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A DECADE OF PHYTOPLASMA RESEARCH: STOLBUR IDENTIFICATION IN MOLDOVAN TOMATO VARIETIES

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KEYWORDS

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ABSTRACT

Tomato crop in Moldova is threatened by '*Candidatus* Phytoplasma solani', a phloem-limited pathogen transmitted by insect vectors. Local tomato varieties were screened by nested-PCR during ten years (2016–2024). Results showed clear variety-dependent susceptibility. Variety Cerasus displayed stable partial resistance (<35% of infected plants). Elvira and Desteptarea were highly vulnerable (50–85%) while Mary Gratefully showed variable incidence (33–92%) influenced by climate conditions of the year. The summarized infection data fluctuated widely between years from epidemic levels (83% in 2016, 72% in 2023) to minimal incidence (10% in 2020). Sequencing of isolates confirmed the persistence of identical strains of phytoplasma over the growing seasons (2018–2020). Ten-year study provides a comprehensive overview of epidemiological situation of '*Ca.* P. solani' in the Republic of Moldova and offers valuable information for tomato breeders as well serving a basis for disease control strategies.

INTRODUCTION

Tomato presents an agri-food crop of global importance, playing an essential role in the diet of the population of the Republic of Moldova (Mihnea, 2016). At the global level, the annual tomato production in 2024 is estimated at approximately 190 million tonnes (FAO, 2024). The productivity and quality of tomato are influenced by numerous biotic and abiotic factors among which are the activity of pathogenic agents including phytoplasmas (Botnari, 2021). Solanaceous plants are mainly affected by '*Candidatus* Phytoplasma solani', with a significant impact on tomato crops. In addition, this pathogen has also been detected in the Republic of Moldova in other agricultural crops such as potato, grapevine, pepper, and some perennial plant species (Bahsiev et al., 2023; Bahsiev et al., 2024; Haustov et al., 2024).

The study of phytoplasmas remains relevant because, although the symptoms of diseases caused by these microorganisms have been known since antiquity, the nature of the pathogen was identified relatively recently. For many years phytoplasmas were considered as viral agents (Namba, 2019). Only with the development of electron microscopy, in the 1960s, it was demonstrated that phytoplasmas are bacteria without a cell wall. The phytopathogen is transmitted mainly by insects from the order Hemiptera, families Cixidae, Cicadellidae, and Psyllidae (Weintraub et al., 2006). Likewise, the spread of phytoplasma can be caused by incorrect agricultural practices, climatic conditions, and wild plants, which act as natural reservoirs where the vector insects overwinter, etc. (Bogoutdinov et al., 2018). The phytoplasma colonizes the plant phloem causing a systemic disease. Although some studies have suggested the possibility of stolbur transmission through seeds, our previous researches on the local tomato varieties have not found the evidence supporting seed-mediated transmission (Bahsiev, 2017; Zamorzaeva et al., 2020). Symptoms of '*Ca.* P. solani' infection in tomato include dwarfism, virescence, abnormal flower development or sterility, and small fruit size. The incubation period from the infection to the symptoms appearance is long. It makes difficult early diagnosis of the disease (Bertaccini et al., 2010). Likewise, the pathogen detection may be limited in the case of mixed infections that mask the symptoms of phytoplasma infection (Xu et al., 2013).

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Disease control is closely linked to the biological cycle of ˈ*Ca.* P. solaniˈ and focuses on limiting its spread through the use of insecticides and herbicides. Another control method is the use of habitat antagonists (Kumari et al., 2019). Thus, it is important to prevent the spread of infection through early diagnosis. Many diagnostic methods have a number of limitations in phytoplasma detection. Morphological methods are limited by the asymptomatic period. The use of electron microscopy is challenging due to phytoplasma cell polymorphism, while immunological methods are limited because some phytoplasmas have no specific antisera available for detection (Fluit et al., 2001; Romanazzi et al., 2009; Zamorzaeva et al., 2020). The emergence and development of molecular methods made a revolution in phytoplasma diagnostics, with the determination of the pathogen taxonomic status. At present, the PCR method remains a useful and rapid tool for identifying the pathogen ˈ*Ca.* P. solaniˈ (Mero et al., 2016).

MATERIALS AND METHODS

Molecular diagnostics of ˈ*Ca.*

P. solaniˈ was performed on local Moldovan tomato varieties cultivated under field conditions. Over a nine-year period (2016–2024), a comparative assessment of phytoplasma distribution across these varieties was conducted. For analysis, tomato fruits with peduncles were collected from each variety at the end of the growing season (late August – early September). This timeframe was identified as optimal for assessing infection levels, as established in a previous study (Zamorzaeva et al., 2019).

In addition, symptomatic stolbur plants were registered in the tomato field during growing seasons and plant material was collected to confirm causative agent of the disease by sequencing ribosomal gene fragments specific to ˈ*Ca.* P. solaniˈ. Sequencing of 16S rRNA gene fragment of phytoplasma was performed using primers P1/P7 and R16F2n/R16R. DNA extraction for sequencing was carried out using DNA-zol.

DNA extraction for nested-PCR was carried out using a rapid boiling method, where thin sections of each peduncle were boiled in 10 µl of 0.3 N NaOH for 5 minutes, followed by neutralization with 10 µl of 0.3 N HCl and centrifugation at 10,000 rpm for 3 minutes (Guo et al., 2003). A 1 µl aliquot of the resulting solution served as a DNA template for PCR analysis.

The molecular diagnosis was mainly conducted via nested-PCR using ˈ*Ca.* P. solaniˈ specific primers (cpn421F/R and cpn200F/R), which were designed in the Molecular Genetics Laboratory of IGPPP, MSU (Chisinau, Moldova) based on the unique chaperonin (*groEL*) gene sequence (Zamorzaeva et al., 2016). In addition, other specific to ˈ*Ca.* P. solaniˈ primers were used in the study (Table 1).

The nested-PCR programs were as follows: initial denaturation at 94°C for 5 minutes followed by 30 cycles (first PCR) and 35 cycles (second PCR) of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension was performed at 72°C for 10 minutes, with the reaction terminating at 4°C.

Table 1. Primers used in diagnosis of 'Ca. P. solani' in tomato

Nr	Primer	F/R	5'-3' sequence	Length bp	T_m C°
1.	16Sr396F	F	TAGGGAAGAGCTTGCGTCAC	20	61
2.	16Sr396R	R	CGTTGAGCGTTGCACTTAGA	20	60
3.	16Sr245F	F	GTAATGGCCTACCAAGACGATG	22	61
4.	16Sr245R	R	TTAGCCGGGGCTTATTCAT	19	61
5.	cpn421F	F	AGCGCAAAGTATGATTCATCGTGG	24	60
6.	cpn421R	R	AAGAGGTAAAATTTCTTGGATCGTGC	26	61
7.	cpn200F	F	TTAAAGAAGGGATCGAACTTGC	22	59
8.	cpn200R	R	AAAACTTTTGGACTCATCGACA	22	60

Amplification results were visualized under UV light after electrophoresis of ethidium bromide-stained PCR products in a 1.5% agarose gel (1×TBE buffer). The amplicon size was determined using the "O'Gene 100 bp DNA Ladder Ruler Plus" molecular marker. Statistical data processing was performed according to the Fisher's criterion.

RESULTS AND DISCUSSION

The molecular diagnosis of phytoplasma presence in local tomato varieties during the period of study demonstrates a huge difference in infection rates between years and varieties (Table 2). The first analysis of stolbur presence was performed in 2016 on Elvira and Cerasus. Nested-PCR analysis revealed a significant

difference in infection loading between these two varieties. In Elvira phytoplasma infection was detected in 83% of plants by the end of the growing season, whereas Cerasus manifested complete resistance to the infection. This initial observation allows to suggest a strong degree of genetic tolerance or immunity in Cerasus.

In 2017, analyses of the same varieties confirmed the difference in susceptibility. Infection incidence in Elvira decreased to 66.6%, while Cerasus remained largely resistant, with only 16.6% of infected plants. These results indicated that the resistance of Cerasus was stable across two growing seasons, while Elvira continued to show high vulnerability.

In 2018, the number of tested varieties was expanded to four: Elvira, Cerasus, Desteptarea, and Mary Gratefully. Assessments conducted in late August demonstrated that Cerasus maintained a low susceptibility to ˈ*Ca.* P. solaniˈ infection (35%). By contrast, widespread stolbur infection was recorded in Elvira (85%) and Desteptarea (80%), while Mary Gratefully showed an intermediate infection level (50%). These results highlighted variety-dependent variation in susceptibility, with Cerasus consistently showing lower infection pressure compared to the other tested varieties.

Table 2. ˈ*Ca.* P. solaniˈ distribution in some local tomato varieties

Variety / Year	Cerasus	Elvira	Mary Gratefully	Desteptarea	Prestij
2016	0%	83%			
2017	16.6%	66.6%			
2018	35%	85%	50%	80%	
2019	25%	50%	92%	58%	
2020	0%	8%	33%	0%	
2022			8.3%	25%	25%
2023			83.3%	66.6%	66.6%
2024	10%		80%	90%	

The 2019 season was likewise characterized by a high incidence of phytoplasma infection. Under the climatic conditions of this year, Mary Gratefully exhibited a very high infection level of 92%, suggesting an environmental influence on its susceptibility. In Cerasus, infection was detected in 25% of plants, which, although higher than in 2018, still demonstrated a comparatively low infection rate. Meanwhile, Elvira and Desteptarea showed intermediate values of 50% and 58%, respectively. These results confirm that while Cerasus is not entirely immune, it consistently displays a higher degree of tolerance relatively to other varieties.

In 2020, molecular diagnostics revealed a low level of ˈ*Ca.* P. solaniˈ infection across all analyzed tomato varieties. Both Cerasus and Desteptarea demonstrated complete immunity at the end of the tomato growing season. In Elvira, only 8% of plants were infected, whereas the highest incidence was observed in Mary Gratefully, with 33.3% of infection under the conditions of the year. These results indicated a notable reduction in phytoplasma pressure compared to previous years, suggesting that environmental factors may have limited pathogen spread during the 2020 season.

Monitoring of phytoplasma distribution in local tomato varieties continued in 2022. The obtained data showed a similar trend to that of 2020, with infection levels remaining low across all tested varieties. In 2023, molecular analysis of tomato varieties revealed a significant increase in the proportion of infected plants across all tested varieties. The highest incidence was observed in Mary Gratefully (83.3%), while Desteptarea and Prestij both reached infection levels of 66.6%.

In 2024, the high prevalence of stolbur infection persisted. In Mary Gratefully, infection remained at a similar level of 80%, while in Desteptarea the proportion of infected plants also reached 80%. On the other hand, Cerasus again demonstrated a remarkable degree of resistance to stolbur, with only 10% of plants tested positive for infection.

Overall, the multi-year analysis (2016–2024) highlights strong variety-dependent variation in susceptibility to stolbur phytoplasma. Mary Gratefully and Desteptarea consistently showed high infection rates under favorable conditions for disease spread, while Elvira displayed unstable moderate susceptibility. In contrast, Cerasus consistently demonstrated the highest level of resistance, with infection rarely exceeding 35% even under severe epidemic conditions. These results confirm Cerasus as a valuable source of resistance for breeding programs and emphasize the importance of variety selection as part of integrated stolbur management strategies in tomato production.

In addition, long-term research confirms the suggestion that the epidemiology of ˈ*Ca.* P. solaniˈ in tomato is strongly influenced by year-to-year climatic and ecological conditions. Abiotic factors such as temperature, humidity and others directly impact to the plant health including the possibility to the immune response (Zamorzaeva et al., 2019). These factors also include to the activity and reproductive capacity of insect vectors which are intermediate hosts in the life cycle of phytoplasma playing an important role in this pathogen spread

(Weintraub et al., 2006; Orlovskis, 2017). Comparison of the data obtained from 2016 to 2024 demonstrates a significant difference in the spread of phytoplasma infection between years of study (Figure 1).

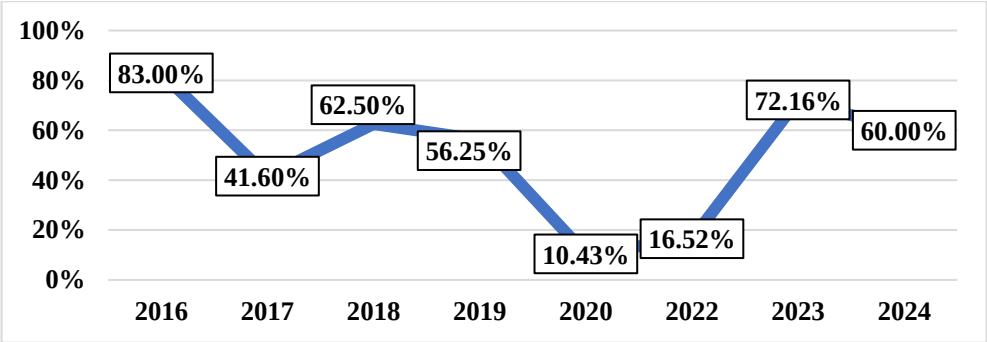

Figure 1. Summary data on the spread of '*Ca.* P. solani' in tomato field in 2016 – 2024

Thus, between 2016 and 2024, molecular diagnostics revealed pronounced year-to-year variation in the incidence of stolbur in tomato crops. The first year of monitoring (2016) was in fact characterized by an epidemic outbreak when 83% of plants in the field were infected. It is possible, the climatic conditions of the growing season were favorable for '*Ca.* P. solani' spread. In 2017, the situation improved considerably, with only 42% of tested to phytoplasma positive tomato plants. This indicates that environmental or agronomic factors have temporarily limited the spread of the pathogen and its vectors. Nevertheless, the downward trend did not persist. At the end of the 2018 growing season, incidence rates escalated again, with 72.5% of infected plants, suggesting a rapid rebound of pathogen or vector activity. In 2019, infection level remained elevated, affecting 66.7% of tomato plants and thus maintaining a high epidemiological risk.

A sharp reduction of stolbur spread was observed in 2020, when infection level dropped to 15%, marking the lowest incidence across the entire study period of ten years. This low prevalence persisted in 2022 (16.7%) supporting the assumption that unfavorable for phytoplasma infection climatic conditions or reduced vector populations played a decisive role in limiting disease transmission. However, this temporary relief was followed by a sudden resurgence: in 2023, infection level spiked dramatically to 72.2%, while in 2024 a persistently high incidence of 60% was recorded. Taken together, these data underline the pronounced temporal variability of '*Ca.* P. solani' epidemiological situation and illustrate an undulating pattern of stolbur distribution.

During the tomato growing seasons tomato plants showing symptoms of stolbur were observed and registered (Figure 2). There were found characteristic disease symptoms as concretion of sepals (Figure 2, photo 6, 11), virescence (Figure 2, photo 2, 5, 8, 10), sepal proliferation (Figure 2, photo 4, 7, 12), reduced or absent inflorescences (Figure 2, photo 3), and fruits with lignified phloem (Figure 2, photo 9).

Figure 2. Morphological symptoms of stolbur on tomato plants: 1 – health plant (fruit); 2 - 12 – stolbur plants

The presence of '*Ca*. P. solani' in stolbur symptomatic tomato plants was confirmed by nested-PCR analysis which results are presented on Figure 3. The pathogen was not detected in DNA extracted from health tomato plant (lane 1 on Figure 3).

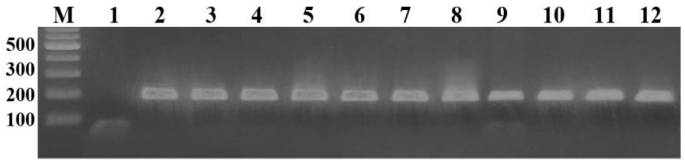

Figure 3. Results of nested-PCR at DNA extracted from health (1) and stolbur symptomatic (2-12) tomato plants

Finally, an important milestone of the research was the identification of the '*Ca.* P. solani' strain infecting tomato in Moldova. For this purpose, fruits from stolbur symptomatic tomato plants were collected in different years (2018, 2019, and 2020) for sequencing of 16S rRNA gene fragment of phytoplasma. It is important to note that the infection rates varied significantly across these three years. Stolbur infection was high in 2018 and 2019 but dropped sharply in 2020 (see Fig. 1). Except for the influence of climatic conditions of the growing season

this may be related to the various aggressive strains. However, the hypothesis about the different strains responsible for the observed discrepancies was rejected. Sequence analysis revealed 100% identity among the compared fragments (accession numbers OQ275003, OQ275004). Moreover, full similarity was established with more than 100 ribosomal gene sequences from NCBI obtained in more than 10 countries (Zamorzaeva et al., 2022). Thus, it was confirmed that the same strain was present in all analyzed plants for three years. In order to better understanding the interaction of phytoplasma infection with tomato in-depth study of the transcriptome response of tomato plants was initiated. Preliminary analysis of gene expression profiles in infected plants aimed to elucidate the molecular mechanisms involved in the differential defense response to ˈ*Ca.* P. solaniˈ infection depending on the tomato variety (Mitina et al., 2024). This approach seems very important.

CONCLUSIONS

The decade research highlights the complex epidemiological situation of ˈ*Ca.* P. solaniˈ in tomato crops of the Republic of Moldova. Multi-year data confirm that phytoplasma susceptibility is strongly genotype-dependent. The variety Cerasus consistently displayed stable partial resistance, making it a promising genetic source for breeding programs aimed at developing resistant varieties. By contrast, Elvira and Desteptarea remained highly vulnerable, while Mary Gratefully showed variable susceptibility, reflecting the interaction between genetic background and environmental conditions.

Temporal variability in incidence underscores the decisive role of climatic and ecological factors. Years with low infection (2020, 2022) suggest that unfavorable conditions can limit pathogen spread and vector activity. However, the re-emergence of infection in 2024 indicates pathogen persistence and the continuing vulnerability of tomato production systems. Molecular diagnostics (nested-PCR) proved reliable pathogen detection confirming, in particular, the phytoplasma presence in stolbur symptomatic tomato plants. Sequence analysis has demonstrated genetic identity of ˈ*Ca.* P. solaniˈ strains across three years of study assuming the pathogen stability, with significant implications for disease management.

The long-term study suggests that effective control of stolbur phytoplasma requires integrated measures: reliable molecular diagnosis of the pathogen presence in tomato plants, continuous tomato field monitoring, and deployment of stolbur-resistant varieties. The development of effective agroecological and biological strategies to reduce vector pressure is also an important part of stolbur management. Under climate change, sustainable control of ˈ*Ca.* P. solaniˈ remains a critical challenge for maintaining tomato yield and stability.

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