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FIRST DETECTION OF 'CANDIDATUS PHYTOPLASMA AUSTRALASIA' IN ROBINIA PSEUDOACACIA: MOLECULAR CHARACTERIZATION AND TISSUE-SPECIFIC DISTRIBUTION

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Abstract: *Candidatus* phytoplasma affects a wide range of host plants. In recent years, the severity and distribution of the disease have increase many fold. The present study isolated the pathogen from leaves (chlorosis), dried branches, and the other tissues of the infected tree. Acacia trees (n=15) showing severe yellowing and desiccation in the landscape areas of Şanlıurfa province were sampled from the buds (V-shaped) in three different parts of the branches, backward from the top of the branches during the flowering period, and from the middle veins of the leaves on the same branch during the leafing period. Samples were initially amplified using the universal primer pair R16F1/R16R0 and subsequently subjected to nested PCR with the primer pair R16F2n/R2. The results indicated that, on average, 2 out of 15 buds collected from the apical parts of the branches, 11 out of 15 from the middle, and 6 out of 15 from the basal parts were infected with 'Candidatus Phytoplasma australasia'. Moreover, all samples collected from the midribs of these 15 trees were found to be infected with Ca. P. australasia.

Keywords: *Acacia,* Phytoplasma, Virtual RFLP, Yellowing

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1. Introduction

North America is the homeland of the *Acacia* (*Robinia pseudoacacia* L.) tree. The genus *Robinia,* belonging to the Fabaceae (Baliates) family, has 843 species, and these species have been naturalized in 154 different regions around the world (GloNAF database; van Kleunen et al., 2015; Wojda et al., 2015). Acacia trees can live in various climatic conditions. Acacia trees produce essential oils and alkaloids; therefore, they have been used in different industries such as paint, adhesives, fragrance, syrup, cologne, confectionery, and pharmacy (Luis et al., 2012). It has become one of the preferred trees because of cultivation under adverse conditions. They have been preferred in landscaping areas in Turkey for some years. However, severe yellowing and drying of branches in acacia trees have been observed in recent years. Preliminary studies were conducted on the etiology of the disease (Korkmazoğlu, 2018). However, in the study conducted by Korkmazoğlu (2018), although laboratory analyses such as PCR and RFLP were performed to identify the pathogen, subgroup determination could not be completed due to the absence of precise DNA sequencing data. Nevertheless, based on the increasing severity of pathological symptoms and their spread among trees, initial laboratory findings confirmed that the causal agent was phytoplasma. For phytoplasma detection, various plant tissues such as the leaf midrib, petiole, young shoots, and floral tissues are commonly used. The aim of this study is to evaluate the effectiveness of pathogen detection in leaves and buds collected from young, intermediate, and old shoots of the tree, and to examine the year-based distribution of phytoplasma within the plant.

2. Materials and Methods

2.1. Collection of samples

A total of 15 symptomatic trees were sampled through a guided sampling approach. Sampling was performed on three different parts (top, middle, and bottom) of a branch of each tree during the leafless period (January-February). The period also corresponded to the budding period. Trees that were sampled had shown severe symptoms of the characteristic phytoplasma disease in the previous year. The province (Sanliurfa), location, date, characteristics, symptoms, and plant parts were recorded and labeled during sampling. In the PCR analysis, a positive control derived from a cactus plant, which produced a 1,400 bp fragment specific for phytoplasma identification, and a negative control (ddH_2O) were used (Ayvacı et al., 2022).

2.2. DNA Extraction

DNA isolation was performed from buds and leaves of

healthy and infected acacia trees according to the total DNA extraction method prepared by Ahrens and Seemüller (1992). Two or three bud samples were taken from three different parts (upper, middle, and lower) of a branch (n=15) of each tree during the budding period, and the samples were used for DNA extraction. A 1 g sample was homogenized using CTAB buffer (2% w/v cetyltrimethylammonium bromide, 1.4 mol L^{-1} NaCl, 0.2 %2-β-mercaptoethanol, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} Tris-HCl, 2% polyvinyl pyrrolydone, pH 8.0) and incubated for 30 min at 65°C. After incubation, the samples were centrifuged at 13,000 *g* for 10 min to remove plant tissue residues, and chloroform-isomyl alcohol (24:1) was added to the resulting supernatant to precipitate the residues (2 times). Then, isopropanol (-20°C) was added to the obtained supernatant, and the samples were incubated at -20°C for 1 h. At the end of the incubation period, nucleic acids were precipitated by centrifugation at 13,000 *g* for 15 min, washed with 70% ethanol, and dried at room temperature. Then, 100 µl of Tris-EDTA (1X) buffer was added to the obtained DNA samples and stored at -80 °C until use.

2.3. Determination of phytoplasmas by PCR

16S rRNA-based PCR analysis was used to identify phytoplasmas. Total DNA obtained from extraction was used for sequencing after two-step PCR amplification. The first of these steps is the direct-PCR step and the other is the nested-PCR step, while the R16F1/R16R0 (Davis and Lee, 1993; Lee et al, 1994) universal primer pair giving 1400 bp product was used in the direct-PCR step, and the R16F2n/R16R2 (Gundersen and Lee, 1996) primer pair giving 1250 bp product was used in the nested-PCR step.

Thermocycler conditions in the reproduction of direct-PCR products: initial denaturation (1 cycle) at 94°C for 3 min; denaturation at 94°C for 1 min; primer binding (35 cycles) at 50°C for 2 min; elongation at 72°C for 3 min; final elongation (1 cycle) at 72°C for 10 min. Thermocycle conditions for nested-PCR were as follows: initial denaturation (1 cycle) at 94°C, 3 minutes; denaturation at 94°C, 1 minute; primer binding (35 cycles) at 55°C, 2 minutes; elongation at 72°C, 3 minutes; final extension (1 cycle) at 72°C, 10 min. Then, 10 µl of the PCR amplification products was taken and electrophoresed at 80 V electric current for 80 min in 1 Tris-boric acid-EDTA (TBE) buffer (67 mmol L^{-1} Tris-HCl, 22 mmol L^{-1} borik asit, 10 mmol L^{-1} EDTA, pH 0.8) medium containing 1% agarose gel, using a 1-kb DNA marker (Fermentas, Life Science, Milan, Italy). Following electrophoresis, the gel was stained with ethidium bromide (EtBr, 10 mg mL^{-1}) and visualized in a UV transilluminator (Lemmetty et al., 2001).

2.4. Sequence and phylogenetic analysis

After the nested PCR process, the pathogenic agent was related to phytoplasma. The nested PCR products were then sequenced in both directions with primers 16F2/R16R2 (Sequencing – SeqScape® Software v2.7-MedSanTek Laboratory Materials Industry. and Tic. Ltd. Sti. Istanbul, Türkiye). Both sequences obtained were combined into a single sequence with the complete sequence (1.25 kbp). The sequences were compared with representatives of 43 phytoplasma groups using the neighbor-joining method with 1000 bootstrap replicates in MEGA X software, and the phylogenetic analysis was subsequently completed (www.megasoftware.net).Two representative isolates were selected and recorded in GenBank under the accession numbers ON062086.1 (Topakasya-1) and ON062085.1 (Topakasya-2).

Table 1. Primer sequences used in direct and nested PCR

R16F1	5'-AAGACGAGGATAACAGTTGG-3'
R16R0	5'- GGATACCTTGTTACGACTTAACCCC-3'
R16F2n	5'-GAAACGACTGCTAAGACTGG-3'
R16R2	5'-TGACGGGCGGTGTGTACAAACCCCG-3'

2.5. Virtual RFLP

Sequencing of the ribosomal phytoplasma group detected in the infected samples was virtually analyzed using *AluI, BamHI, BfaI, BstUI, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI, MseI, RsaI, SspI, HhaI*, and *TaqI* enzymes via *i*PhyClassifier software (Zhao et al., 2009; https://plantpathology.ba.ars.usda.gov).

3. Results and Discussion

It was previously observed that ball acacia trees show yellowing and drying symptoms in leaves and branches (Korkmazoğlu, 2018). However, the pathogenic agent was not identified. To determine if the pathological agent was *Ca*. pytoplasma, the diagnostic process was performed via PCR based on 16S rRNA. Since Koch's postulate is insufficient for diagnosing phytoplasmas (Dermastia et al., 2017), conducting molecular studies on rRNA is imperative. Because phytoplasma infection has a very complex structure, isolation is not always reproducible, as with fungal and bacterial agents. DNA isolation of samples from the leaf midrib and buds in the branches (top, middle, and bottom) during the budding of 15 trees with severe yellowing and drying. Previously marked symptoms gave positive results for phytoplasma (Figs. 1,2,3, and 4). During the budding period, we examined the top, middle, and bottom buds of branches taken from 15 trees (Fig. 6). We obtained positive results from 2/15 top buds, 11/5 middle buds, and 6/15 bottom buds of branches taken from 15 trees. During the vegetative period (leafy period), all leaf midribs collected from the 15 healthy trees gave positive results (Fig. 4 and 7). The trees' characteristics, symptoms, and status were visible from a distance (Figs. 5, 6 and 7).

Based on molecular tests, we determined that the pathological agent causing severe yellowing and drying in acacia trees belonged to the phytoplasma group *Ca.* Phytoplasma australasia'. The presence of this pathogen in ball acacia trees was made for the first time with the study in the world.

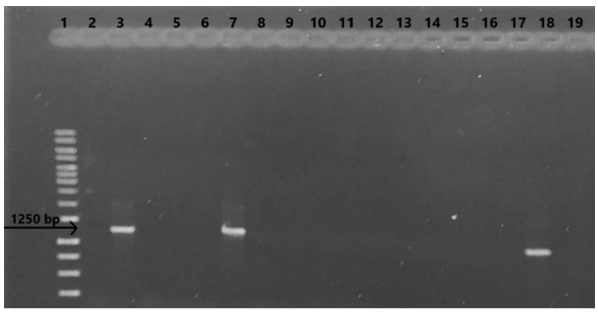

Figure 1. Image of DNA gel electrophoresis of phytoplasmas isolated from top buds of branches from ball acacia trees after nested-PCR amplification. Columns 2–16 represent infected trees; columns 17 represent healthy acacia and columns 18 represent positive controls (a cactus plant that produces 1250 bp specifically for phytoplasma characterization), and columns 19 represent negative control (ddH_2O) (1% agarose gel).

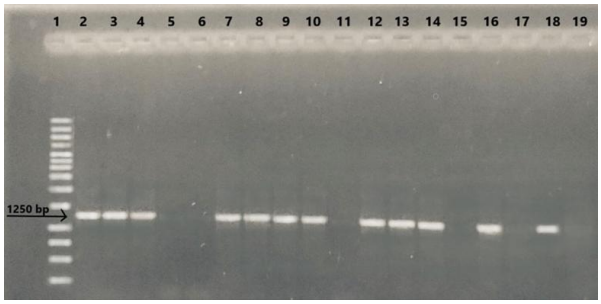

Figure 2. Image of DNA gel electrophoresis of phytoplasmas isolated from middle buds of branches from ball acacia trees after nested-PCR amplification. Columns 2–16 represent infected trees, columns 17 represent healthy acacia, columns 18 represent positive control (a cactus plant that produces 1250 bp specifically for phytoplasma characterization), and columns 19 represent negative control (ddH_2O) (1% agarose gel).

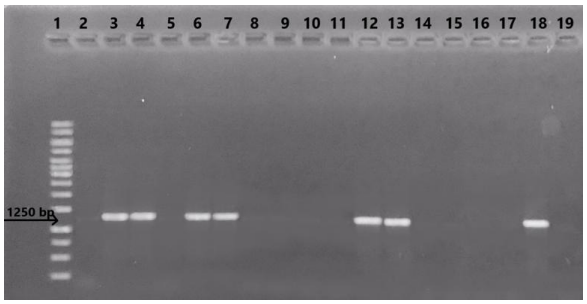

Figure 3. DNA gel electrophoresis of phytoplasmas isolated from bottom buds of branches from ball acacia trees after nested-PCR amplification. Columns 2–16 represent infected trees, columns 17 represent healthy acacia, and columns 18 represent positive control (a cactus plant that produces 1250 bp specifically for phytoplasma characterization), and columns 19 represent negative control (ddH_2O) (1% agarose gel).

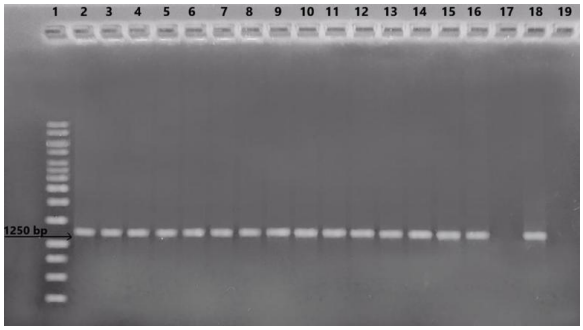

Figure 4. Image of DNA gel electrophoresis of phytoplasmas isolated from leaf midribs from ball acacia trees after nested-PCR amplification. Columns 2–16 represent infected trees, columns 17 represent healthy acacia, while columns 18 represent positive control (a cactus plant that produces 1250 bp specifically for phytoplasma characterization), and columns 19 represent negative control (ddH_2O) (1% agarose gel).

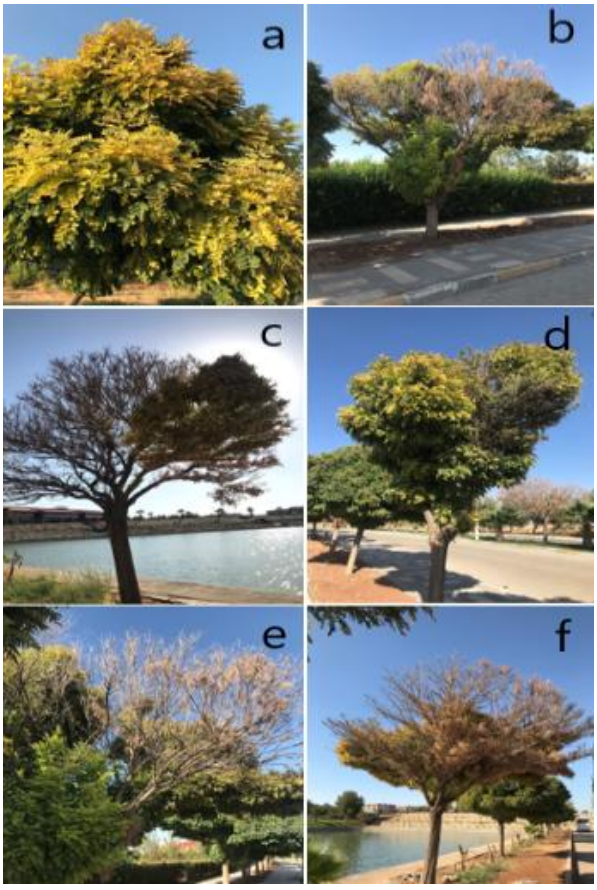

Figure. 5. Symptomatic of ball acacia (*Robinia pseudoacacia* L.) trees infected with *Ca.* Phytoplasma australasia' a) ball acacia showing signs of severe yellowing, b, and c) Ball acacia trees showing signs of severe drying, d) trees showing signs of yellowing and drying, e) partial trees showing drying, and f) trees showing severe drying.

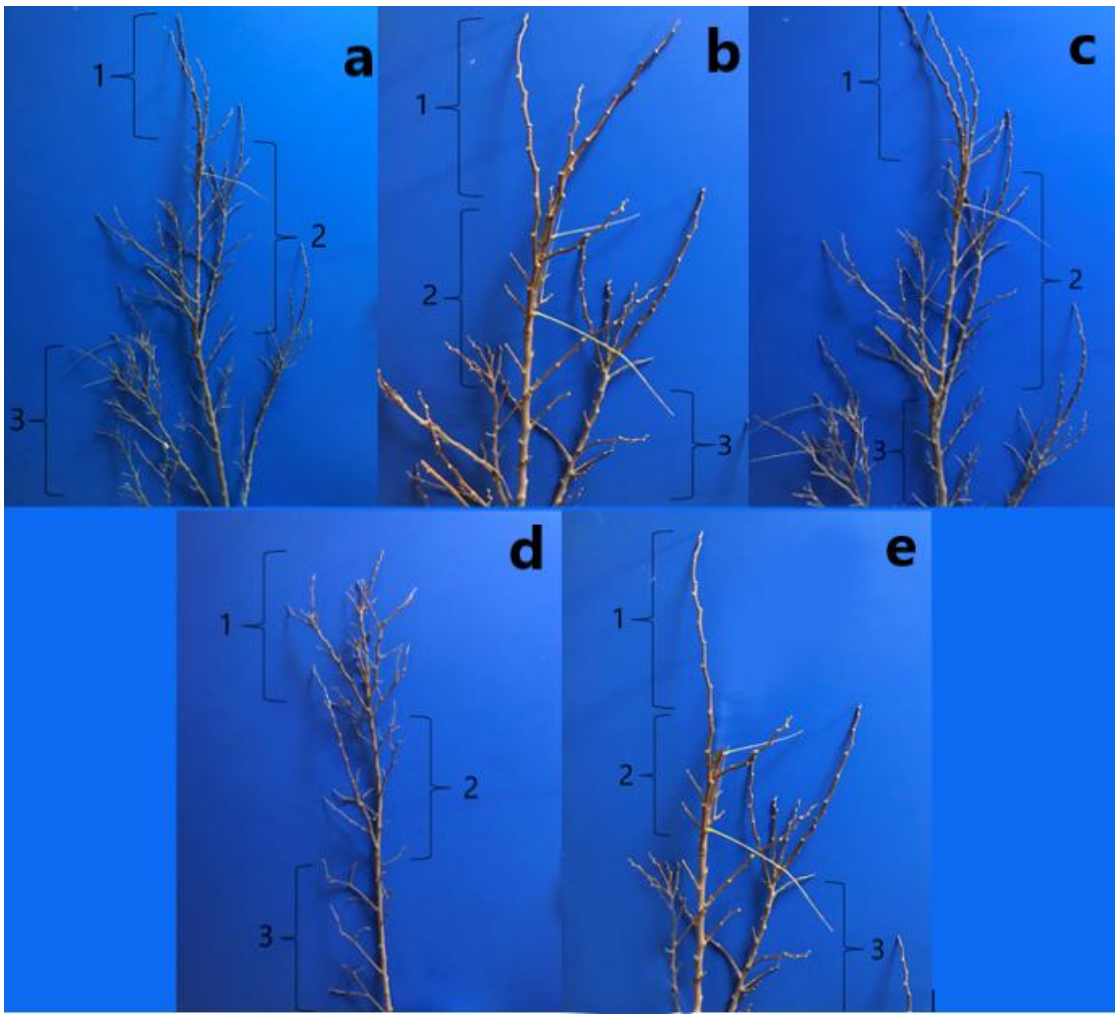

Figure 6. Branch samples taken from five ball acacia trees (a, b, c, d, and e) during the budding period were divided into 3 parts (1=top, 2=middle, 3=bottom). Two or three buds were sampled from each part of the branch.

Figure 7. Yellowing symptoms on leaves of different ball acacia trees (a, b, c, and d).

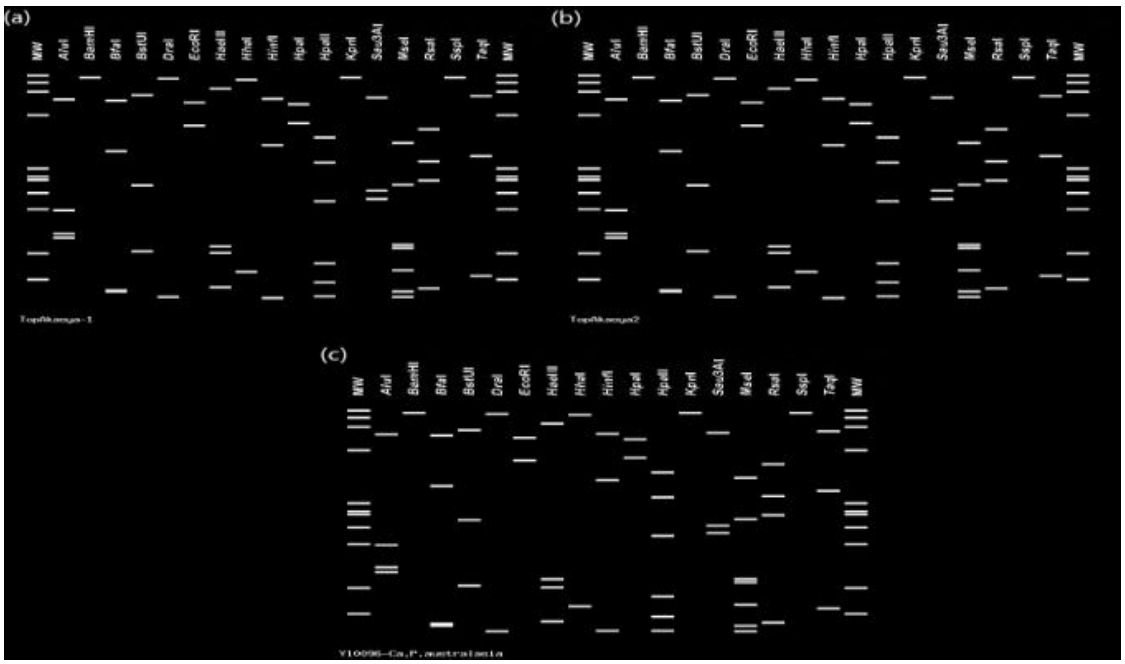

Figure 8. Sequences of phytoplasma-infected leaf and bud isolates were determined by virtual RFLP using the *i*PhyClassifier software. (a) Ball Acacia-1 (leaf), (b) Ball Acacia-2 (bud), and (c) '*Ca.* Phytoplasma australasia (Y10096).

4. Discussion

Ca. Phytoplasma australasiae is widely found in grapevine vineyards and fruit trees globally, causing significant agricultural damage in various countries (Bertaccini et al., 2014). In Turkey, this pathogen also spreads in plants such as tomatoes, lucerne (alfalfa), and Pyracantha angustifolia, with different strains contributing to diseases and economic losses across the country (Usta et al., 2023; Ayvaci et al., 2020; Kilic et al., 2022).

Acacia trees infected with phytoplasma have recently been reported in other countries. For example, acacia (*Robinia pseudoacacia*) trees showing witches' broom and dieback symptoms in Iran have been reported to be infected with the 16SrII phytoplasma (D subgroup) group (Esmaeilzadeh-Hosseini et al., 2019). In another study, it has been reported that acacia (*Acacia mangium*) trees showing yellowing symptoms in the northern region of India are infected with 16SrII (subgroup C) phytoplasma (Rao et al., 2020).

In our study, we determined that the agent was '*Ca.* Phytoplasma australasia'. In addition, we stated that the most healthy sampling could be made from the leaf midrib to isolate the pathogen. However, we could state that a sufficient amount of pathogen was present for isolation and diagnosis during the budding period of the trees. During the budding period, we determined that middle buds should be preferred for sampling to detect the pathological agent. In this study, it was noticed that the agent presence of the pathogen in the upper shoots was very low compared with that in other parts. One of the main reasons for this could be attributed to the high protein activity in the top buds of the branches and the host protein synthesis. High metabolic activities through protein synthesis could inhibit DNA replication of the pathogen (Eisenreich et al., 2019). Even if high metabolic processes in top buds did not directly inhibit or slow down pathogen propagation, they could increase the defense barrier and mechanics and result in the accumulation of phenolic substances. These jasmonic or salicylic acid contents could reduce the complication of the pathogen population within the plant system (Ayvacı et al., 2022).

There are quite a few publications on protein density inactivating pathogen growth and causing lysis on the pathogen's cell wall and DNA molecule, making it difficult to detect the pathogen because of the fragmentation of the DNA molecule (Musidlak et al., 2017). Plants generally have various mechanisms against pathogen attacks. Plant proteins synthesized inside the cell are important in suppressing viruses and bacteria. Pathogen-related (PR) proteins are activated when the pathogen enters the cell. Ribosome-inactivating proteins (RIPs) inactivate pathogens by binding to RNA molecules of the virus or phytoplasm. The agent's presence in the buds in the lower part of the branches was approximately 50% lower than in the buds in the middle part. Therefore, we concluded that the obligate pathogen might have lost its viability as the tissues began to undergo necrosis, and therefore, success in the isolation was low. For example, considering that the defense mechanism of asymptomatic plants is impaired because of abiotic and biotic stress, it has been observed that pathogens in the latent position cause symptoms in the host tissue (Bez et al., 2021). We could also conclude that the translocation of pathogen propagules within the phloem system depends on seasonal precipitation and temperature.

Jiang et al. (2004) stated that the mulberry dwarf (MD) phytoplasma varied in various organs of trees. MD

phytoplasma was found in the roots of infected trees from winter to spring, but it could not be detected in the winter buds of the trees. They also detected MD in flowers, fruits, and seed pods, but irregular organ distribution has been reported. Such an uneven distribution is also observed in fruit and forest trees. For example, an irregular distribution was observed in trees infected with walnut witches' broom phytoplasma (Chen et al., 1992) or paulownia witches' broom phytoplasma (Sahashi et al., 1995). They stated that winter buds are an important place for MD phytoplasma to spend the winter (Jiang et al., 2004). In our study, rather than the tendency of phytoplasma to proliferate in buds, we confirmed that the phytoplasma was transferred to buds from the previous year. Generally, phytoplasma infection affects buds, leaves, roots, stems, seeds, and fruits. It has been reported that the disease causes premature buds, fruit deformation, and early ripening (Maust et al., 2003). Maust et al. (2003) and Marcone et al. (2014) stated that physiological, biochemical, and molecular changes in roots and leaves occur before visible symptoms, and these might affect the pathogen distribution within the plant system, affecting the development stages of plants and the environmental conditions.

Not only do protein synthesis and enzyme activities play significant roles in the concentration and distribution of pathogens within the plant system, but carbohydrate accumulation in leaves and roots might also have a significant impact on the pathogen population. Carbohydrate depletion within the plant organs due to abiotic, biotic, or both stress conditions could result in reduced plant defense responses and increased pathogen dissemination within the plants, leading to the onset of earlier symptom appearances if pathogens are already established from the previous years (Sood et al., 2021).

An important aspect of the isolation of phytoplasma from buds is that it allows the isolation and identification of latent pathogens during the dormant period of the disease (Brzin et al., 2003).

It is important to analyze the sucrose or glucose transfer mechanism in detail and provide sucrose transfer to the symptomatic areas to prevent phytoplasma diseases or at least reduce the severity of symptoms and slow down the course of the disease. However, the solution will not be as easy as envisioned. Energy metabolism accelerates with carbohydrate accumulation, and plants quickly consume nutritional elements with increased respiration (Xue et al., 2018). However, phytoplasmas stimulate chloroplast starch degradation by disrupting sugar metabolism (Xue et al., 2018). The initial accumulation of sugar in the phloem, leaves, buds, or other organs temporarily causes phytoplasmas' proliferation; however, phytoplasmas at later stages cause structural damage to the plant. Trees infected with phytoplasma are estimated to have increased respiration, thickened cell walls, and increased synthesis of some defense-related metabolites, for example, increased amounts of carbohydrates and free amino acids, which may provide energy under biotic conditions (Hou, 2012). Since genes regulate the increase in sucrose in plants or trees infected with phytoplasma, the increase in phytoplasma-based gene expression might increase sucrose synthesis. Because of constantly increasing gene expression, the sucrose level reaches a plateau level. Starch and sucrose are quickly broken down into fructose and glucose, which may obstruct phloem vascular bundles (Andre et al., 2005). This mechanism is accepted to explain why the sucrose and glucose contents of diseased plants increase significantly. However, intense sugar accumulation or development of parenchymatic tissues are not accepted for many reasons, including drying of the tissues. One of the main reasons for this is that the phytoplasm and starch density are not effective enough to cause obstruction, and the parenchymatic tissues cannot develop densely enough to block the phloem. The genes producing these metabolites are upregulated because of mRNA expression of sugar and starch production. A high rate of sugar and starch is synthesized, and the excess synthesized sugar accumulation to prevent the pathogen or over-dissemination may disrupt energy metabolism, premature aging, and early death of trees.

5. Conclusion

This study identified *Ca. Phytoplasma australasia* as the pathogen responsible for yellowing and wilting symptoms in *Robinia pseudoacacia* (black locust) trees. The most reliable method for pathogen isolation was found to be from the leaf midvein, with phytoplasma presence being lowest in upper shoots and highest in middle buds. The disease disrupts sugar transport in the phloem, leading to carbohydrate deficiency and eventual tissue desiccation. Since 2018, it has been spreading rapidly, posing a serious threat to *R. pseudoacacia* populations. If left uncontrolled, it could lead to significant losses. Moreover, as the disease may also affect other cultivated plants, identifying transmission vectors and implementing effective control strategies is crucial.

Author Contributions

Percentages of the authors' contributions are present below. All authors reviewed and approved final version of the manuscript.

	H.A.	M.E.G.	M.D.
C	50	20	30
D	50		50
S	50		50
DCP	100		
DAI	40	20	40
L	100		
W	80		20
CR	30	20	50
SR	50	20	30
PM	20	40	40
FA		70	30

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Since no studies involving humans or animals were conducted, ethical committee approval was not required for this study.

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