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DETECTION OF PHYTOPLASMA INFECTIONS IN VARIOUS FRUIT TREES IN BULGARIA, 2018-2024

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Abstract

This article covers research conducted between 2018 and 2024 aimed at detecting phytoplasma infections in various fruit crops in Bulgaria. Phytoplasma diseases, such as 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma pyri', and 'Candidatus Phytoplasma prunorum', represent a serious threat to agriculture, leading to significant losses in yields and fruit quality. The studies described in the article include the diagnosis of phytoplasma infections in different fruit crops. Molecular methods, including PCR (Polymerase Chain Reaction) and RT-RFLP analysis, were performed to identify pathogens, enabling precise and rapid detection of phytoplasmas in affected plants. More than 3,294 samples were analyzed, of which 4 tested positive for 'Candidatus Phytoplasma prunorum' and 6 for 'Candidatus Phytoplasma pyri', while 'Candidatus Phytoplasma mali' was not detected. The article emphasizes the importance of early detection and management of phytoplasma infections in fruit growing to minimize economic losses and ensure sustainable fruit production in the future.

***Key words**: Phytoplasma infections, Fruit trees, Bulgaria*

INTRODUCTION

Pomiculture is one of the main sectors in agriculture, playing a key role in the Bulgaria's economy, ensuring food security, and improving the quality of life.

Among the most widely grown fruit plants in the country are apples, plums, cherries, apricots, peaches, and strawberries. These crops not only satisfy the domestic market but are also exported abroad, contributing to the increase in agricultural product exports.

The yields in horticulture depend on numerous factors, including climatic conditions, the technologies used, soil quality, and the application of modern agronomic practices.

In recent years, the areas occupied by apple orchards have remained relatively stable, ranging between 12 and 15ha. Apple yields vary significantly depending on climatic conditions. In years with favorable conditions (such as 2019 and 2022), yields reached 150,000–160,000 tons. In 2021, yields were lower (around 120,000 tons) due to spring frosts and drought.

The areas occupied by pears are relatively small in Bulgaria, ranging between 2 and 3ha. There is a slight downward trend in these areas, as pears are a less popular crop compared to apples and plums. Pear yields range between 10,000 and 15,000 tons annually.

Plums are one of the most widely grown fruit crops in Bulgaria. The areas occupied by plum orchards are around 20–25ha. There has been a slight decrease in these areas in recent years, as some old orchards have been abandoned. Plum yields vary significantly, ranging from 50,000 to 90,000 tons annually. In 2020, yields were lower (around 50,000 tons).

The areas occupied by apricots are around 5-7 ha. There has been a slight decrease in these areas, as apricots are more sensitive to climate changes and diseases. Apricot yields range between 15,000 and 25,000 tons annually.

The areas occupied by peaches range between 5 and 7ha during the period 2020–2023. A slight decrease in these areas has been observed due to their sensitivity to climate changes and diseases, making them less attractive to producers. In years with favorable conditions (such as 2022), yields reached 25,000–30,000 tons (Agrostat, 2021).

Fruit trees from the Rosaceae family can be seriously affected by phytoplasmas from the Apple Proliferation (AP 16SrX group). The AP group includes 'Candidatus Phytoplasma mali', which causes apple proliferation (AP), ‘Candidatus Phytoplasma prunorum’, associated with European stone fruit yellows (ESFY), and ‘*Candidatus* Phytoplasma pyri’, associated with pear decline (PD) (Seemüller & Schneider, 2004; Marcone et al., 2010). These are among the most economically important diseases in many fruit-growing regions of Europe, as well as in Bulgaria.

Phytoplasmas belong to the class Mollicutes and are considered putative pathogens of the so-called "yellows diseases," which affect at least 1,000 plant species worldwide (McCoy et al., 1989; Seemuller et al., 2002; Trivellone and Dietrich, 2020). These microscopic, intracellular prokaryotes colonize the phloem of infected host plants, as well as various tissues and organs of their insect vectors.

Phytoplasmas are transmitted after a latent period of several days to months to plants during the feeding of their vectors, primarily cicadas and leafhoppers from the order *Hemiptera* (IRPCM, 2004; Thébaud et al., 2009).

Infection by ‘*Ca.* P. mali’ occurs primarily in plants of the genus *Malus*, but it has also been sporadically detected in other non-typical host plants, such as stone fruits, European pear (*Pyrus communis*), and Asian pear (*Pyrus pyrifolia*) (Lee et al., 1995; Del Serrone et al., 1998; Seemüller & Schneider, 2004; Mehle et al., 2007). ‘*Ca.* P. pyri’ is mainly associated with the genus *Pyrus* (Seemüller & Schneider, 2004). On the other hand, ‘*Ca.* P. prunorum’ causes economically significant diseases in apricot (*Prunus armeniaca*), Japanese plum (*Prunus salicina*), and peach (*Prunus persica*) (Carraro & Osler, 2003). European plum (*Prunus domestica*), as well as some wild species of the genus *Prunus* (*Prunus spinosa, Prunus cerasifera, Prunus insititia*), are also susceptible to infection but typically do not show symptoms, making them hidden sources of infection (Carraro et al., 1998a, 2004; Carraro & Osler, 2003).

Leafhoppers play a significant role in the spread of phytoplasmas from the AP group (Tedeschi & Alma, 2004).

‘*Ca.* P. prunorum’ is transmitted to plants of the genus *Prunus* by the insect vector *Cacopsylla pruni* (Carraro et al., 1998b). Additionally, the cicada *Asymmetrasca decedens* (also known as *Empoasca decedens*) is considered a potential carrier of this phytoplasma (Pastore et al., 2004).

‘*Ca.* P. pyri’ is spread by two main vectors, *Cacopsylla pyricola* (Davies et al., 1992) and *Cacopsylla pyri* (Carraro et al., 1998c).

Among the known leafhopper vectors of ‘*Ca.* P. mali’ are *Cacopsylla picta* (also called *Cacopsylla costalis)* (Frisinghelli et al., 2000; Jarausch et al., 2003) and *Cacopsylla melanoneura* (Tedeschi & Alma, 2004).

In addition to leafhoppers, other insects are also associated with the transmission of ‘*Ca.* P. mali’, such as *Philaenus spumarius*, *Artianus interstitialis* (Hegab & El-Zohairy, 1986), and *Fieberiella florii* (Krczal et al., 1988).

Phytoplasmas, which cause significant economic damage to fruit trees, are widely distributed.

Pear decline was first reported in North America in the late 1940s but is believed to have European origins (Seemüller & Schneider, 2004). The infection is widespread in almost all EU countries.

‘*Ca.* P. mali’ has been reported in the EPPO region, (Tunisia, Syria), and North America (USA and Canada). ESFY (European stone fruit yellows) is also a widely spread infection in the EPPO region. The disease occurs with varying frequency in each country. ESFY is a serious problem in countries bordering the Mediterranean Sea (Spain, France, Italy, the Balkans), where the cultivation of susceptible and sensitive species of the genus Prunus is widespread (Steffek et al., 2012).

In Bulgaria, ‘*Ca.* P. prunorum’ and ‘*Ca.* P. pyri’ were first detected near the city of Plovdiv (Topchiiska et al., 2000; Topchiiska & Sakalieva, 2001, 2002).

In 1960, Apple proliferation was reported in Bulgaria by Sen and Jun Blattny, as cited by Trifonov (1972).

In 2012, the first summarized results from the official phytosanitary monitoring program conducted between 2007 and 2011 by the Bulgarian Food Safety Agency were published (Etropolska & Laginova, 2012).

The summarized results show that AP was detected in the regions of Blagoevgrad, Kyustendil, Pazardzhik, Plovdiv, and Veliko Tarnovo. PD was identified in 10 regional directorates of BFSA, including Blagoevgrad, Kyustendil, Pazardzhik, Plovdiv, Veliko Tarnovo, Sliven, Silistra, Burgas, Haskovo, and Yambol. ESFY was detected in 6 regions –

Pazardzhik, Lovech, Sliven, Dobrich, Burgas, and Yambol.

At that time, to control the spread of phytoplasmas, over 200 infected mother trees, more than 5,000 first-year nursery trees, and over 22,000 second-year nursery trees were uprooted and destroyed under the supervision of phytosanitary inspectors. In infected orchards, more than 0,2ha were destroyed (Avramov et al, 2013).

In 2016, an analysis for the presence of phytoplasmas was conducted in the Kyustendil region of Bulgaria. The results showed that diseases from the AP group were present in 85.0% of the tested apple trees, 62.0% of the tested pear trees, and all tested plum trees, along with the observation of many symptomatic trees. This indicates a high prevalence of phytoplasmas in and around the studied orchards in the Kyustendil region (Borisova & Kamenova, 2016).

At present, these phytoplasma diseases have had their status changed in Bulgaria from quarantine to non-quarantine regulated pests.

Additionally, all species of the genus *Cacopsylla* were described in Bulgaria by entomologists as early as the 1960s (Harizanov, 1966a, 1966b, 1982), long before they were recognized as vectors of phytoplasmas.

In 2011, the first report of the detection of '*Ca.* P. prunorum' through PCR analysis in individuals of the vector *C. pruni,* collected from Sofia and Kyustendil, was published (Etropolska et al., 2011a).

During a study conducted between 2011 and 2013, AP, ESFY, and PD were detected in commercial or non-cultivated orchards in five of the most important fruit-growing regions of Bulgaria: Sofia, Blagoevgrad, Plovdiv, Kyustendil, and Sliven regions (Etropolska et al., 2015).

The main symptoms of AP disease in apples, as described by Nemeth (1986), include "witches' brooms," enlarged stipules, small and poor-tasting fruits, and reddish discoloration of the leaves. The fruits of infected trees are deformed and smaller in size compared to those of healthy trees.

In pear trees, slow growth is observed. The leaves are fewer in number, smaller, light green, and have curled edges. The growth of shoots is very weak. The most characteristic symptom of PD infection is the reddish discoloration of the leaves and their premature fall in early autumn.

The symptoms described for ESFY include early bud development, followed by reddish discoloration and curling of the leaves, which are smaller in size (Carraro et al., 1992; Jarausch et al., 2000a; 2000b; Carraro and Osler, 2003).

There is no evidence of transmission through seeds or pollen (Seidl & Komarkova, 1974). *Cuscuta sp*. can transmit '*Ca*. P. mali' between apple, *Catharanthus roseus*, and other hosts.

To date, only hawthorn (*Crataegus sp*.) has been identified as a wild plant reservoir in the pathogen's epidemic cycle (Janik et al., 2020).

Over longer distances, *'Ca.* P. mali' is mainly spread through the movement of infected trees, cuttings, and rootstock material of apples.

'Ca. P. prunorum' is easily transmitted both in summer and winter (Riedle-Bauer et al., 2012).

Imported plant material is considered the primary way of introducing '*Ca*. P. prunorum' into Turkiye (Ulubaş Serçe et al., 2006). The local spread of '*Ca*. P. prunorum' is primarily driven by insect vectors and infected *C. pruni* can transmit phytoplasmas to Prunus species over distances of 27–50 km (Marie-Jeanne et al., 2020).

Due to dynamic ecological, climatic and anthropogenic factors, phytoplasma infections in fruit orchards are inconsistent.

Given the scarcity of data, this study aims to identify spreading of these infections and support future preventive measures to limit their spread in Bulgaria.

MATERIALS AND METHODS

Annually, phytosanitary inspectors from the Regional Food Safety Directorates conduct surveys across the entire country.

The focus of the research is on nurseries producing fruit planting material and high-risk fruit plantations. The species examined during this period were *Malus L., Prunus domestica, Prunus avium, Prunus cerasus, Pyrus L., Prunus armeniaca, Prunus persica* and *Cydonia* L. Samples were collected from 21 regions cover all over Bulgaria and sent to the laboratory of the University of Forestry.

In 2018, a total of 718 samples, primarily from fruit planting material, were processed and analyzed; in 2019, the number of samples was

637; in 2020 – 532 samples; in 2021 – 565 samples; in 2022 – 485 samples; in 2023 – 354 samples; and in 2024 – 116 samples, the total number of samples was 3,407 samples.

Malus L. and *Prunus domestica* were the two species with the highest number of samples in almost all years.

Prunus avium was also a frequently encountered species, particularly in regions such as Kyustendil and Plovdiv. In the past, before molecular techniques were developed, diagnosing phytoplasma diseases was challenging and primarily relied on symptom observation.

The method used to detect phytoplasmas was PCR. Total DNA was extracted from plant tissue (Doyle and Doyle, 1990) or a predetermined individual in CTAB buffer following the protocol by Marzachi et al. (1998).

Laboratory analysis was performed using Nested PCR with two pairs of universal primers, P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1996) and U3/U5 (Lorenz et al., 1995), as well as specific primers f01/r01 (Lorenz et al., 1995), followed by RT-RFLP analysis using *Rsa*I/*Alu*I restriction enzymes for final identification and species classification (Lee et al., 1998).

RESULTS AND DISCUSSIONS

Between 2018 and 2024, 3,431 laboratory analyses were conducted on 3,407 plant samples, was performed us following: for PD 541 analysis, for ESFY 2,157 analysis and for AP 733 analysis.

Phytoplasma infections were detected and confirmed in 12 number of samples. Out of all the samples examined during the period from 2018 to 2024, Apple proliferation phytoplasma was not detected in Bulgaria.

In 2018, none of the laboratory analysis showed positive reaction for the presence of phytplasmas. In 2019, positive samples for ESFY and PD were identified.

ESFY infection was detected in the Blagoevgrad region in mother trees of *Prunus domestica* (Table 2), while PD was found in the Kyustendil region in mother trees of *Pyrus communis* (Table 1).

Table 1. Confirmed PD phytoplasma infection, 2018-2024

Regions	Confirmed phytoplasma infection						
	2018	**2019**	**2020**	**2021**	**2022**	**2023**	**2024**
Total № analysis PD	**94**	**106**	**105**	**99**	**63**	**58**	**16**
Vidin	-	-	-	**PD**	-	-	**PD**
Vratsa	-	-	**PD**	-	-	-	-
Kyustendil	-	**PD**	-	-	-	-	-
Montana	-	-	-	**PD**	-	-	-
Razgrad	-	-	-	-	-	-	**PD**
Ruse	-	-	-	-	-	**PD**	-
Stara Zagora	-	-	**PD**	-	-	-	-

PD = positive result; “-“= negative result

Table 2. Confirmed ESFY phytoplasma infection, 2018-2024

Regions	Confirmed phytoplasma infection						
	2018	**2019**	**2020**	**2021**	**2022**	**2023**	**2024**
Total № analysis ESFY	**489**	**463**	**316**	**309**	**301**	**214**	**65**
Blagoevgrad	-	**ESFY**	-	-	-	-	-
Vidin	-	-	-	**ESFY**	-	-	-
Pazardjik	-	-	-	-	**ESFY**	-	-
Plovdiv	-	-	-	-	**ESFY**	-	-

ESFY = positive result; “-“ = negative result

In 2020, we identified one positive sample for PD in the Vratsa region and another positive sample for PD in the Stara Zagora region (Table 1).

In 2021, the highest number of positive samples for PD were recorded - 2 positive samples in the Vidin region from fruit planting material of

Pyrus L. (Table 1), and 6 positive samples in the Montana region from *Pyrus domestica* (Table 1). We also detected one positive sample for ESFY infection in *Prunus domestica*, again in the Vidin region (Table 2).
In 2022, we identified 2 cases of ESFY in the Pazardzhik region on *Prunus persica* and in the Plovdiv region on *Prunus domestica* (Table 2).
For 2023, there was only one positive sample for PD in the Ruse region (Table 1).
During the period 2018–2024, a gradual spread of PD and ESFY phytoplasmas was observed in various regions of Bulgaria.
The highest activity of PD was in 2021, while ESFY was most active in 2022. In 2023, a significant decrease in positive samples was observed, which may be attributed to implemented control measures.
Further research and measures are necessary to limit the spread of these diseases, particularly in the Vidin, Montana, Pazardzhik, and Plovdiv regions.
After conducting laboratory analyses of all samples, according to the nature of the material, the infection found in mother trees was 1.8% of 450 samples, in fruit nurseries, the infection rate was 1.4% of 250 samples, and in functioning orchards throughout the country it was about 1% of 300 samples (Figure 1).

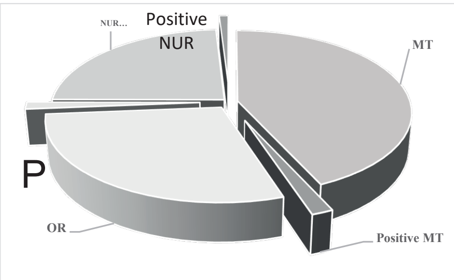

Figure 1. Spreading of infections according to testing material: MT – mothers trees, NUR – nursery, OR – orchards.

CONCLUSIONS

Fruit growing is a key sector in Bulgarian agriculture, contributing to the economy, food security, and quality of life. The most widely cultivated crops include apples, plums, cherries, apricots, peaches, and strawberries, which are also exported to international markets. Yields in fruit growing depend on climatic conditions, technologies, soil quality, and the application of modern agronomic practices.
In recent years, the areas dedicated to apple cultivation have remained stable, but yields have varied significantly depending on the climate. Phytoplasmas, which cause diseases such as Apple Proliferation (AP), Pear Decline (PD), and European Stone Fruit Yellows (ESFY), are a serious problem for fruit growing. They are primarily spread through insect vectors such as leafhoppers and psyllids.
In recent years, a gradual spread of PD and ESFY has been observed in various regions of Bulgaria, with the highest activity of PD recorded in 2021 and ESFY in 2022. In 2023, a decrease in positive samples was observed, which may be attributed to the control measures implemented.
The results were provided to the Bulgarian Food Safety Agency (BFSA). Based on these results, directives and various measures for the eradication of infections were prepared. The measures taken upon the detection of PD infection in the Kyustendil region were as follows: an additional sample was taken from the second mother tree of the same variety. Two mother trees of the "Paskresan" variety were uprooted and destroyed by burning before the results from the second tree were obtained, and a protocol was drawn up.
The additional sample from the second mother tree, which had already been destroyed, tested negative. In 2020, measures were taken in the regions of Vratsa and Stara Zagora, where infections were detected, including the destruction of fruit mother trees by the end of 2020. For 2021, the following measures were taken for the detected infections in the Vidin region for PD and ESFY: issuance of directives for the implementation of phytosanitary measures, including the purchase of healthy fruit planting material, regular treatment with approved plant protection products against vectors, strict hygiene practices such as washing and disinfecting used tools and machinery, and disinfection of pruning equipment with suitable agents (soaking in bleach or a 5-10% formalin solution or washing with a 70% alcohol solution).
In the Montana region, three "Abate Fetel" pear trees and one "Santa Maria" pear tree were

removed and burned from the mother orchard, and in the second-year nursery, 49 "Abate Fetel" pear trees and 53 potentially infected "Santa Maria" pear trees were destroyed.

Timely measures were prescribed to control the pear psyllid, and planting in the area of the destroyed trees was prohibited until the next growing season.

For 2022, upon detection of infections, the same measures as the previous year were taken: uprooting and destruction by burning, treatments to control the vector, and the use of grafts from healthy mother trees during grafting.

In 2023, in the Ruse region, where a PD infection was detected, a ban was imposed on the collection of grafts, and the infected trees were removed from the nursery's varietal composition.

In 2024, we identified 2 positive samples for PD - one in Vidin district and one in Razgrad district. Despite the decrease in positive samples in 2023-2024, continued monitoring and research are necessary, particularly in regions such as Vidin, Montana, Pazardzhik, and Plovdiv, to limit the spread of phytoplasma diseases.

Based on the obtained results, it can be concluded that the absence of AP is likely due to years of research and the measures implemented by state authorities and farmers. On the other hand, the limited number of detected infections should heighten our attention, focusing it on regions with large fruit orchards.

Fruit growing in Bulgaria faces challenges related to climate change, the susceptibility of certain crops to diseases, and the need to adopt modern technologies and practices to increase productivity and improve the quality of produce.

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