Study of Sudden Decline of Lavender in Bulgaria Caused by `*Candidatus* Phytoplasma solani`

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Abstract
The beneficial properties of medicinal and essential oil crops and medicinal and aromatic plants have been studied for thousands of years. Their cultivation corresponds to their growing demand, which leads to an annual increase in areas and an increase in the amount of herbal collection. In Bulgaria, the cultivation of some crops such as oil-bearing rose, lavender, and mint, has old traditions and world recognition and fame. Lavender as an intensive crop is often accompanied by the appearance of diseases and pests that can cause serious damage and in some cases even compromise the harvest. It is inadmissible in the production and its derivatives, the presence of even traces of the use of plant protection products. On the basis for the improvement of the applied methods of plant protection is an achievement of a good knowledge of the diseases in lavender plantations. An important point is the accurate, fast and reliable identification of pathogens in laboratory conditions of new and unknown phytoplasma diseases for Bulgaria.Examination of the lavender fields revealed symptoms of lavender disease with marked yellowing, reduction, straightening or sagging of the leaves, reduction and abortion of the inflorescences. A laboratory analysis total number on 91 lavender plant samples, 32 cicadas and 16 weed samples were performed. Phytoplasma infection in five samples from the Chirpan and Dobrich regions belonging to the Stolbur phytoplasma group, showing identical profiles was identified by PCR and RFLP analysis. For confirmation qPCR and sequencing was performed. The results are evidence that the established infection is of Sudden wilting of lavender caused by the phytoplasma 'Candidatus Phytoplasma solani' identified for the first time in Bulgaria.

Keywords: Lavender; '*Candidatus* Phytoplasma solani'; Bulgaria

INTRODUCTION

People started to study the beneficial properties of medicinal and essential oil crops (MEOCs) and medicinal and aromatic plants (MAPs) thousands of years ago. Having accumulated experience, they administered them more appropriately and efficiently, resulting in their widespread use. Growing demand led to an increase in the amount of herbs collected annually. Even with the best conservation and consequent storage of medicinal plants, their effects weaken over time. Therefore, for each herb you need to know how long after its preparation it is suitable for use. Improper drying can result in complete loss of their curative effect. Following those requirements ensures the effectiveness of phytotherapy. There is an old tradition of cultivating some essential oil crops, such as oil rose, lavender, peppermint, etc., in Bulgaria and the country is known worldwide for this, while other, relatively newer ones, such as silibum (milk thistle), sage, yellow poppy, etc., are showing potential and their production is expanding. At the same time, according to FAO data, many plant species are going extinct across the world every year. Therefore, in order to address the deficit and satisfy growing consumption, technologies for their cultivation have been and are being created.

The cultivation of lavender as an intensive crop is often accompanied by diseases and pests that can cause serious damage and, in some cases, even destroy the harvest. As a crop for medical and cos-

metic purposes, if plant protection products are used on lavender, the final product and its derivatives must not contain even minimal residues. At the heart of improving the methods and approaches used to protect plants is good knowledge of the species, as well as the diseases and damages they cause to lavender plantations. An important part is to accurately, quickly and reliably identify the pathogens of new and unknown in Bulgaria phytoplasma diseases in laboratory conditions. Projects 21/2016 and B-1070/2020 of the Research Department of the Sofia Forestry University supported this study of phytoplasma pathogens in aromatic and medicinal crops and their vectors in Bulgaria.

Historical Overview of Phytoplasma Diseases

The first literature data on phytoplasma diseases, commonly known as "yellowing diseases", date back to 1000 years ago in China, during the Song Dynasty (960-1227) (Maramorosch et al., 1988). It was then that the most beautiful peony tree, called "*yao-yellow*", was described for the first time. It had beautiful yellow-green flowers but produced no seeds. The emperor ordered that it be moved to the imperial courtyard, so that only he could enjoy its beauty. For centuries, the peony's mysterious green colour has been very popular in China. It has also taken the same amount of time to show, using the electron microscope, that the cause of this beauty is phytoplasma (Aster yellows phytoplasma, group 16SrI). The term "mycoplasma" was adopted after 1897, when Erickson discovered membrane-free particles at a development stage of rusts – fungal plant diseases. It was later found that there was no such stage and the term "mycoplasmas" was abandoned. Historically, many scientists have looked for the causes of yellows and have accepted the source of infection as a virus or other filterable cause of plant diseases. The problem of the mysterious transmission of Aster yellows was discovered in the United States in 1924, when the Boyce Thompson Institute in Yonkers (USA) discovered that the *Macrosteles fascifrons* (Stål) cicada was the disease vector (Kunkel, 1926). Since no bacterium or fungus was found in diseased plants, the study author suggested the disease was caused a virus. He believed the virus multiplied in the cicada's body and proved his theory by serial passage techniques where the causative agent was inoculated by injecting the vector (Maramorosch, 1952). In 1946 in Moscow, Sukhov and Vovk found experimentally that the vector of the disease, then considered viral and known as stolbur on plants of the potato family (*Solanaceae*), was the *Hyalesthes obsoletus* Signoret cicada (Sukhov et al., 1949). Another key point about the nature of causative agents was established in 1940 by Bennett, who first proved viral transmission using a diseased cuscuta plant to infect a healthy one. The transmission of diseases through cuscuta thus became an important and characteristic diagnostic sign in viruses (Bennett, 1944). Advances in phytoplasma research began to be made in 1967, when Japanese scientists identified the cause of mulberry dwarfing. The causative agent was found accidentally in the phloem of diseased plants and named "mycoplasma-like organism, MLO". Their research deepened and found similar "mycoplasma" pathogens that caused witches'-broom diseases on potatoes and *Paulownia* and Aster yellows. Until then, those diseases were considered viral based on the symptoms observed and the impossibility of mechanical transmission. Their studies confirm that all mycoplasmas are transmitted by cuscuta, vectors and grafting, but they are also clear that mycoplasmas are different species and cause a variety of signs in host plants (Doi et al., 1967). Subsequently, in 1994, at the 10th Congress of the International Organisation for Mycoplasmology (Bordeaux, France), the name "phytoplasmas" was adopted for mycoplasma pathogens in plants. About 1,000 phytoplasma diseases are currently known (Bertaccini, 2007).

Phytoplasmas are highly pleomorphic organisms, which means that, within the same species, there are individuals of different shape and size – spherical, ovoid, filamentous, branched or otherwise. Their size rangers from 60-80 to 800 nm in diameter (Kuske & Kirkpatrick 1992; Lee et al., 2000). Filamentous shapes are often observed in the conducting vessels of plants. This suggests they are migratory phases in their life cycle. The conducting tubes are wide enough to allow even the largest phytoplasma shapes to pass through. They do not have a cell wall, but a three-layer cytoplasmic membrane (Sears &Kirkpatrick, 1994). This is how they differ from bacteria (McCoy, 1981). The cytoplasm contains two types of nucleic acids (DNA and RNA), protein molecules, bacteria-like ribosomes with a diameter of 10-15 nm, and nuclear material. The nucleus of phytoplasmas does not have a mem-

brane, which is why it is called a nucleoplasm. This feature defines phytoplasmas as prokaryotes (Murray, 1984). Their DNA is a double-stranded ring-shaped molecule with a molar mass of 4.10^8-1.10^9. Guanine (G) – Cytosine (C) content is 23 to 41% (Dikinson, 2003). The phytoplasmic genome is very small (530-1,350 kb) and contains a small number of genes (Kirkpatrick, 1992; Marcone et al., 1999). Phytoplasmas have their own metabolism, which is no different from those of other single-celled microorganisms. They do not develop and reproduce in a nutrient medium. Sterols need to be present in nutrient media for isolated cases of cultures developed *in vitro*. On dense agar media, they form small specific colonies resembling egg yolk. Phytoplasmas are very sensitive to high temperatures and are inactivated at 40-50°C (Sears & Kirkpatrick, 1994).

Symptoms of Phytoplasma Diseases. The signs observed in the course of development of phytoplasmas depend on the host plants, the mode and time of infection and weather conditions. In vector insects, the infection causes premature death (McCoy et al., 1989). According to Nienhaus &Sikora (1979), there are several types of signs in plants, which are subsequently summarised as groups of syndromes caused by phytoplasma infection and having characteristic symptoms (McCoy et al., 1989; Kirkpatrick, 1992; Lee et al., 2000): yellows; dwarfing, rusts, leaf curl, epinasty and wilt, known as "plant stolbur"; witches'-brooms; phyllody; virescence; colour sterility; rubbery wood and others. Very often one phytoplasma species causes symptoms in a variety of hosts. The opposite is also possible – the same type of symptoms in different hosts. Such is the case with stolbur. The general taxonomy of the species is systematically assigned to the ***Mollicutes*** **class** (Lee et al., 1998; Kuske et al., 1992; Namba et al., 1993; Sears & Kirkpatrick, 1994), with more than 30 species belonging to the *Candidatus* Phytoplasma genus and transmitted by insect vectors (Bergey's Manual of Systematic Bacteriology, 2011).

Symptoms and transmission of phytoplasma infection in lavender. Understanding crop epidemics requires focusing on the direct relationship between the host plant and the pathogen by various parameters such as virulence resistance / pathogen host and interbreeding. In vector-borne diseases, a third player participates in the system, who allows the pathogen to spread and encounter its host. Therefore, the biology of insect vectors needs to be considered and the study of insect vectors of plant pathogens needs to receive more attention (Almeida, 2008; Chuche et al. 2017; Purcell &Almeida, 2005). The vector and the pathogen may or may not share the same host plant. Nettles (*Urtica dioica*) allow the development of *Ca*. P. solani and *H. obsoletus*, while vines (*Vitis vinifera*), for example, can be affected by the pathogen, but cannot be vector host plants (Johannesen et. al., 2008). In this situation, the vine is the ultimate phytoplasma host. *H. obsoletus* acquires its infectivity mainly as a nymph by feeding on the plant in its third generation (e.g. nettle or bindweed) and transmitting the pathogen as an adult when exploring the vines. In contrast to the vine, lavender (*Lavandula angustifolia*) and lavandin (*Lavandula* X *intermedia*) are both hosts to the *Ca*. P. solani phytoplasma and its vector cicadas (Boudon-Padieu & Cousin, 1999).

The disease, called SUDDEN DEATH OF LAVENDER, has been the main threat to lavender production in recent decades, with strong economic consequences. Lavender plants are usually cultivated for 10 to 12 years, but the presence of "sudden death" limits growth and results in the plants being uprooted within 4-5 years of planting (Moreau et al., 1970). Symptoms of lavender death include yellowing, reduction, leaf straightening or sagging, inflorescence shrinkage and abortion (Boudon-Padieuand & Cousin, 1999). As in other phytoplasma diseases, the symptoms may only be located on some branches or affect the whole plant. After yellowing, the affected branches wither, leading to plants with a mixture of dead and green branches. After several vegetation cycles, the plants become completely brown and necrotic (Boudon-Padieu & Cousin, 1999). There is no cure for phytoplasma disease. The main ways to control the epidemic are to suppress the transmission of pathogens from one plant to another by eliminating the vector (Chuche &Thiery, 2014) or destroying the plant vector if the susceptible culture is under threat and is a host of phytoplasma (Kehrli &Delabays, 2012). Insecticides cannot be used to manage the problem in the *H. obsoletus*/stolbur/lavender system because the crop is very attractive to pollinators (honey bees, bumble bees) and acoustic protection rules prevent the use of insecticides. Cicada nymphs develop in the roots and can be found at a depth of tens of centimetres

(Boudon-Padieu & Cousin, 1999) and therefore cannot be affected by insecticide sprays. The main actions that can be taken are to use healthy planting material and select more sustainable varieties of lavender and lavandin (Sémétey at al., 2018). However, no variety has so far been resistant to phytoplasma infection (Gaudin et al., 2011). Other prevention methods, such as spraying kaolin clay or indoor cultivation of plants, are currently being researched. Most of the resistance to lavender death is associated with the plant's unsuitability and/or poor attractiveness for the *H. obsoletus* cicada. Thus, the varieties less susceptible to the disease, Diva (lavender) and Grosso (lavandin), have a very low number of adults and nymphs, in contrast to the most sensitive varieties, C15/50 (lavender) and Abrial (lavandin), (Yvin, 2013). A surprisingly high rate of symptom decline has been observed in recent years in south-eastern France on the less sensitive Grosso variety. At the same time, an increase in the number of *H. obsoletus* has been observed in those areas. Tests on insects close to these lavender fields have shown that recently cultivated plots of sage, S.sclarea, host a high population of insects close to *H. obsoletus*. A study by a team of researchers (Chuche et al., 2018) showed that another source of phytoplasma infection in lavender (*Lavandula angustifolia*) and lavandin (*Lavandula* X *intermedia*) in France is sage (*Salvia sclarea*). The study authors found that *S. sclarea* is also a host plant of *Hyalesthes obsoletus* with full development of the vector and may be a source of Stolbur infection caused by *Ca.* Phytoplasma solani. The study authors found a new host of phytoplasma infection with as yet unclear and unstudied phytosanitary status, but threatening lavender plantations in France. This information can be very useful to Bulgarian producers, and they should be informed of the potential infection, so they can take into account the risks of growing in the vicinity of these two crops.

Mycoplasma plant diseases were first reported in Bulgaria in 1970 (Kovachevski, 1971). Only a few studies have been conducted in this area – stolbur on tomatoes and peppers (Kovachevski et al., 1964) and its vector, the *H. obsoletus* cicada (Arabadzhiev, 1964). Mycoplasma diseases have been reported in fruit crops (Trifonov, 1965; Hristova, 1973). Other diseases include strawberry green petal (Hristov et al., 1970), strawberry aster yellows (Kacharmazov, 1972), rose wilt (Hristova, 1973). Also, a new vine disease has been reported – golden yellow (Abrasheva, 1977). As the methods used to identify phytoplasmas improved, new ones were found across Bulgaria, such as Pear decline phytoplasma (Topchiiska et al., 2001), European fruit stone yellows phytoplasma (Topchiiska et al., 2002), vine yellows (Avramov, 2014) and Fruit crop phytoplasmas (Etropolska, 2015).

In Bulgaria, information on phytoplasma diseases is scarce and lags behind the modern level of knowledge (Dobrev 1909, 1910; Trifonova et al., 1952; Kovachevski., 1971; Abrasheva, 1977; Kovachevski et al., 1999). The disease known as sudden decline of lavender has not been detected and reported in Bulgaria. This means targeted research should be conducted to obtain new information about this disease, its vectors and host plants. The update is necessary due to changes in varietal composition in new lavender plantations in Bulgaria, as well as due to increased imports and exports of seedlings from/into the EU and other countries that have controls to prevent the transmission of phytoplasma infection from known outbreaks. Eliminating the uncertainty about the spread of diseases in medicinal crops will be beneficial to science and practice, which is why this study had to be developed.

The main goal of this study is to find, detect and identify the phytoplasma causing the sudden death of lavender (*Lavandula augustifolia* Mill.) in Bulgaria. To show how it is spread and why it is present in lavender fields.

To achieve this goal, we set ourselves the following tasks: Review literature sources and prepare a literature overview of phytoplasma pathogens that cause lavender diseases; Detect and identify the cause of sudden wilt of lavender – the ***Candidatus* Phytoplasma solani** phytoplasma in Bulgaria; Analyse different species of cicadas and weeds to show transmission and existing reservoirs of new infections; Identify phytoplasma and fungal pathogens and differentiate them by visual signs; Summarise study data and make recommendations to producers to help through preventive measures against disease vectors and to reduce the risk to the volume and quality of products obtained. Eliminating the uncertainty about the spread of phytoplasma diseases in lavender will benefit science and practice, which is why this study had to be developed.

MATERIALS AND METHODS

The study covers plant materials from industrial lavender plantations (areas in the stage of full fruiting, vineyards, including stock nurseries, parent vines and rootling nurseries) in Bulgaria in the period 2016-2020[1].

Plant samples. A total of 139 plants were subjected to laboratory analysis, distributed as follows: 52 plants, 32 cicadas and 16 weed samples were subjected to PCR testing to identify phytoplasma pathogens in plants and detect already established pathogens; 39 samples were tested by humidity chamber, nutrient medium and microscopy methods for mycological analysis purposes. The results from the identified phytoplasma pathogens were confirmed at the USDA after we sent 31 DNA extracts to the United States.

Positive controls from extracted DNA and vinca plants (*Catharanthus roseus L.*) infected with phytoplasmas that cause black wood (bois noir, BN) and flavescence dorée (FD) were used in the course of the study[2]. In the initial stage of the study, we had to conduct parallel testing for viral and bacterial infection.

Cicadas, vectors of phytoplasmas, causative agents of death of lavender. Along with the plant samples, the study includes the cicada insects *Hyalesthes obsoletus* Signoret, *Reptalus* spp., *Fieberiella florii*, *Lepyronia* spp., carriers (vectors) of phytoplasmas causing vine stolbur or yellows, as well as other species from the country[3]. Isolates and benchmark strains of phytoplasmas. Phytoplasma isolates were distinguished by groups using standard isolates[4] on grafted vinca plants (*C. roseus*) from the collection of INRA, Bordeaux, France. According to the classification of Seemüller et al., 1998 and Lee et al., 1998, the standards belong to different groups: phytoplasma (FD-92; FD-70); Stolbur PO; phytoplasma (AP-15). When performing biological and identification tests, we used vine, tomato and pepper plants naturally infected with stolbur[5]. The tomato and pepper plants were sent by inspectors at the Plovdiv Regional Food Safety Directorate (RFSD)[6] and the vines by their counterparts at the Sliven RFSD. Affiliation to the Stolbur group (16Sr XII - Stolbur group) was determined by PCR, and Real Time PCR was used for verification. We put some of the plant materials in storage in refrigerators at -80°C for future research, and we stored the rest until the final release of the results.

1 When implementing the projects NIS 21/2016 and NIS-B-1070 of the Forestry University.

2 Provided by Jacques Gillet, LNPV, Colmar, France.

3 Identified by I. Ivanova, Entomology Department, CPQL and confirmed by Prof. R. Tomov of the Forestry University.

4 Kindly provided by Dr. Xavier Foissac.

METHODS

The methods we used were aimed at establishing the presence in Bulgaria of phytoplasmas causing the sudden death of lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula X intermedia*) in industrial plantations. We used those methods to survey industrial lavender plantations, thanks to Project НИС 21/2016 of the Forestry University titled: "Diseases of medicinal and aromatic plants cultivated in Bulgaria – types of pathogens and prevalence in Bulgaria." We collected samples of lavender, weeds and other hosts that are potential sources and reservoirs of infection. We also captured cicadas, phytoplasma carriers, adult *H. obsoletus*, *Reptalus* spp., *Fieberiella florii*, *Lepyronia* spp. Individuals, or other cicadas collected as part of Project NIS-B-1070/16.03.2020 titled: "Assessment of the impact of foreign insect species and the risk of their impact in agrocenoses in Bulgaria." We collected samples of vectors while performing surveys (May to late June) or by applying yellow sticky traps (early June to late August).

Morphological diagnosis. In the course of visual diagnostics, we examined lavender plants, weeds and other vegetation that, according to literature data, is host to phytoplasmas causing stolbur in agricultural crops. We compared the signs we observed with literature sources.

Molecular testing.

We ran the tests in Phytopathology Laboratory 19 of the Forestry University and in the Central Laboratory for Plant Quarantine (CLPQ) of the Bulgarian Food Safety Agency. PCR testing was performed according to the Kary Mullis method (1989).

Preparation of tested samples of plant parts and vectors

Leaves and leaf stalks. We processed the leaves by the Doyle & Doyle method (1990). We used a

scalpel to remove the tissue between the veins of 5 to 10 leaves with stalks from each sample. We weighed 1.2 g on an analytical balance and then placed the amount in a plastic bag and ground it with a 3% CTAB buffer (Daire et al., 1997). We collected the weed samples from lavender plantations near the villages of Partizanin and Zetyovo (Chirpan), Kozloduytsi (Dobrich), Hasarya, Sopot, Karlovo and Plovdiv. To determine the presence of phytoplasma infection in the weeds, we tested bindweed stems, roots, leaf stalks and leaf veins (*Convolvulus arvensis* L.) by the Doyle & Doyle method (1990). Cicadas were captured using yellow sticky traps of a specific wavelength (580-600 nm), i.e. fluorescent yellow in the visible spectrum (SZz, SCALOMON, Hungary), a colour that attracts insects. The captured insects were submitted to the CPQL between 2016 and 2017. After determining the species of cicadas, specimens of *H.obsoletus*, *Repthalus* spp., *Fieberiella florii*, *Lepyronia* spp., we tested for viruliferous for Stolbur phytoplasmas by PCR. For total DNA extraction, we placed each insect in a 1.5 ml Eppendorf tube and ground it with 500 µl CTAB buffer heated to 60°C. **Isolation methods.** We used three different methods to isolate total DNA depending on the material being tested and the assumed phytoplasma concentration. When isolating DNA from the bodies of cicadas and weeds, we used the Doyle & Doyle method (1990), adapted by Marzachì (1998). In routine plant sample tests, we used the CTAB method of Boudon-Padieu et al. (2003), which is faster. This method was also used to isolate nucleic acids from vinca, but at lower concentrations of Tris and CTAB (2%) (Kollar & Seemüller, 1989). In this study, we adopted the use of a 3% CTAB concentration in 1M Tris buffer (Boudon-Padieu et al., 2003), which is suitable for vines and other woody plants containing large amounts of phenols, tannins and acids.

Molecular methods for phytoplasma identification. We used a variety of PCR techniques depending on whether we were looking for a group-wide sequence or a subgroup-specific sequence in conserved regions, such as 16S and 23S rDNA. We also used universal primers. (Raplay, 2000).

RFLP (Restriction Fragment Length Polymorphism) **analysis**. The method essentially proves the polymorphism in the sequences of amplified products. After restricting the amplified DNA with endonucleases (*Alu*I, *Rsa*I, *Trul*I), we obtained fragments of different lengths, which, after visualisation in agarose or polyacrylamide gel electrophoresis, formed strain- and species-specific restriction profiles (Gerstein, 2001). We used molecular methods to identify phytoplasmas causing vine yellows (BN = stolbur = Lavender decline, according to OEPP/EPPO (2007).

PCR method with universal primers, followed by nested group-specific PCR (Lee et al., 1993). We used two pairs of universal primers to identify lavender phytoplasmas by 16S rDNA analysis. The first pair of primers was P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995). For group identification of phytoplasmas from the stolbur group, we used fU5 and rU3 (Lorenz et al., 1995).

Electrophoresis and visualisation. We used 1.5% agarose gel in TAE 0.5x. electrolyte buffer at 110V for 20 minutes. After 30 minutes of incubating the DNA products in the agarose gel in ethidium bromide solution (1 µg/1 ml), we visualised them using UV light. We used a DNA marker: Sigma's 1Kb Ladder Eurogentech or 10Kb DirectLoad Wide Range.

Distinction of stolbur phytoplasmas. We used the restriction analysis technique (RFLP) with the *Alu*I or *Rsa*I enzymes on all positive samples and controls. We separated part of the amplicon in tubes (15µl) and added restriction enzyme (2µl). We incubated at 37°C for 4 hours for *Alu*I (Amersham Biosciences) and 3 hours at 37°C for *Rsa*I (*Afa* I, Amersham Biosciences). We used the *Taq*I enzyme in RFLP analysis of 16r758f/M23Sr for subgroup affiliation (in the 16SrV group), and we performed the visualisation after electrophoresis in 10% polyacrylamide gel incubated in ethidium bromide (Angelini et al., 2001).

Real Time PCR method. We followed a Real-Time PCR protocol to detect the sudden death of lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula* X *intermedia*) using universal primers to amplify phytoplasma 23S rDNA (Hodgetts et al., 2010). For confirmation and direct detection of Stolbur infection the qPCR method of Hren et al. (2007) was performed. The results were recorded

using the MxPro (Statagene) software designed to detect fluorescent signals during the decay of the fluorescent probe at the limit of detection (*Ct value*). For this calculation, the limit value (Threshold (dRn) = 0.0695dR) was automatically set and the fluorescent values of the different amplicons were calculated for each well.

RESULTS AND DISCUSSION

2016 RESULTS

A total of 87 plant samples were subjected to laboratory analysis. Out of those, 24 lavender samples were tested by humidity chamber, nutrient medium and microscopy methods for mycological analysis purposes. The aim was to morphologically differentiate between fungal and phytoplasma diseases (Fig. 1).

Infection with a fungal pathogen of the *Septoria* genus was detected in three of the samples. The pathogen was identified as *Septoria lavandulae* Desm (Fig. 2).

Figure 1. Spots on lavender leaves from Chirpan region (*Pict. Zh. Avramov*)

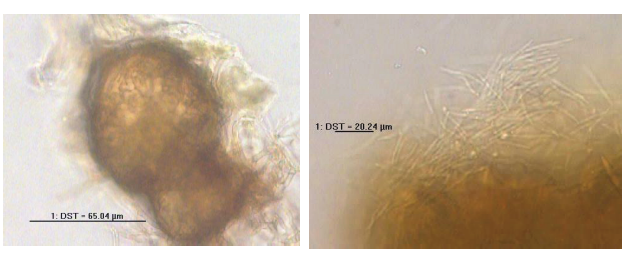


Figure 2. Picnidium and picnidiospores of *Septoria (Pict. Zh. Avramov)*

When re-examining the stems of infected plants, we found pseudothecia (fruiting bodies) with asci and ascospores (Fig. 3).

We focused our attention on those plants because we could see they had suppressed growth and healthy and diseased (dead) branches.

A fungal pathogen belonging to the *Phoma* genus was identified in three lavender plant samples from an area near Kazanlak with symptoms of wilt and tuft reduction. (Fig. 4).

After laboratory examination, the *Phoma lavandulae* Cabot species was identified by morphological features (Fig. 5).

Figure 3. Ascus with ascospores on a microscope magnification of x40 (*Pict. Zh. Avramov*)

Figure 4. Symptoms of lavender plants infected with *Phoma lavanduleae* (*Pict. Zh. Avramov*)

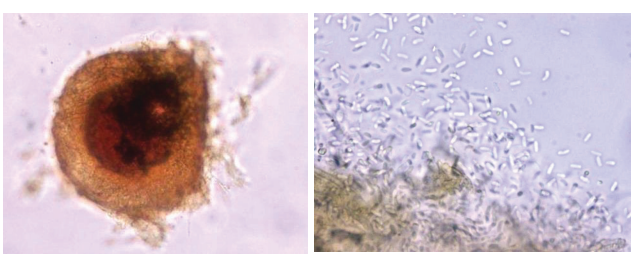


Figure 5. Pycnidia and pycnidiospores of *Phoma lavandulae* Cabot (*Pict. Zh. Avramov*)

Molecular analysis results

After laboratory identification of phytoplasma plant pathogens by PCR using universal primers P1/P7 and subsequent U3/U5 testing, 42 plant, 10 cicada and 11 weed samples were tested. A phytoplasma infection was found in a sample of lavender from an area near the village of Partizanin, Chirpan (Fig. 6).

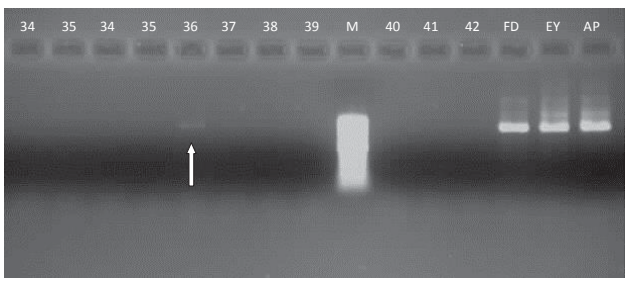


Figure 6. PCR positive result - line 36; M – marker (1 kb DNA Ladder, Sigma); FD – positive FD control; EY – positive Elm yellows ufntrol; AP – positive Apple proliferation control (universal primers U3 / U5, Lorenz et al., 1995)

No such phytoplasma infection was detected in the cicada and weed samples (Fig. 7).

To differentiate and determine the group affiliation of the results, sample 36 was subjected to RLFP analysis with restriction enzymes (Fig. 8).

The results of the RLFP analysis showed the same profiles and that the phytoplasma infection detected (line 7) belonged to the Stolbur group (line St).

This gave us a reason to continue the identification by performing real-time PCR using universal primers to confirm the nature of the causative agent, specifically whether it is phytoplasma. We selected a set of samples with a negative PCR result, controls and Sample 36 originating from the area around Chirpan (Table 1).

The results of qPCR confirmed the infection initially detected in Sample 36 (Fig. 9) from an area near Chripan (Partizanin Village).

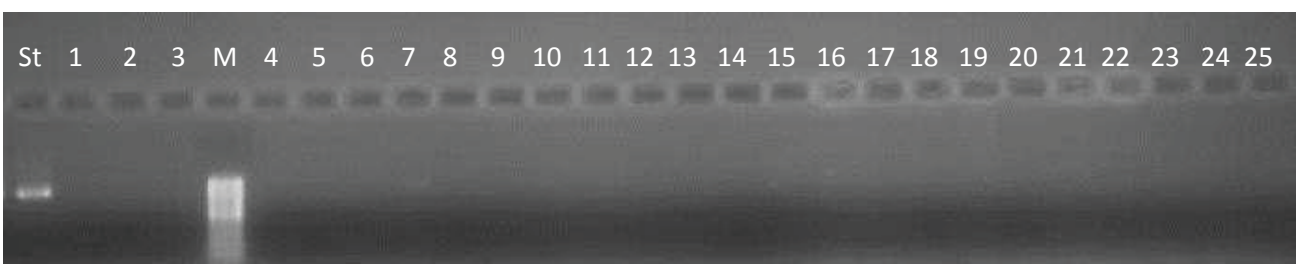


Figure 7. PCR negative results in numbered lines; St – positive Stolbur control; M – marcker (1 kb DNA Ladder, Sigma); (primers U3 / U5, Lorenz et al., 1995)

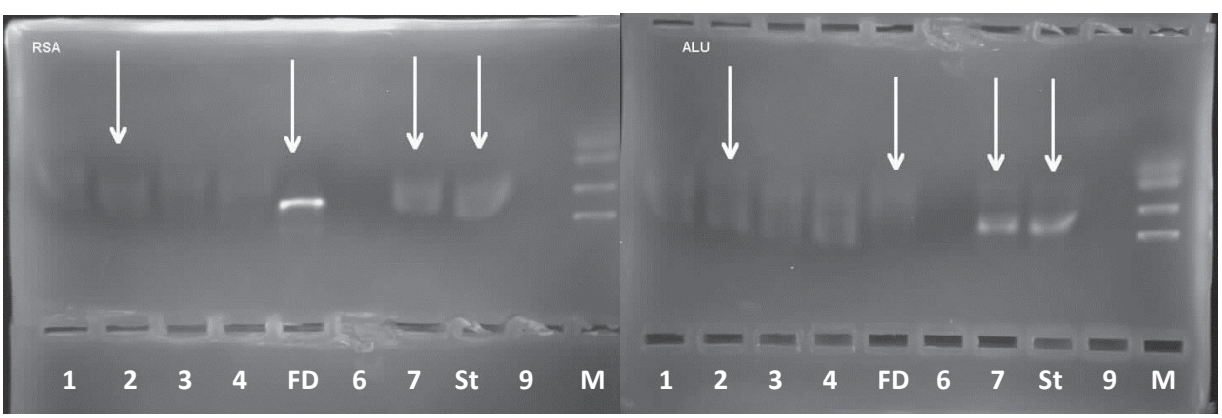
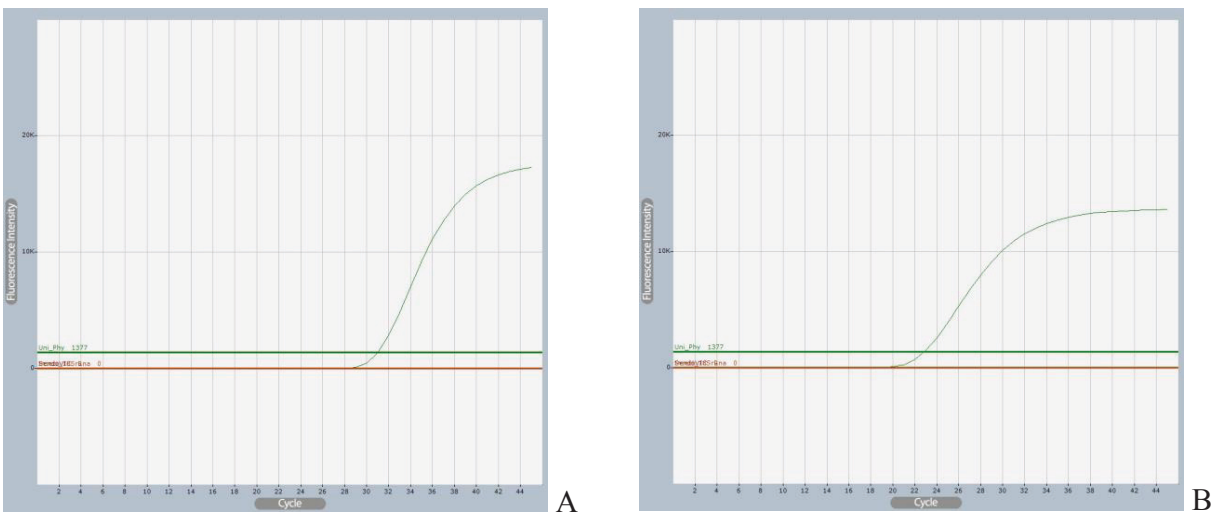


Figure 8. Results of RLFP assay with R*SA* (A) and A*lu* I (B). Line 1 - Negative control (K-); 2 - *H.obsoletus*; 3 - *Conv. Arvense*; 4 - *Lepironia* spp.; FD - ppositive control for FD; 6 - K-; 7 - sample 36; St - control for Stolbur phytoplasma; 9 - mix; M - marker (500 bp, Sigma)

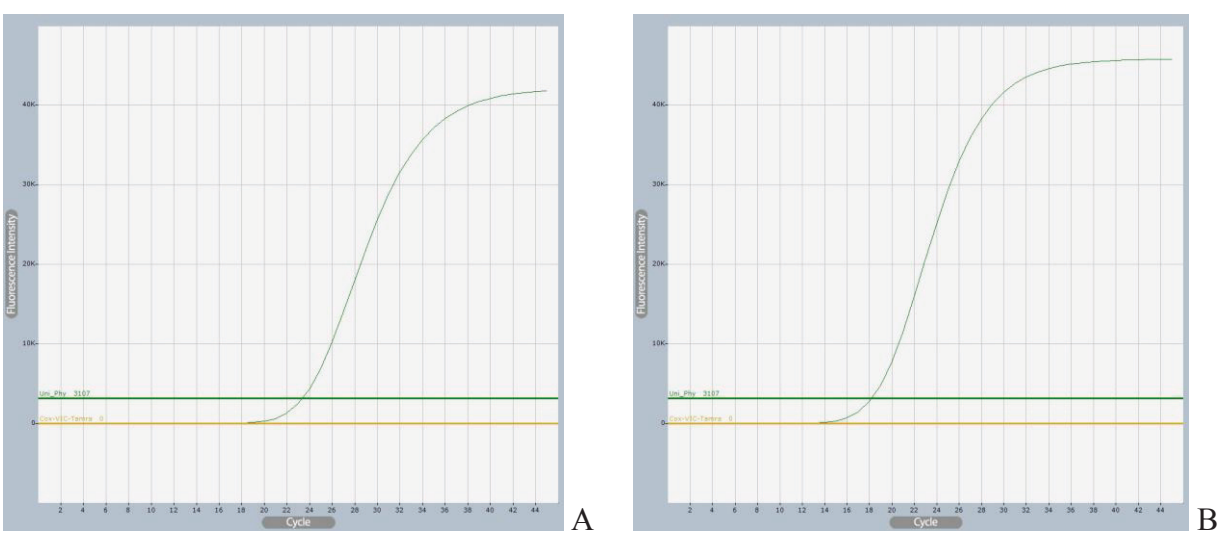
Table. 1. Real Time PCR results in 2016

Positive controls		Plant samples		Insect samples	
	qPCR		qPCR		qPCR
L22 BG- AP	+	A13- LavenderSpasovo	-	M22- Phylaenus spulmarius	+
L23 BG- ESFY	+	A14- LavenderSpasovo	-	M23- Lepitonia	-
12 PD+	+	A15- LavenderSpasovo	-	M21- Seelanos	-
15 FD+70	+	A16- LavenderSpasovo	-	A22- Cicadella Lavender	-
C5- cicada	+	A17- LavenderSpasovo	-	Partini	-
		A28- LavenderPartizani	-	A23- CicadellaWoods	-
		A29- LavenderPartizani A30-	-	D91803- Reptalus	-
		LavenderPartizani	-	O8- Reptalus	-
		A36- LavenderPartizani	+	O6- Reptalus	-
		10ST- Grapevine BN?	+	G13- Reptalus	-
		16ST- Grapevine BN?	+	G14- Phylaenus spulmarius	-
		24ST- Vinca Stolbur+	+	G15- Phylaenus spulmarius	-
		D12- bindweed	-	G16- Phylaenus spulmarius	-



A B

Figure 9. Results of qPCR (Hodges, 2009): A – lavender plant sample 36; B – Positive Stolbur control



A B

Figure 10. Results of qPCR (Hodges, 2009): A – insect sample M22; B – Positive Stolbur control.

In the course of the laboratory examination, a new phytoplasma infection was found in another sample of cicada (*Phylaenus spulmarius,* sample M22) captured near Spasovo Village, Chirpan (Fig. 10).

Discussion of 2016 Results

The results mean we can confirm that the identified infections by fungal pathogens of the *Septoria* and *Phoma* genera produce similar symptoms of wilt and decline in lavender plants. It is quite possible to describe those mixed infection symptoms as sudden wilting caused by *Candidatus* Phytoplasma solani. For the first time, a phytoplasma infection belonging to the Stolbur group (16Sr XII Stolbur group) was identified in Bulgaria. The possibility of transmitting this infection through cicada vectors has been proven. The *Phylaenus spulmarius* species had not been reported in Bulgaria as a carrier of phytoplasma infection.

The molecular methods used to identify phytoplasmas in vines (EPRO Protocols) are suitable to identify the phytoplasma pathogen of sudden wilting in lavender. The use of protocols for total DNA extraction in lavender, which we followed in 2016 (CTAB), needs to be accompanied by protocols for the purification of DNA material to avoid rapid inactivation.

2017 RESULTS

After laboratory examination, 10 plant, 22 cicada and 5 weed samples were subjected to PCR testing to identify phytoplasma pathogens in plants and detect already established pathogens. We found phytoplasma infection on samples of lavender, bindweed and *Lepironia coleoptrata* originating from the village of Zhitnitsa near Dobrich (Fig. 11). The infection was confirmed by performing qPCR.

Discussion of 2017 Results

We found an infection manifesting as sudden wilt in lavender and caused by the *Candidatus* Phytoplasma solani phytoplasma near Dobrich.

We failed to confirm the 2016 infection found near the village of Partizanin. During the survey, we found the plantation had been irreparably damaged due to decline and wilting, probably as a result of extremely low temperatures. This fact supports and confirms that phytoplasmas suppress normal plant aging and makes plants unable to withstand low temperatures, as a result of which lavender plants die (Fig.12).

An infection was found in bindweed, which proves its importance as a reservoir in the spread of Sudden Decline of Lavender caused by phytoplasma from the Stolbur group.

In the second year of the study, the *Lepironia coleoptrata* cicada was shown to be the phytoplasma vector.

The PCR and qPCR methods used to identify the Sudden Decline of Lavender are reliable, but their simultaneous use is necessary to produce a complementary effect that rules out any errors from false negative and positive results.

The study results obtained over two years provided us with sufficient evidence for the existence of phytoplasma infection in lavender. Having assessed this potential risk to lavender production, we prepared a brochure to inform our farmers of the dangers of the spread of the disease known as SUDDEN DECLINE OF LAVENDER.

CONCLUSIONS

1. For the first time in Bulgaria, a phytoplasma infection belonging to the Stolbur group (16Sr XII Stolbur group) was identified in lavender.

2. We found an infection manifesting as sudden decline in lavender and caused by phytoplasma near Dobrich and Chirpan.

3. An infection was shown in bindweed, which confirms its importance as a reservoir in the spread of the phytoplasma of the Stolbur group, causing the sudden decline of lavender.

4. The possibility of transmitting this infection by cicada vectors has been proven. The *Phylaenus spulmarius* and *Lepironia coleoptrata* species are carriers of phytoplasma infection.

5. When used concurrently, the PCR and qPCR methods used to identify the Sudden Decline of Lavender are fast, accurate and reliable in detecting and identifying phytoplasma pathogens.

6. Fungal pathogens of the *Septoria* and *Phoma* genera produce similar symptoms of wilt and death in lavender plants. It is quite possible to describe those mixed infection symptoms as sudden decline caused by the *Candidatus* Phytoplasma solani phytoplasma.

Given the potential risk to lavender production and the need to prevent the spread of Sudden de-

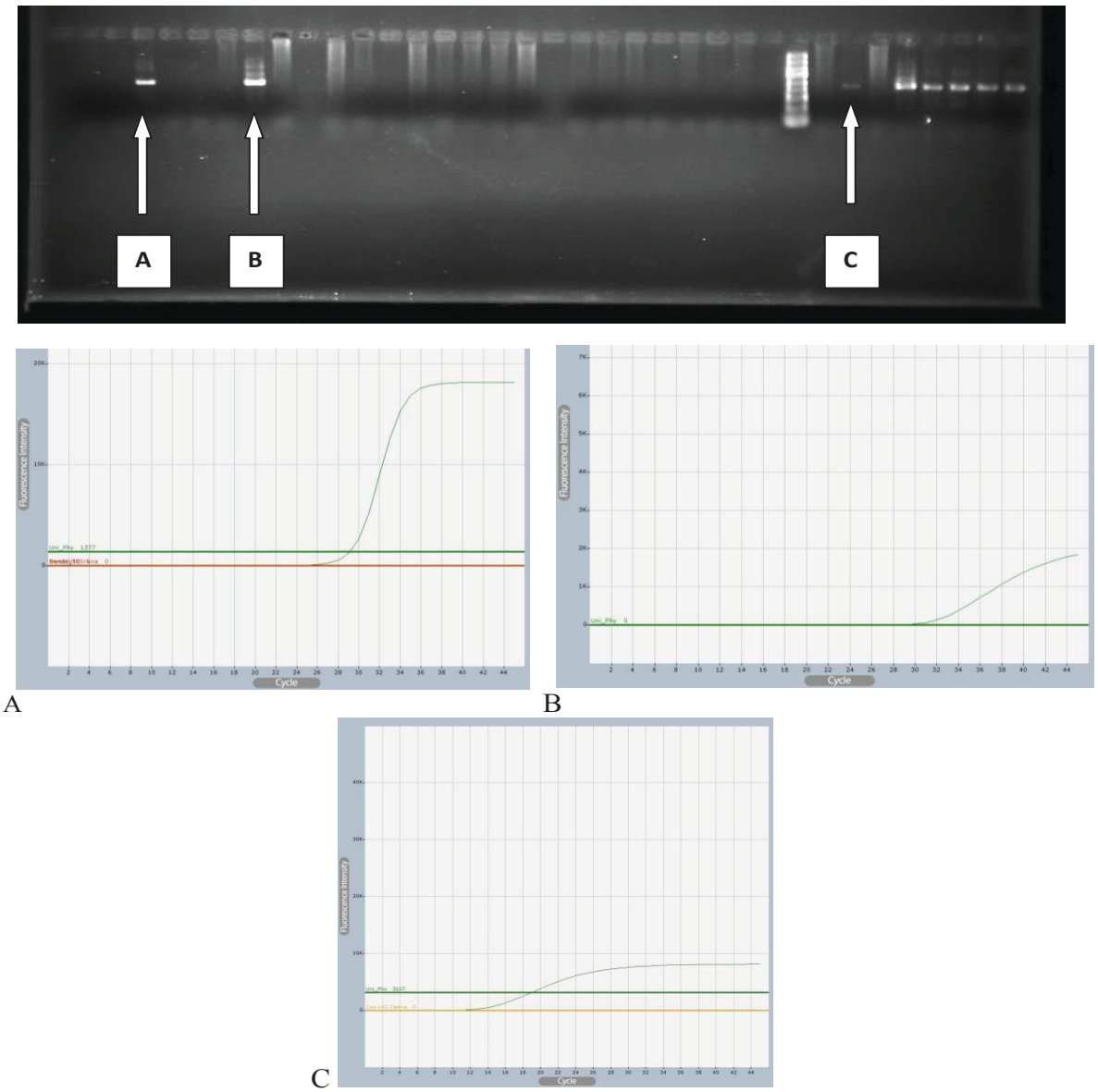


Figure 11. Results of PCR and qPCR: A - *Lepironia coleoptrata;* B – bugweed; C – lavender (Hren et al., 2007)

Figure 12. The 2016 infection found near the village of Partizanin and eradication of lavender plants in 2017 (*Pict. Zh. Avramov*)

cline of lavender, this work must continue and all stakeholders have to be fully informed.

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