associated with the increased adoption of the "second crop season" and use of irrigation, practices that have routinely been adopted by maize growers. The presence of plant hosts during several months of the year favors the population of insect vectors, which have higher chances to acquire and disseminate both types of pathogens. However, it would be interesting to evaluate, in a future study, the reduction of crop yields caused by only one of the pathogens and the level of damage present in the mixed infections. The results generated in the present study contribute to extend the understanding of this relevant pathosystem, mainly in relation to the influence of the temperature in the geographic distribution of the pathogens.

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Author contributions All authors were involved with the elaboration of the research project. Material and data collection were performed by SRG and EOS; analyses and interpretation were conducted by SRG and IPB. The manuscript was initially written by SRG and complemented by EOS and IPB. All authors read and approved the final manuscript.

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