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MOLECULAR DIAGNOSIS OF PHYTOPLASMA IN DIFFERENT LOCAL TOMATO VARIETIES

162.01. Plant genetics

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The thesis was developed within the Molecular Genetics laboratory, Institute of Genetics, Physiology and Plant Protection, Moldova State University.

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The defense will take place on 15th November 2024, time 3 PM during the Meeting of the Commission for public defense of the doctoral thesis within the Doctoral School of Natural Sciences, MSU. Headquarters – Moldova State University (http://www.usm.md), M. Kogalniceanu 65 A, block 3, room 332, MD-2009, Chisinau, Moldova.

The doctoral thesis and the summary can be consulted at the National Library of the Republic of Moldova, Central Library of the Moldova State University (MD 2009, Chisinau, 60 Alexei Mateevici str.), on the ANACEC website (http://www.anacec.md).

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CONTENT

CONCEPTUAL MARKINGS OF THE RESEARCH

Actuality and importance of the chosen topic. Phytoplasmosis (stolbur) is a current issue in tomato cultivation, both in the Republic of Moldova and globally. This disease causes significant economic losses by reducing both the quality and quantity of production, with losses reaching 70-100% [1]. As a result of the pathogenic process in tomatoes, affected fruits manifest enlarged phloem, its lignification, reduced sugar content, and a decrease in organoleptic qualities [2]. The spread of stolbur has reached extremely high values globally, especially in the Euro-Mediterranean area. In the European Union, it is considered an endemic disease [3]. The causative agent of stolbur is '*Candidatus* Phytoplasma solani'. Insects from the order Hemiptera, families Cicadelidae, Cixidae, and Psilidae, play a primary role in transmitting the phytoplasma [4]. Weeds, on whose roots vector insects overwinter, also contribute to the spread of '*Ca*. P. solani'. The incubation period of the phytoplasma in plants after infection lasts from 6 to 30 days.

Traditional methods of detecting phytoplasma have many limitations. These are based on observing typical symptoms in field conditions, and studying the morphological characteristics through macro and microscopic analyses. Disease diagnosis based on symptom appearance is only possible in the late stages of infection, approximately after a month [5]. Similarly, morphological study is quite difficult as the infection may occur simultaneously with other diseases that can mask the symptoms [6]. Detecting the pathogen using electron microscopy is challenging because phytoplasma is polymorphic from a morphological perspective. The *in vitro* cultivation method became feasible only recently, in 2012, and requires the use of commercial media, but it is not possible to obtain specific colonies [7, 8]. Meanwhile, reliable and timely diagnosis is possible using molecular diagnostic methods. The advantages of molecular diagnosis include determining the presence or absence of the pathogen before symptom appearance, its specificity, as well as greater accuracy. Early diagnosis of phytoplasma in tomatoes, before symptoms appear, can be valuable in studying plants with mixed diseases. It's also important to note that molecular methods can allow the rapid identification of the differentiated resistance of tomato genotypes to '*Ca*. P. solani' [9]. The use of resistant genotypes will help reduce potential crop losses while maintaining environmental safety. Timely and properly dosed plant treatment will ensure quality production without, or with minimal use of, antibacterial agents and mineral fertilizers [10]. This is crucial given the increasing demand in several European and American countries for high-quality agricultural products [11].

The molecular study using PCR techniques of the resistance of local tomato varieties was conducted for the first time in the Republic of Moldova. Morphological-level research on the attack degree of stolbur in tomato plants was previously carried out by researchers Ciobanu V. and

Munteanu N. in 2014 [12]. Similarly, research on stolbur infection rates based on morphological symptoms was conducted by Vlasov I. and Samsonova L. in 2000 [13].

Research aim: To determine the sensitivity of local tomato genotypes to '*Ca*. P. solani' infection and study its spread using molecular methods.

Research objectives:

1. Molecular detection of the distribution and degree of phytoplasma infection in tomato plants of some local varieties during the vegetation period.
2. Identifying the stage at which differences in sensitivity to phytoplasma infection are evident.
3. Determining phytoplasma in 4 wild species of tomatoes.
4. Developing and optimizing a diagnostic system for '*Ca*. P. solani' in tomatoes, vector insects, and weeds.
5. Comparative analysis of methods (nested-PCR, Real-time PCR, PCR through serial DNA dilution) to determine the sensitivity of local tomato varieties to phytoplasma infection.
6. Sequencing fragments of the 16S rRNA gene to identify '*Ca*. P. solani' strains in experimental plots.
7. Determining the possibility of stolbur transmission through seed material in the analyzed varieties.
8. Detecting phytoplasma infection in insects from the Hemiptera order (vector insects) and weeds to monitor the epidemiological status of '*Ca*. P. solani' in experimental fields.

Research hypothesis: The sensitivity of tomato plants to the phytopathogen '*Ca*. P. solani' varies depending on the genotype and under the influence of climatic factors.

Summary of methodology and justification of the chosen research methods. The scientific study and research methodology included a wide range of methods and techniques that justified achieving the proposed aim and objectives. Various DNA isolation methods were applied to determine the fastest and most efficient method for phytoplasma diagnosis. Molecular methods such as PCR, nested-PCR, and Real-time PCR were used because they allow the specific identification of '*Ca*. P. solani' compared to other methods. Particularly, DNA sequencing enabled the identification of the strain of '*Ca*. P. solani' infecting the agricultural crops and vector insects. The application of modern research methods demonstrated the varied sensitivity of local tomato varieties to '*Ca*. P. solani' infection.

Summary of the thesis chapters The thesis includes annotations written in Romanian, English, and Russian. A list of abbreviations, the introduction, five chapters, general conclusions, practical recommendations, the bibliography, the declaration of responsibility, and the CV of author. The

work is written on 99 pages of main text, contains 59 figures, 14 tables, a bibliography with 156 titles, and 4 appendices.

Publications on the thesis topic. During the study, the data obtained were published in 39 scientific publications: 9 publications in Scopus and Web of Science databases, 4 articles in the national profile register, 2 publications in databases accepted by ANACEC, 6 articles in international scientific proceedings, 1 article in scientific proceedings with international participation, 8 articles in international scientific proceedings (abroad), 1 abstract presented at international scientific event, 4 abstracts at national scientific events with international participation, and 4 abstracts in national scientific proceedings. The research conducted and the data obtained were presented annually at the scientific council meetings of IGPPP (MSU), as well as at 21 national and international scientific meetings.

CONTENT OF THE THESIS

The **Introduction** summarizes and briefly presents information about the current state of phytoplasma diagnosis, highlights the relevance of the problem proposed for resolution, defines the aim and objectives of the work, states the hypothesis and research methodology, justifies the selected analysis methods, and provides an overview of the thesis chapters.

I. GENERAL ASPECTS OF '*CANDIDATUS* PHYTOPLASMA SPP.'

This chapter contains an analysis of current research on the general aspects of phytoplasma morphology, genome, and evolution. It presents possible transmission ways, listing the main natural reservoirs and vector insects that facilitate the spread of phytoplasma. The specific symptoms of the disease caused by '*Ca*. P. solani' in tomatoes are described, along with the negative consequences that affect the quality and quantity of production. The interaction of phytoplasma with host plants and insects is explained. Key measures for controlling and preventing the spread of the pathogen are outlined. The chapter also emphasizes the main diagnostic methods for phytoplasma, including the advantages and disadvantages of each method.

II. MATERIALS AND METHODS

The molecular diagnosis of the presence of '*Ca*. P. solani' was performed on the following tomato genotypes: Elvira, Cerasus, Mary Gratefully, Desteptarea, from the collection of the Institute of Genetics, Physiology, and Plant Protection (IGPPP) of MSU. Additionally, four other wild tomato species were studied: *Solanum habrochaites*, *S. pimpinellifolium*, *S. chilense*, and *S. peruvianum*. Twelve species of weeds: *Convolvulus arvensis*, *Calystegia sepium*, *Daucus carota*, *Chenopodium album*, *Setaria viridis*, *Solanum nigrum*, *Potentilla reptans*, *Artemisia vulgaris*, *Urtica dioica*, *Sonchus oleraceus*, *Polygonum convolvulus*, *Polygonum aviculare*, were tested as potential reservoirs of phytoplasma infection. The research was conducted based on material collected between 2018-2020. Various DNA isolation methods were used depending on the

sample: DNA-zol, boiling in alkaline solution, K-acetate, Na-acetate, and microwave techniques. The molecular diagnosis for the presence of the pathogen was conducted using PCR, nested-PCR, RT-PCR, and DNA sequencing. Twenty-two primers designed within the Molecular Genetics laboratory were used for the identification of '*Ca*. P. solani'. Statistical analysis was performed using Fisher's criterion.

III. DEVELOPMENT AND EVALUATION OF THE EFFICIENCY OF MOLECULAR DIAGNOSTIC PROCEDURES FOR PHYTOPLASMA IN TOMATOES, WEEDS, AND INSECTS

The need to develop and optimize the procedures for determining the pathogen '*Ca*. P. solani' in various hosts is due to several factors: 1) In molecular diagnosis, there is a risk of obtaining false-positive and false-negative results. Optimizing the methods helps minimize this risk. 2) The use of molecular methods on various subjects, organs, and tissues requires selecting specific conditions for material collection, DNA extraction, PCR execution, etc. 3) To speed up, simplify, and reduce the cost of molecular analyses, a comparison of relatively simple procedures was made, such as isolation by alkaline solution boiling, microwave methods, PCR, PCR with serial DNA dilution, nested-PCR, and RT-PCR. In subsequent studies, optimized methods with proven reliability were used.

3.1. Development and evaluation of molecular diagnostic methods for phytoplasma in tomato plants

The selection of the most optimal and reliable molecular diagnostic methods for phytoplasma in tomatoes was carried out in the following stages:

- DNA isolation from various subjects and organs;
- Amplification of phytoplasma DNA fragments;
- Electrophoresis of PCR products.

3.1.1. Selection and comparison of DNA extraction methods from tomatoes

The molecular analysis of '*Ca*. P. solani' infection in tomato plants to evaluate the percentage of infected plants from different varieties was performed using DNA extraction from the peduncle via the alkaline express method. This method is fast, low-cost per sample, and allows for the evaluation of a large sample of plants.

The effectiveness of DNA extraction using the express method was evaluated by comparing the molecular diagnosis results of phytoplasma infection in ripened tomato fruits, where DNA was isolated using the K-acetate method and the DNA-zol method. All three methods gave similar results, with 80% of the Elvira tomato variety plants infected with '*Ca*. P. solani' (Table 3.1).

Table 3.1. Identification of the infection '*Ca*. P. solani' in Elvira variety tomato plants using various DNA extraction methods

DNA extraction methods	Tomato organ	Number of tomato plants		% infected plants
		analyzed	infected	
Express	peduncles	20	16	80
K-acetate	fruits	10	8	80
DNA-zol	fruits	10	8	80

3.2. Selection of PCR conditions for tomato analysis

To select the appropriate PCR conditions that allow for accurate molecular diagnosis of '*Ca*. P. solani' in tomatoes, the following were compared:

1) Data obtained from PCR and nested-PCR analyses;
2) The number of amplification cycles;
3) Different sets of specific primers.

3.2.1. Comparison of results obtained through PCR and nested-PCR analysis using chaperonin primers

The comparison of data obtained from PCR and nested-PCR analysis leads to a clear conclusion that nested-PCR is necessary for precise and accurate diagnosis of phytoplasma infection in tomatoes. This is well illustrated in the molecular diagnosis of '*Ca*. P. solani' infection in the Elvira tomato variety (Figure 3.1 A, B).

Using PCR analysis, 4 infected plants were identified when using DNA isolated by the K-acetate method, and 5 infected plants were identified using the DNA-zol method (Figure 3.1 A). However, nested-PCR analysis revealed 8 infected plants using both DNA isolation methods (Figure 3.1 B). Thus, PCR results were partially false-negative, detecting only 40% or 50% of infected plants compared to the 80% detected by nested-PCR.

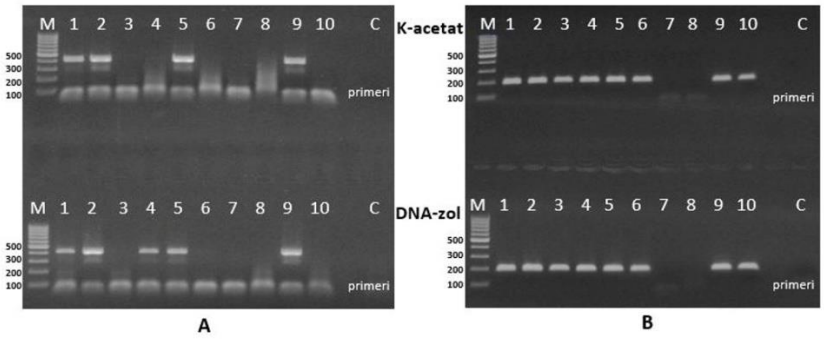


Figure 3.1. Results of PCR and nested-PCR based on DNA isolated from Elvira tomato variety using the K-acetate or DNA-zol method

(A – 421 bp fragment, chaperonin-specific primers for '*Ca*. P. solani') (B – 200 bp fragment obtained in the second round), M – DNA fragment length marker; C- – negative control

The number of amplification cycles for nested-PCR was also optimized. It is important to note that while a high number of cycles increases diagnostic sensitivity, it can lead to the appearance of non-specific fragments, including primer dimers. For example, with 45 amplification cycles, there is a high intensity of synthesized dimers, whereas with 35 cycles, the number of dimers is reduced. As a result of a series of experiments, 30 amplification cycles were selected for the first round and 35 cycles for the second round.

The conditions for performing nested-PCR were established through the above-described experiments, allowing for stable and accurate data to be obtained in the molecular diagnosis of phytoplasma infection in tomatoes. Both rounds of amplification should be performed in a 25 µl reaction mixture containing: ×1 buffer for Taq polymerase, 1U Taq polymerase, 0.2 mM dNTP, 0.25 µM F primer, 0.25 µM R primer, ultrapure water, and 1 µl DNA for the first round, or 1 µl of the diluted 1/20 first-round mixture in the second round. The following amplification program can be used: Round I: I: 94°C for 5 minutes, II: 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds × 30 cycles, III: 72°C for 10 minutes, IV: 4°C hold. Round II: I: 94°C for 5 minutes, II: 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds × 35 cycles, III: 72°C for 10 minutes, IV: 4°C hold. Specific primer pairs for '*Ca*. P. solani' used in the first round: cpn421F, cpn421R; for the second round: cpn200F, cpn200R.

3.2.2. Testing the efficiency of ps chaperonin primers specific for '*Ca*. P. solani' in PCR analysis

To develop a new technique for evaluating tomato resistance to phytoplasma, it is essential to have reliable molecular methods for detecting '*Ca*. P. solani' in plants. Previously, we developed a reliable method for identifying '*Ca*. P. solani' infection through nested-PCR in plants. At this stage, we compared the performance of different primer pairs using PCR and nested-PCR to optimize the process.

During this stage, several primer combinations were tested. A particular focus was placed on optimizing the conditions of the PCR analysis to achieve reliable results with the primer pairs used. The advantage of PCR over nested-PCR is that it is faster, less expensive, and requires less sample handling. However, it has some disadvantages compared to nested-PCR in terms of sensitivity and specificity. A well-designed set of primers, along with optimized reaction conditions, can improve the sensitivity and specificity of PCR analysis while maintaining its accessibility and simplicity.

In the study, six ps primers (3 Forward and 3 Reverse in different combinations), specific to the '*Ca*. P. solani' pathogen and based on the chaperonin gene sequence were analyzed. The amplification program was as follows: I: 5 minutes at 94°C, II: 30 seconds at 94°C, 30 seconds at 58°C, 30 seconds at 72°C × 30 cycles, III: 10 minutes at 72°C, IV: 4°C hold.

Figure 3.1 presents the summary of PCR analysis results for the same samples as in Figure 3.2 (DNA extracted using the DNA-zol method) with different combinations of ps primers.

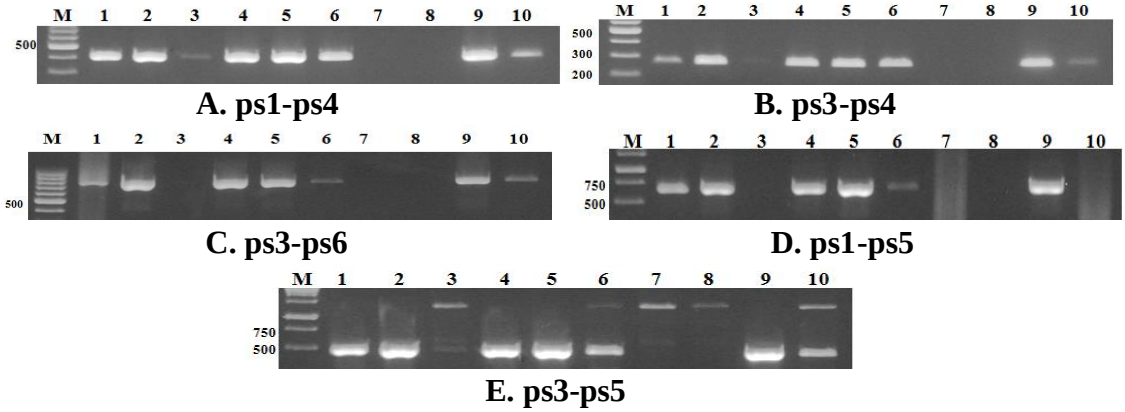


Figure 3.2. Results of PCR analysis with ps primer pairs, based on DNA extracted from tomato plants of the Elvira variety for the presence of the phytopathogen '*Ca*. P. solani'.

M is the DNA marker.

The results suggest that the ps1-ps4 and ps3-ps4 primer pairs are suitable for use in PCR analysis. However, samples with a low infection titer may produce weak bands on the gel and inconclusive results, requiring further analysis using nested-PCR.

3.4. Selection of conditions for the diagnosis of '*Ca*. P. solani' in vector insects

The molecular diagnostic procedures for vector insects include the following steps: collecting leafhoppers in the field, isolating DNA from individual insects, nested-PCR using two pairs of primers specific for '*Ca*. P. solani', and electrophoresis of nested-PCR products.

Two methods of insect collection were used: trapping with yellow sticky traps in the tomato field or greenhouse, and catching insects by mowing with an entomological net near the field. It is important to note that the second method offers more varied results in terms of species diversity and the number of insects captured. We guess that yellow color is only attractive to a limited number of species.

Two methods of DNA extraction from an individual leafhopper were compared: DNA isolation with DNA-zol and the alkaline express method. Positive results for the detection of phytoplasma infection were only obtained using the DNA-zol isolation method.

Nested-PCR (both rounds of amplification) with chaperonin primers should be carried out according to the following amplification program: I – 94°c for 5’; II – 94°c for 30’’, 58°c for 30’’, 72°c for 30’’ (×45 in round I and ×35 in round II); III – 72°c for 10’; IV – 4°c ∞.

Using this program helps avoid false-negative results in the molecular diagnosis of phytoplasma in leafhoppers. The challenges arise from the varying sizes of insects analyzed, which results in the extraction of different amounts of DNA. False-negative data were obtained when 30 cycles of amplification were used in round I and 45 or 50 cycles in round II.

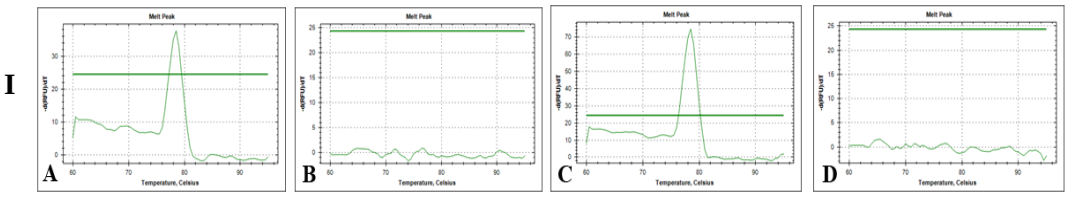
3.5 Quantitative analysis using Real-time PCR

In this study, '*Ca*. P. solani' DNA was detected and quantified in tomato DNA samples using Real-time PCR based on SYBR-Green. The number of DNA copies of the phytoplasma isolated from four tomato varieties was quantified. These varieties were previously reported to have contrasting resistance to phytoplasmic infection. Therefore, it was expected that they would present a different number of copies of the chaperonin gene of '*Ca*. P. solani'. The less resistant variety would have more copies of '*Ca*. P. solani' DNA compared to the more resistant variety [14]. Twelve plants of each variety were collected for DNA extraction, and DNA was isolated using the DNA-zol method. The concentration of purified DNA was measured with a Nanodrop spectrophotometer, and 9 ng of purified DNA was used. For the Real-time PCR analysis, two pairs of primers were created based on the chaperonin gene of '*Ca*. P. solani'.

The PCR conditions followed the manufacturer's recommendations for SYBR-Green: initial incubation at 50°C for 2 minutes, initial denaturation at 95°C for 2 minutes, followed by 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. Reactions were performed in the BIORAD CFX96 touch Real-time PCR system. Detection was performed on the SYBR channel. Primer efficiency was calculated using the Real-time PCR efficiency calculator [15].

For the Real-time PCR standard, a fragment was amplified using conventional PCR with the primer pair qfys7 and qfys8. The fragment was then visualized on an agarose gel, excised, and purified. The DNA concentration of the purified fragment was determined using a Nanodrop spectrophotometer. Given the fragment size of 160 bp, the number of copies in 1 ng of fragment DNA was determined using the online calculator [16].

First, the primers were experimentally tested to evaluate their specificity, efficiency, and optimal concentration in the reaction. For specificity evaluation, the dissociation curve was obtained for both primer pairs. Figure 3.3 presents the dissociation curve of the fragment amplified with the qfys5-qfys6 primer pair, using DNA from tomatoes infected with '*Ca*. P. solani' as the template. To determine the optimal primer and DNA concentration, the fragment was amplified containing 200 nM (Figure 3.3 I-A, B) and 400 nM (Figure 3.3 I-C, D) of each primer, both without dilution (Figure 3.3 I-A, C) and with a 10-fold dilution of the template DNA (Figure 3.3 I-B, D). As can be seen, the higher primer concentration resulted in more intense fluorescence without causing primer dimer formation (Figure 3.3 I-C), indicating a single distinct peak. However, this primer pair failed to amplify the fragment from a more diluted template (Figure 3.3 I-B, D).



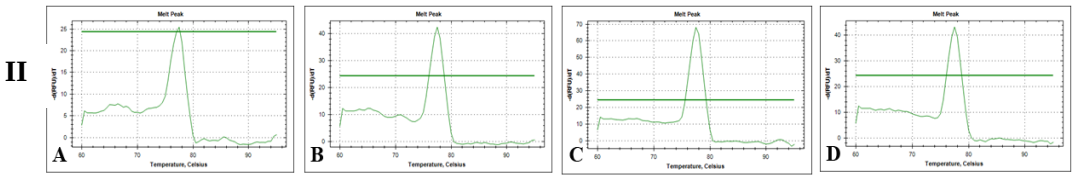


Figure 3.3. Dissociation curve of the fragment amplified by the qfys5-qfys6 primer pair (I) and qfys7-qfys8 primer pair (II). Primer concentration: 200 nM each (A, B); 400 nM each (C, D). Template dilutions: undiluted (A, C), 10-fold dilution (B, D).

The higher concentration of the qfys7-qfys8 primers led to increased fluorescence and did not cause primer dimer formation (Figure 3.3 II-C), resulting in a single pronounced peak. This primer pair successfully amplified the fragment even in the reaction with the diluted template (Figure 3.3 II-B, D). Therefore, this primer pair was used for further analyses at a concentration of 400 nM each. Subsequently, the efficiency of the qfys7-qfys8 primer pair was evaluated. The DNA fragment was amplified by the same primer pair in a conventional PCR and then diluted to be used as a template for the RT-PCR reaction. Four dilutions (10, 100, 1000, and 10,000-fold) of the initial PCR reaction DNA were used as templates in the RT-PCR reaction. The Ct values were measured, and a concentration curve was constructed to calculate the primer efficiency.

The primer efficiency graph, created from serial dilutions, is shown in Figure 3.4 a. A slope of -3.3183 indicates an efficiency of nearly 100%, suggesting that this primer pair can be used in Real-time PCR assays for measuring concentrations of '*Ca*. P. solani'. Thus, the qfys7-qfys8 primer pair was used for the quantification of '*Ca*. P. solani' DNA in tomato samples. For this purpose, serial dilutions of DNA with a known copy number were prepared and analyzed using Real-time PCR. The creation of a standard curve was based on the recorded Ct values. Figure 3.4 b illustrates the correlation between the Ct value and the initial number of template copies in the reaction.

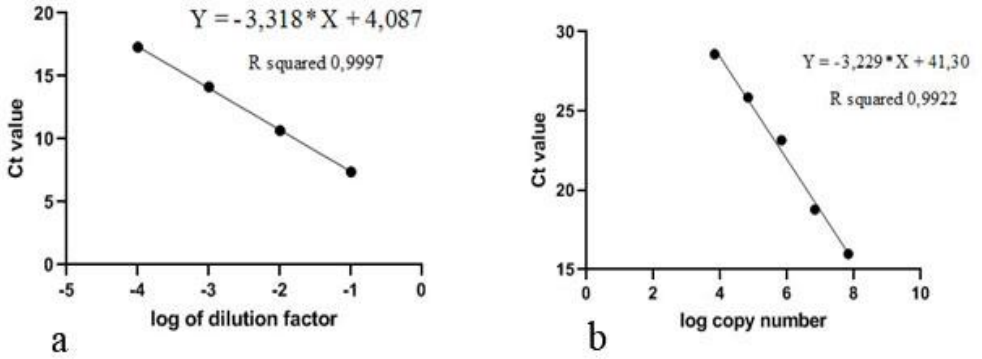


Figure 3.4. Graph of the efficiency of the qfys7-qfys8 primer pair (a) and the correlation between the Ct value and the initial number of template copies in the reaction (b).

IV. MOLECULAR DIAGNOSIS OF '*CA*. P. SOLANI' IN DIFFERENT TOMATO GENOTYPES FOR THREE YEARS OF VEGETATION

Molecular diagnosis of '*Ca*. P. solani' in tomatoes was performed in three stages of plant development: at the beginning of fruit ripening (July), at the stage of mass fruit ripening (August), and at the end of the vegetation season after harvesting a significant part of the fruit crop (September).

4.1. Molecular diagnosis of phytoplasma in tomato plants in the field in 2018

Molecular determination of the presence of '*Ca*. P. solani' in four tomato varieties (Cerasus, Elvira, Desteptarea, and Mary Gratefully) was carried out based on DNA isolated from peduncles collected in July, August, and September. For analysis of phytoplasma infection, ten plants were selected in two repetitions for each of the four analyzed varieties. The analysis revealed differences in the rate of plant infection among certain varieties. According to the obtained data, the first symptoms of infection appeared in July. The percentage of infection with '*Ca*. P. solani' in the Elvira variety was 35%, while the Desteptarea variety showed similar indices of 30%. A lower sensitivity was demonstrated by the Cerasus variety, with only 5% of plants infected with phytoplasma. The molecular analysis of '*Ca*. P. solani' in August showed similar trends. The lower infection rate was established in the Cerasus variety - 35%, and half of the plants were infected in the Mary Gratefully variety. The more pronounced infection was in the Elvira and Desteptarea varieties, with 80% of infected plants.

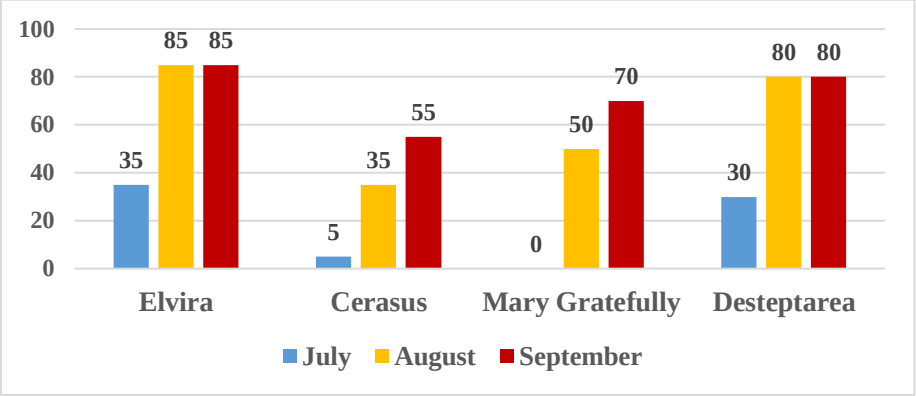


Figure 4.1. Spread of phytoplasma infection in different tomato varieties in 2018

The nested-PCR analysis performed in September, at the end of the vegetation period, in the four autochthonous tomato varieties, reconfirmed the trends of phytoplasma abundance. However, the differences between varieties were not as pronounced. The percentage of affected plants in the Cerasus variety was 55%, in Elvira 80%, Mary Gratefully 75%, and Desteptarea 85% (Figure 4.1).

4.2. Molecular diagnosis of phytoplasma in tomato plants in the field in 2019

The results of the molecular diagnosis of phytoplasma infection in tomato plants of the four varieties grown in the field are presented in Figure 4.2. Comparison of the obtained data was performed using statistical processing according to the Fisher criterion.

In July, at the beginning of tomato fruit ripening, half of the plants of the Desteptarea and Mary Gratefully varieties were infected with '*Ca*. P. solani'. In the Cerasus and Elvira varieties, only a quarter of the analyzed plants were determined to have phytoplasma infection. The number of plants infected with phytoplasma in the Desteptarea variety increased insignificantly in the following stages of fruit ripening, reaching 58% in August and September. Half of the plants of the Elvira variety were infected with stolbur in August, and the percentage increased to 58% in September. Phytoplasma infection in Mary Gratefully plants increased significantly at the stage of mass fruit ripening in August, reaching over 90% and remaining at this level in September.

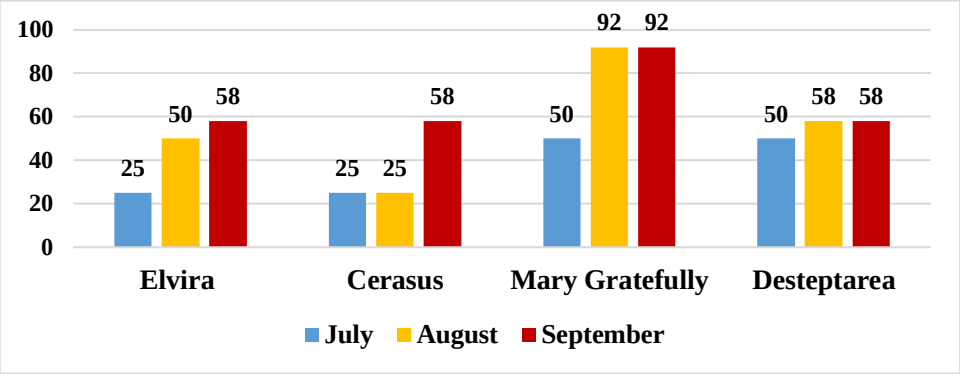


Figure 4.2. Spread of '*Ca*. P. solani' in the tomato field in 2019

Only a quarter of the plants of the Cerasus variety were infected with '*Ca*. P. solani' in August, at the stage of mass fruit ripening. At the end of the vegetation season, in September, the level of phytoplasma infection in this variety increased insignificantly, representing 58%.

The molecular analysis of phytoplasma infection in tomato plants of the analyzed varieties demonstrated that in the conditions of 2019 (summer with high temperatures and low precipitation), the maximum values of infection were recorded at the stage of mass fruit ripening in August and did not increase significantly until the end of the vegetation season (mid-September). The first signs of infection were identified in the first decade of July, at the beginning of fruit formation. At the end of July, half of the plants of the Elvira and Desteptarea varieties were infected with 'Ca. P. solani'. We guess that this mode of phytoplasma infection distribution in the tomato field is associated with the cycle of reproduction and activity of insect vectors. The warm weather increased the activity of cicadas at the beginning of the vegetation season but negatively

influenced their reproduction. Similarly, the number of plants infected with phytoplasma did not increase significantly from August to September. Comparison of the sensitivity of the studied varieties in 2019 demonstrated that the Mary Gratefully variety was the most sensitive to '*Ca*. P. solani' infection, while the Desteptarea and Elvira varieties showed intermediate values. The Cerasus variety presented the highest resistance.

4.3. Molecular diagnosis of phytoplasma in tomato plants in the field in 2020

The scheme of molecular diagnosis for the presence of '*Ca*. P. solani' infection in autochthonous tomato varieties in the conditions of the 2020 was similar to that in 2018, 2019. Thus, fruits were collected from 12 plants of each of the four varieties.

According to the obtained results, a low spread of phytoplasmic infection was observed in the field (Figure 4.3). In July, the presence of the '*Ca*. P. solani' infection was not detected in the tomato field. The infection of tomatoes with phytoplasma began at the ripening stage, but the level of infection was low. The presence of stolbur was confirmed in two tomato genotypes analyzed. In the Elvira variety, the percentage of plants infected with '*Ca*. P. solani' was 8%, and in the Mary Gratefully variety, it was 33%.

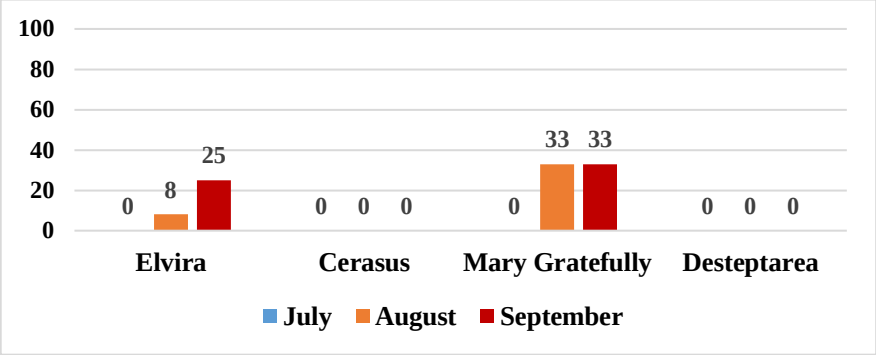


Figure 4.3. Spread of '*Ca*. P. solani' infection in different tomato varieties in 2020

The percentage of phytoplasma infection in the Mary Gratefully variety remained at the same level at the end of the vegetation season. In the Elvira variety, an increase in the level of phytoplasma infection was observed, reaching 25%. In the Cerasus and Desteptarea varieties, the presence of '*Ca*. P. solani' was not determined throughout the entire vegetation season. Thus, the results obtained indicate a low distribution of phytoplasma in the field in the conditions of 2020. The percentage of '*Ca*. P. solani' infection in the field reached only 14.5% at the end of the vegetation season. The data do not allow for a clear statistical determination of which variety is more or less resistant to '*Ca*. P. solani' infection. However, some trends were established in the conditions of 2022 - greater sensitivity in the Elvira and Mary Gratefully varieties and lower sensitivity in the Cerasus and Desteptarea varieties.

4.4. Comparative analysis of the degree of phytoplasma distribution in tomatoes for three years of vegetation

A detailed analysis of the distribution of stolbur among tomato plants of the analyzed varieties revealed a significant difference in the sensitivity of these varieties to phytoplasma infection. This difference was visible in all three vegetation seasons (Figure 4.4, Table 4.1).

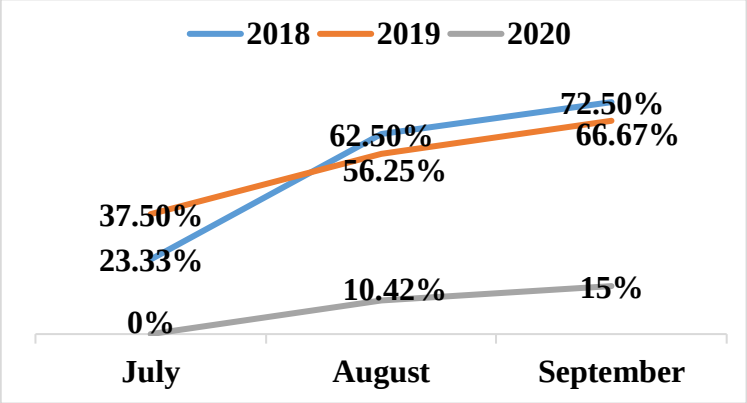


Figure 4.4. Summary data on the spread of '*Ca*. P. solani' in the tomato field in 2018, 2019, and 2020

Molecular diagnosis of phytoplasma infection in the analyzed tomato varieties during the years 2018-2020 revealed significant differences. In July 2019, at the beginning of fruit ripening, half of the plants of two tomato varieties (Desteptarea and Mary Gratefully) were affected by stolbur. In contrast, in the Elvira and Cerasus varieties, the percentage of infected plants was significantly lower, at 25%. In the conditions of 2018, the Cerasus variety showed a very low infestation (5%), and the Desteptarea variety showed a lower percentage of infected plants (30%) compared to 50% in 2019. In July 2020, the '*Ca*. P. solani' infection was absent in the tomato field.

Table 4.1. Results of molecular identification of phytoplasma infection in plants of different tomato genotypes in 2018, 2019, and 2020

Variety	Spread of stolbur in the field (%)								
	2018			2019			2020		
	I	A	S	I	A	S	I	A	S
Elvira	35	85	85	25	50	58	0	8	25
Cerasus	5	35	55	25	25	58	0	0	0
Mary Gratefully	XX	50	70	50	92	92	0	33	33
Desteptarea	30	80	80	50	58	58	0	0	0

Note: I-July; A-August; S-September. XX - molecular diagnosis of phytoplasma infection in these varieties was not performed.

The largest differences in the degree of phytoplasma infection of plants of different tomato varieties were observed during the period of mass fruit ripening, in August. The influence of climatic conditions in the years of study on the response of plants of the analyzed varieties was ambiguous (Table 4.1, Figure 4.5).

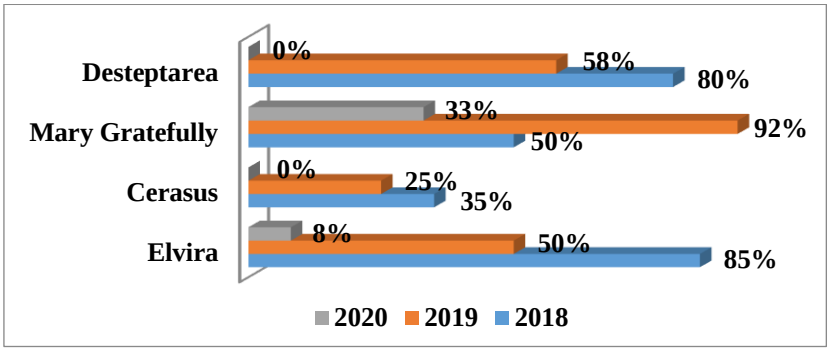


Figure 4.5. Distribution of '*Ca*. P. solani' in the tomato field during the period of mass fruit ripening in 2018, 2019, and 2020 (August)

The studies carried out made it possible to identify the genotypes that are more sensitive and more resistant to '*Ca*. P. solani' infection in 2018, 2019, and 2020. Firstly, it was shown that Cerasus manifests the greatest resistance to '*Ca*. P. solani' infestation among the studied varieties, a relatively higher level of damage to plants of this variety by phytoplasmic infection was observed only at the end of the vegetation season, in September, while slightly more than half of the plants were infected. Thus, the Cerasus variety can be recommended to breeders as genetic material for creating phytoplasma-resistant genotypes (hybrids, varieties). Additionally, the use of this more phytoplasma-resistant variety in agricultural production can be economically viable for farmers to increas the quality and quantity of harvested fruits.

The Elvira variety had a higher sensitivity to phytoplasma infection in all study years, regardless of climatic conditions. Similarly, the Desteptarea variety demonstrated similar indices to the Elvira variety in the vegetation seasons of 2018 and 2019. However, in the conditions of 2020 with high temperatures and extreme drought, this variety demonstrated complete immunity to phytoplasma infection. Nevertheless, summing up the data obtained over the study years, we can conclude that these two varieties showed a much greater sensitivity to phytoplasma infection compared to the Cerasus variety, the difference in the spread of infection at the stage of mass fruit ripening was very evident (2018 and 2019).

Genotypes that were susceptible to '*Ca*. P. solani' infection depending on the climatic conditions of the growing season were found to be Mary Gratefully and Desteptarea. It was observed that the Mary Gratefully variety, under the unfavorable climatic conditions of the 2019 and 2020 seasons, was the most sensitive to phytoplasma, with its infection level being

significantly higher compared to other varieties. This was already noticeable in August, during the mass fruit ripening stage. In contrast, in August 2018, this variety was less sensitive to phytoplasmic infection, with a smaller number of infected plants recorded during this period only for the Cerasus variety, which is the most resistant variety to phytoplasma. Similarly, the Desteptarea variety in the 2018 conditions was more sensitive to *'Ca*. P. solani', while with rising climatic temperatures, the infestation level of this variety decreased [17].

In summary, molecular diagnostics are a useful tool in the process of breeding tomato varieties or hybrids resistant to phytoplasma infection. Additionally, molecular analysis provides valid results that have been confirmed in the analysis of plants with morphological symptoms of stolbur (Figure 4.6).

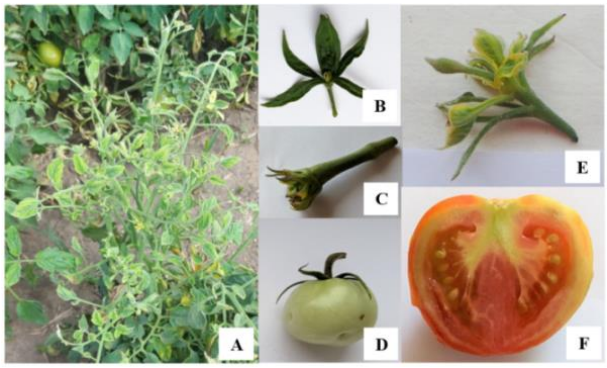
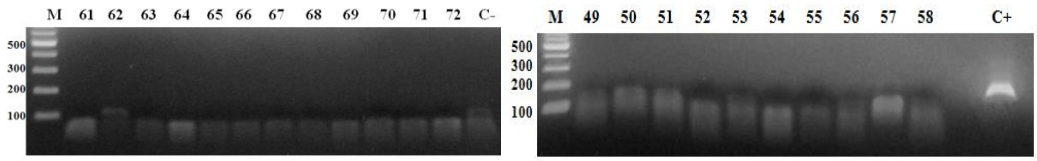


Figure 4.6. Phytoplasmosis symptoms in tomatoes

A. Symptomatic plant, B. Flower without petals, C. Flower with reduced petals, D. Reduced fruit, E. Flower with virescence symptoms, F. Lignified fruit

4.2. Analysis of wild tomato species for the presence of phytoplasma

Molecular diagnosis of the wild tomato form *Solanum habrochaites* for the presence of the pathogen '*Ca*. P. solani' was carried out during the 2019 and 2020 growing seasons. Phytoplasma determination was conducted on 10 individual plants. Molecular analysis did not identify the presence of '*Ca*. P. solani' in the wild *S. habrochaites* form in both growing seasons in the field (Figure 4.7). Thus, we can conclude that *S. habrochaites* plants exhibit high resistance to stolbur.



2019 **2020**

Figure 4.7. Determination of '*Ca*. P. solani' in *Solanum habrochaites* in September 2019 and 2020. M – Marker, C- – Negative control. C+ – Positive control.

Additionally, in 2020, three other wild tomato species were studied: *S. pimpinellifolium*, *S. chilense*, and *S. peruvianum*. Molecular analysis using the primers cpn 200 F/R did not detect the presence of '*Ca*. P. solani' in the tomato plant species analyzed during the growing season. Therefore, phytoplasma infection was not detected in the plants of these four wild tomato forms. We believe that wild tomato forms have reduced sensitivity to phytoplasma compared to cultivated tomato varieties. This should be considered by breeders when developing tomato varieties resistant to stolbur.

4.3. Quantitative analysis of phytoplasmic infection in tomato fruits

4.3.1. Quantitative Real-time PCR analysis

The results obtained from Real-time PCR demonstrated that the tomato fruits with the highest concentration of '*Ca*. P. solani' belonged to the Elvira and Mary Gratefully varieties (Figure 4.8). The Cerasus variety had approximately half the amount of phytopathogen compared to the Elvira or Mary Gratefully varieties. These data are consistent with previous results reporting higher resistance of the Cerasus variety. The lowest concentration of phytoplasma in tomato fruits was found in the Desteptarea variety, which was twice as low as in the Cerasus variety [18].

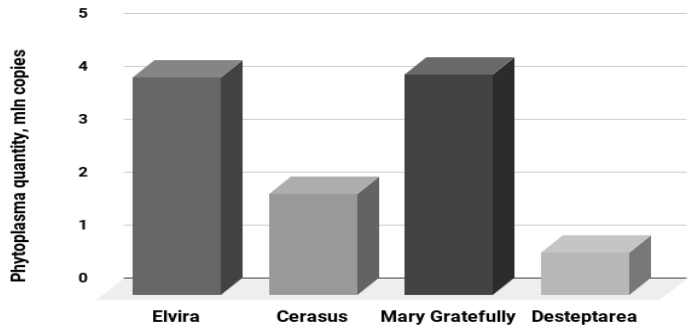


Figure 4.8. Quantification of '*Ca*. P. solani' in four tomato varieties by Real-Time PCR

In addition to quantifying '*Ca*. P. solani' in tomato fruits and its contribution to decreased fruit quality, the spread of phytoplasma infection in the tomato field during the mass fruit ripening period, as well as the productivity of the studied tomato varieties, was analyzed. The proportion of infected plants among the four tomato varieties was calculated and compared using Fisher's criterion. A significant difference in the infection rate of '*Ca*. P. solani' was established among the four tomato varieties, with a notable difference between the most resistant variety (Cerasus, 25.0% of infected plants) and the most sensitive (Mary Gratefully, 91.7% of infected plants) ($P < 0.05$). The Elvira (50.0% of infected plants) and Desteptarea (58.3% of infected plants) varieties were positioned intermediate in terms of infection distribution.

Comparing the data on '*Ca*. P. solani' quantification in fruits from different tomato varieties and the distribution of phytoplasma in the field demonstrated a partial correlation of results. Both the concentration of phytoplasma and the proportion of infected plants were low in the Cerasus

variety and high in the Mary Gratefully variety, indicating a correlation. For the Elvira variety, these indicators showed a relative correlation. On the other hand, although the Desteptarea variety fruits manifested very low phytoplasma concentration, a relatively high proportion of infected plants was observed in the field.

Thus, although a large proportion of Desteptarea plants were infected with '*Ca*. P. solani', the infection level in the fruits of this variety was low. The low level of '*Ca*. P. solani' in the fruits of this variety could be due to a plant immune response to this infection. Therefore, the nature of such a response requires further study. This response could be associated with certain genetic and metabolic traits of the genotype, such as hormone signaling pathways known for stolbur disease in tomato plants or the ability of phytoplasma-infected plants to recover. The yield of marketable fruits in the Desteptarea variety was very high, suggesting that fruits collected from plants with low pathogen concentration are of good quality (Table 4.2) [19].

Table 4.2. Productivity indices for the analyzed tomato varieties in 2018 and 2019

Variety	Productivity, t/ha				Percentage of marketable fruit,%	
	Total		Marketable fruit			
	2018	2019	2018	2019	2018	2019
Elvira	58,0	23,1	40,1	20,3*	69,1	87,9
Mary Gratefully	73,0*	31,5*	59,5*	28,2*	80,2*	89,5
Cerasus	74,4*	31,7*	60,9*	29,6*	84,6*	93,4*
Desteptarea	67,8*	30,2*	50,9*	29,8*	75,0*	98,7*

Significant difference with Elvira, $P < 0.05$

The Cerasus variety demonstrated the highest resistance indicators to '*Ca*. P. solani' under the unfavorable conditions of 2019. The Elvira and Mary Gratefully varieties were more susceptible to phytoplasma, resulting in lower fruit quality. A strong correlation between the low concentration of phytoplasma in fruits and the yield of high-quality fruits was observed in the Desteptarea variety. Correlation analysis was performed using Excel. The proportion of marketable fruits was highest in this variety, despite the widespread infection in the experimental field. So, the selected primer pair can be used for quantifying '*Ca*. P. solani' in tomatoes, with previously established optimal conditions, for testing four tomato varieties with contrasting resistance to phytoplasma infection. The Real-time PCR method can be successfully used for detecting and quantifying phytoplasma infection and for comparing the resistance of tomato varieties to phytoplasma. There is potential for using this method to detect and quantify the pathogen '*Ca*. P. solani' in other crops as well, not just in tomatoes.

4.3.2. PCR analysis of phytoplasma presence by serial dilution of isolated from tomatoes DNA

PCR analysis via serial dilution of DNA was conducted to quantify the pathogen in tomatoes of the Elvira and Mary Gratefully varieties. This method involves applying a series of successive dilutions of DNA isolated from tomato fruits. According to the obtained results, the amount of DNA in the Elvira variety samples is lower compared to the DNA samples isolated from the Mary Gratefully variety. These results are consistent with the Real-time PCR analysis for quantitative determination of the '*Ca*. P. solani' pathogen in tomato varieties. Therefore, the PCR technique via serial dilution of DNA is reliable for quantifying the pathogen in certain varieties. Thus, we can conclude that PCR analysis via serial dilution of DNA can be used as an alternative method for quantifying '*Ca*. P. solani' in tomato plants. Additionally, we considered that this method is an efficient and relatively inexpensive approach, especially when mass analysis is needed.

4.4. Comparison of nucleotide sequences of phytoplasma and identification of '*Ca*. P. solani' strains present in tomatoes

Based on the obtained data, three DNA samples with the necessary concentrations for sequencing were selected (Figure 4.9). The PCR products amplified from these three samples collected for three years (2018, 2019, 2020) were then outsourced for purification through gel extraction and direct sequencing in both directions, using primers R16F2n/R2, to CeMIA company in Greece.

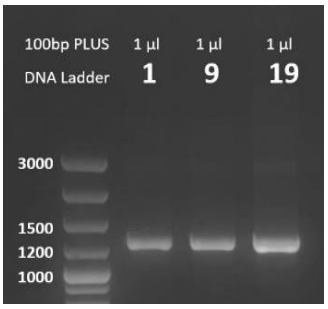


Figure 4.9. DNA samples with optimal concentrations for sequencing

The amplicon sequences of the samples from the three years were found to be identical. These sequences were compared in a BLAST search with sequences from various geographic regions available in GenBank. The comparison revealed that the ribosomal RNA sequence of the phytoplasma infecting tomato plants in 2018 and 2019 in Moldova shared 100% identity with 97 sequences of '*Ca*. P. solani'.

Molecular identification of '*Ca*. P. solani' in tomatoes was performed for the first time in Moldova, and the present study demonstrated the presence of '*Ca*. P. solani' in symptomatic tomatoes in the country. Two nucleotide sequences were registered in the global NCBI GenBank database (accession numbers OQ275003 and OQ275004) [19].

Thus, analysis of the DNA sequences showed complete identity with sequence records available in NCBI from several European countries (Romania, Bulgaria, Serbia, Poland, Czechia, Italy) and from Russia, Turkey, Iran, and China. This suggests that the strain might originate from these countries.

V. ANALYSIS OF THE TRANSMISSION OF PHYTOPLASMA BY THE STUDY OF INFECTION OF SEED MATERIAL AND INTERMEDIATE HOSTS

5.1. Determining the possibility of transmission of phytoplasma through seed material

The possibility of transmission of phytoplasma through seeds was studied on four local varieties of tomatoes. For the study, seeds of four tomato varieties were grown: Elvira, Cerasus, Mary Gratefully, and Desteptarea. Additionally, to these samples, seeds extracted from fruit no. 41 of the Elvira variety infected with phytoplasma were analyzed. Further, the diagnosis of seeds and tomato seedlings grown in a thermostat was performed to detect the presence of phytoplasma. For each sample, 15 seeds were taken. The nested-PCR analysis was performed with primers chaperonin cpn200F/R, and the presence of infection was detected in all seeds of the tomato varieties. However, the analysis of seedlings grown in thermostat conditions showed the absence of '*Ca*. P. solani' infection in all analyzed samples, including sample no. 41 (Figure 5.1).

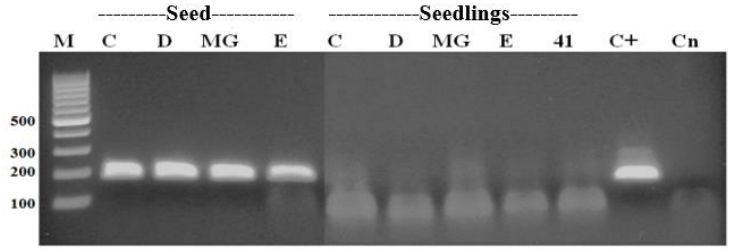


Figure 5.1. Results of nested-PCR for the presence of '*Ca*. P. solani' infection in tomato seeds and seedlings. M - molecular marker; C- - negative control of amplification

The main conclusion of this study is that stolbur is not transmitted by the seeds of the analyzed tomato varieties [20].

5.2. Identification of phytoplasma in insects from the Hemiptera order - potential vectors

The molecular study of the infestation of insects and, simultaneously, of tomatoes with the pathogen '*Ca*. P. solani' was carried out in 4 distinct periods: 1 - at the end of May, in the greenhouse, where the tomato seedlings were grown; 2 - in the first days of June, after planting the plants; 3 - in July and August (the stage of tomato fruit ripening); 4 - in the middle of the second decade of September, at the end of the tomato plant growth season in the field (Table 5.1), [21].

Table 5.1. Determining of the pathogen '*Ca*. P. solani' in insects during the tomato vegetation season

Stage	Vegetation Stage / Month	Number of insects		% infected insects	% infected tomatoes
		Analyzed	Infected		
I	Seedling growth in the greenhouse (end of May)	5	0	0	0
II	Mass flowering (first days of June)	25	0	0	0
III	Fruit maturation (July-August)	48	7	14.6	44.9
IV	End of the vegetation period (September)	12	7	58.3	69.6

The molecular analysis of phytoplasma in 48 insects caught in July and August allowed the determination of the pathogen in 7 insects. The results are illustrated in Table 5.1. The molecular analysis of tomato plants collected in July and August indicated the presence of fitoplasmic infection in 44.9% of the examined plants.

In Figure 5.2, the cicadas in which the phytoplasma infection was diagnosed in the period of analysis in July and August are illustrated.

Figure 5.2. Potential insect vectors of '*Ca*. P. solani', collected in July and August

The nested-PCR analysis revealed the presence of phytoplasma infection in 58.3% of the analyzed insects at the end of the vegetation season (Table 5.1). Molecular diagnosis allowed the determination of a high percentage of infection with stolbur in tomato plants in the studied period, which reached a value of 69.6%.

Insects from the Hemiptera order, infected with '*Ca*. P. solani' in September, are presented in Figure 5.3.

Figure 5.3. Potential insect vectors of '*Ca*. P. solani', collected in September

Examining the obtained results, it is noted that in September (at the end of the vegetation season), there was a considerable increase in the percentage of '*Ca*. P. solani' infection in insects compared to July and August. Respectively, in the molecular diagnosis, 7 insects infected with

phytoplasma were determined in July and August out of 48, and in September, 7 out of 12. Thus, the percentage of phytoplasma infection in insects increased from 14.6% in July and August to 58.3% at the end of the vegetation season.

The data received from the molecular diagnosis suggest the presence of phytoplasma infection on the experimental plots of IGPPP starting from July and to the abundance of infection in September. This trend was observed and confirmed both in the molecular analysis of tomato plants and insects. Those, insects from the Hemiptera order in which the presence of the pathogen was determined can be considered as potential vectors of this pathogen.

5.3. Analysis of weeds for the presence of phytoplasma

An important aspect in the study of phytoplasma spread is the analysis of all elements in the chain of transmission of this pathogen including weeds, on whose roots the insect vectors overwinter. Molecular screening for the determination of phytoplasma was performed on twelve species of weeds: *Chenopodium album*, *Solanum nigrum*, *Daucus carota*, *Setaria viridis*, *Polygonum convolvulus*, *Potentilla reptans*, *Convolvulus arvensis*, *Polygonum aviculare*, *Calystegia sepium*, *Artemisia vulgaris*, *Urtica dioica*, *Sonchus oleraceus*. The plant material was collected near the experimental fields of the institute and near the greenhouse, mainly in last decade of August. The selection of these species for study was based because they are known as natural hosts of '*Ca*. P. solani' in neighbor countries [22].

Following the molecular analysis, optimal conditions for identifying phytoplasma in some weed species were established. The rapid DNA extraction method presented the most reliable results compared to other methods. Although the K-acetate method diagnosed the presence of the pathogen in the *S. nigrum* species, the amplified fragment was not evident. Thus, in the case of low pathogen titers, it is possible to obtain false-negative results. After the optimization of the molecular diagnosis the presence of '*Ca*. P. solani' infection was established in weeds *C. arvensis* and *S. nigrum*. *C. arvensis* is a perennial plant and can therefore be considered as an important reservoir of infection in the region.

Table 5.2. Results of molecular analysis of phytoplasma in some weed species

№	Species	No. plants	Tissue	Presence / absence of infection
1	*Daucus corota*	27	stem	-
2	*Urtica dioca*	20	stem	-
3	*Convolvulus arvensis*	21	stem	+
4	*Artemisia vulgaris*	20	stem	-
5	*Potentilla reptans*	24	leaves	-
6	*Solanum nigrum*	14	stem	+
7	*Polygonum aveculare*	8	stem	-
8	*Polygonum convolvulus*	12	leaves	-
9	*Castygela sepium*	12	leaves	-
10	*Sonchus oleraceus*	6	stem	-
11	*Chemopodium album*	12	leaves	-
12	*Setaria viridis*	12	leaves	-

GENERAL CONCLUSIONS AND RECOMMENDATIONS

1. Molecular analysis of '*Ca*. P. solani' in plants of 4 tomato varieties was performed for three years. The Cerasus variety is more resistant compared to the Elvira variety.
2. Genotypes Mary Gratefully and Desteptarea were susceptible to '*Ca*. P. solani' infection depending on the climatic conditions of the vegetation season.
3. The different sensitivity of the analyzed varieties to '*Ca*. P. solani' is revealed more clearly at the stage of mass fruit ripening, in the material collected in August. So, the period of mass fruit ripening is the most suitable for estimating the resistance of tomato varieties to phytoplasma.
4. Phytoplasma infection was not determined in wild species of tomatoes. The wild species *S. habrochaites* can be used as a control. The plants of this species were not affected by '*Ca*. P. solani'.
5. The different resistance to phytoplasma evaluated by the nested-PCR method was confirmed by Real-time PCR, PCR on serially diluted DNA can be used as an alternative method for quantifying '*Ca.* P solani'.
6. Diagnosis of '*Ca*. P. solani' presence in tomato seeds and seedlings grown in controlled conditions was found no transmission of phytoplasma infection in all analyzed local genotypes.
7. '*Ca*. P. solani' infection was determined in some insects of the Hemiptera order in August and September, in the experimental fields of IGPPP. Insects of the Hemiptera order, in which '*Ca*. P. solani' infection was identified, can be considered as potential vectors of this pathogen.
8. The pathogen '*Ca*. P. solani' was identified in cicadas mainly at the end of the vegetation season, while at the beginning of the vegetation season it was not determined.
9. Nested-PCR analysis of '*Ca*. P. solani' in 12 weed species established the presence of the pathogen in two species, *Convolvulus arvensis* and *Solanum nigrum*. The species *C. arvensis*, being a perennial plant, can be considered as a reservoir of infection.
10. The nucleotide sequences obtained on the base of DNA isolated from tomatoes affected by stolbur in 2018-2020 were completely identical to one another.
11. The sequenced fragments are completely identical to those reported in the NCBI database, which were detected in numerous European countries (Romania, Poland, Bulgaria, Italy, Serbia, Czech Republic), as well as in Russia, Turkey, China, etc.

It is recommended:

1. The Cerasus variety, together with the wild tomato species *S. habrochaites*, can be recommended for inclusion in breeding programs for creating tomato varieties or hybrids resistant to phytoplasma.
2. The method of DNA isolation in alkaline solution can be used in the rapid and reliable evaluation of tomato resistance to stolbur.

3. Molecular diagnosis of 12 samples from a variety allows obtaining statistically reliable data in August. In the case of plant analysis in July and September, a larger sample size of approximately 20 individual samples is necessary to achieve statistical significance at $P \leq 0.05$.

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ADNOTARE

Bahșiev Aighiuni, "Diagnosticul molecular al fitoplasmei la diferite soiuri de tomate autohtone", teză de doctor în științe biologice, Chișinău, 2024.

Structura tezei: Introducere, 5 capitole, concluzii și recomandări, bibliografie din 156 de titluri, 4 anexe, 99 de pagini text de bază, 59 figuri, 14 tabele. Rezultatele cercetării au fost publicate în 39 publicații științifice.

Cuvinte-cheie: '*Candidatus* Phytoplasma solani', diagnostic molecular, tomate, perioadă de vegetație, stolbur, rezistență, *nested*-PCR, insecte vector, plante ruderale.

Domeniu de studiu: 162.01. Genetică vegetală

Scopul lucrării: Determinarea sensibilității genotipurilor locale de tomate la infecția '*Ca.* P. solani' și studierea răspândirii acesteia utilizând metode moleculare.

Obiectivele cercetării: Detectarea moleculară a repartizării şi a gradului de atac al infecţiei fitoplasmice la plantele de tomate ale unor soiuri autohtone pe parcursul perioadei de vegetaţie; Identificarea etapei pentru determinarea diferenței în sensibilitate la infecția fitoplasmică; Determinarea fitoplasmei la 4 forme spontane de tomate; Elaborarea și optimizarea sistemului de diagnostic al '*Ca.* P. solani' la tomate, insecte vector și plante ruderale; Analiza comparativă a metodelor (*nested*-PCR, RT-PCR, PCR) prin diluarea în serie a ADN-ului) pentru determinarea sensibilității la infecția fitoplasmică a soiurilor autohtone de tomate; Secvențierea a fragmentelor a genei 16S ARNr cu scopul determinării tulpinilor '*Ca.* P. solani' pe loturile experimentale; Determinarea posibilității transmiterii stolburului prin materialul semincer la soiurile analizate; Determinarea infecţiei fitoplasmice la insectele din ordinul Hemiptera (insectele vector) și plante ruderale cu scopul analizei stării epidemiologice a '*Ca.* P. solani' pe câmpurile experimentale.

Noutatea şi originalitatea ştiinţifică: Pentru prima dată în Republica Moldova, a fost determinată prin metode moleculare prezența fitoplasmei la soiurile de tomate autohtone. Prin secvențierea ADN-ului patogenului a fost identificată tulpina '*Ca.* P. solani' ce infectează tomatele.

Rezultatul obținut care contribuie la soluționarea unei probleme științifice importante constă în: Determinarea metodelor de minimizare a răspândirii '*Ca.* P. solani' la tomatele autohtone prin utilizarea genotipurilor rezistente.

Semnificația teoretică: Sensibilitatea diferită a soiurilor autohtone la infecția fitoplasmică a fost confirmată prin metode moleculare. S-a determinat că perioada de maturare în masă a fructelor este cea mai potrivită pentru estimarea rezistenței soiurilor de tomate la stolbur. Lipsa transmiterii infecției fitoplasmice prin semințe la genotipurile locale de tomate a fost constatată în condiții controlate. S-a realizat secvențierea fragmentelor genei 16S ARNr a '*Ca.* P. solani'. Două secvențe de ADN specifice pentru '*Ca.* P. solani' identificate în tomate în Republica Moldova s-au plasat în Banca de Gene Mondială (NCBI). Creșterea numerică semnificativă a cicadelor infectate cu '*Ca.* P. solani' a fost determinată la sfârșitul perioadei de vegetație a tomatelor.

Valoarea aplicativă: Rezultatele obținute permit de a propune soiul Cerasus, împreună cu forma spontană *Solanum habrochaites*, pentru includerea în programele de selecție pentru crearea soiurilor sau hibrizilor de tomate rezistenți la stolbur. Metoda alcalină expres de extragere a ADN-ului poate fi utilizată în evaluarea rapidă și sigură a rezistenței tomatelor la fitoplasmoză. A fost elaborată schema diagnosticului molecular al fitoplasmei la tomate.

Implementarea rezultatelor științifice: Datele obținute în lucrare servesc în calitate de material ştiinţifico-didactic la predarea cursului de Fitopatologie. Primerii se recomandă a fi utilizați în testarea sensibilității germoplasmei de tomate la '*Ca.* P. solani'.

АННОТАЦИЯ

диссертация **«Молекулярная диагностика фитоплазмы у различных местных сортов томата»,** представленная **Бахшиев Айгюнь** на сойскание степени доктора биологических наук по специальности – **162.01. Генетика растений**

Кишинев, 2024.

Структура диссертации: Работа включает введение, 5 глав, выводы и рекомендации, библиографию из 156 наименований, 4 приложения, 99 страниц основного текста, 59 рисунков, 14 таблиц. Результаты исследований были опубликованы в 39 научных работах.

Ключевые слова: *'Candidatus* Phytoplasma solani′, молекулярная диагностика, томат, столбур, устойчивость, вегетационный период, нестед-ПЦР, насекомые-переносчики, сорняки.

Цель работы: Определение молекулярными методами чувствительности местных генотипов томата к инфекции '*Ca*. P. solani' и изучение ее распространения.

Задачи исследования: Молекулярная диагностика распространения фитоплазмоы и степени поражения фитоплазмозом растений томатов местных сортов в течение вегетационного периода; Идентификация стадии развития растений для наиболее точного определения разницы в чувствительности к фитоплазменной инфекции; Тестирование фитоплазмы у 4 диких видов томатов. Разработка и оптимизация системы диагностики '*Ca*. P. solani' у томатов, насекомых-векторов и растений-сорняков; Сравнительный анализ методов (нестед-ПЦР, ПЦР в реальном времени, ПЦР с использованием серийного разведения ДНК) для определения чувствительности местных сортов томатов к фитоплазменной инфекции; Секвенирование фрагментов гена 16S рРНК с целью идентификации штаммов '*Ca*. P. solani', присутствующих в растениях томата на экспериментальных участках; Изучение возможности передачи столбура через семенной материал анализируемых сортов; Определение фитоплазменной инфекции у насекомых из отряда Hemiptera (насекомые-векторы) и многолетних растений с целью мониторинга эпидемиологического состояния '*Ca*. P. solani' на экспериментальных полях.

Научная новизна и оригинальность: Впервые в Республике Молдова было доказано молекулярными методами наличие столбура у местных сортов томатов. Также был идентифицирован штамм '*Ca*. P. solani', заражающий томаты, путем секвенирования фрагмента ДНК.

Решённая научная проблема: состоит из определения подходов снижения распространения '*Ca*. P. solani' у местных томатов за счет использования устойчивых генотипов.

Теоретическое значение работы: Различная чувствительность местных сортов к фитоплазменной инфекции была подтверждена молекулярными методами. Было определено, что период массового созревания плодов является наиболее подходящим для оценки устойчивости сортов томатов к фитоплазменной инфекции. Отсутствие передачи фитоплазменной инфекции через семена было установлено в контролируемых условиях у местных генотипов томатов. Было выполнено секвенирование фрагментов гена 16S рРНК '*Ca*. P. solani'. Две специфичных последовательности ДНК '*Ca*. P. solani', идентифицированных на томатах в Республике Молдова, были размещены в Мировом Банке Генов (NCBI). Значительное увеличение численности цикадок, зараженных *'Ca*. P. solani´, было отмечено в конце сезона вегетации томатов.

Прикладная ценность: Полученные результаты позволяют предложить сорт Cerasus и дикую форму Solanum habrochaites для включения в селекционные программы для создания сортов или гибридов томатов, устойчивых к столбуру. Экспресс-метод щелочной экстракции ДНК может быть использован для быстрой и надежной оценки устойчивости томатов к фитоплазмозу. Разработана схема молекулярной диагностики фитоплазмы у томатов.

Внедрение научных результатов: Полученные данные служат в качестве научно-дидактического материала при преподавании курса Фитопатологии. Разработанные праймеры рекомендуется использовать для тестирования чувствительности гермоплазмы томатов '*Ca*. P. solani'.

ANNOTATION

Of the thesis entitled **"Molecular diagnosis of phytoplasma in different local tomato varieties"**. Presented by the candidate **Bahsiev Aighiuni,** for obtaining the degree of Doctor in Biological Sciences with specialty – **162.01. Plant genetics.**

Chisinau, 2024

Structure of the thesis: Introduction, 5 chapters, conclusions and recommendations, a bibliography of 156 titles, 4 appendicies, 99 pages of basic text, 59 figures, 14 tables. The research results were published in 39 scientific papers.

Key words: '*Candidatus* Phytoplasma solani', molecular diagnostics, tomato, vegetative period, stolbur, resistance, nested-PCR, insect-vectors, weeds.

Research purpose: Determine the sensitivity of local tomato genotypes to '*Ca*. P. solani' infection of and to study its spread by molecular methods.

Research objectives: Molecular detection of phytoplasma spread and the degree of phytoplasma infection in tomato plant of local varieties during the vegetation period; Identification of plant development stage for the most reliable determination of the difference in sensitivity to phytoplasma infection. Testing phytoplasma presence in 4 wild tomato species. Development and optimization of a diagnostic system for '*Ca*. P. solani' in tomato, insect vectors, and weeds. Comparative analysis of methods (nested-PCR, RT-PCR, PCR, serial dilution of DNA) to determine the sensitivity of local tomato varieties to phytoplasma infection. Sequencing a 16S rRNA gene fragment to identify '*Ca*. P. solani' strains in experimental plots. Study of the possibility of stolbur transmission through the seed material of analyzed varieties. Determination of phytoplasma presence in insects of the Hemiptera order (insect vectors) and weeds to monitor the epidemiological state of '*Ca*. P. solani' in experimental fields.

Scientific novelty and originality: For the first time in the Republic of Moldova, the presence of stolbur in local tomato varieties was proven by molecular methods. The '*Ca*. P. solani' strain infecting tomato was identified by DNA sequencing.

An important scientific problem solved: Consists of determining aproaches to reduce the spread of '*Ca*. P. solani' in local tomato through using resistant genotypes.

The theoretical significance: The varied sensitivity of local varieties to phytoplasma infection was confirmed by molecular methods. It was determined that the mass fruit ripening period is the most suitable for assessing the resistance of tomato varieties to phytoplasma infection. The absence of infection transmission through seeds was established under controlled conditions in local tomato genotypes. Sequencing of the 16S rRNA gene fragment of '*Ca*. P. solani' was performed. Two specific DNA sequences to '*Ca*. P. solani' identified in tomato in the Republic of Moldova were placed in the Global Gene Bank (NCBI). A significant increase in the number of leafhoppers infected with '*Ca*. P. solani' was noted at the end of the tomato growing season.

The applicative value: The obtained results suggest that the Cerasus variety, and the wild species *Solanum habrochaites* can be included in breeding programs to create tomato varieties or hybrids resistant to stolbur. The alkaline DNA extraction express method can be used for rapid and reliable assessment of tomato resistance to stolbur. The scheme for the molecular diagnostic of phytoplasma presence in tomato was developed.

Implementation of the results: The obtained data serve as scientific didactic material for the teaching Phytopathology course. The developed primers are recommended for testing the sensitivity of tomato germplasm to '*Ca*. P. solani'.

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MOLECULAR DIAGNOSIS OF PHYTOPLASMA IN DIFFERENT LOCAL TOMATO VARIETIES

161. 01. Plant genetics

Summary of the doctoral thesis in biological sciences

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