ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume 72 12 Number 5-6, 2024

ANALYSIS OF '*CANDIDATUS* PHYTOPLASMA PRUNORUM' TITER IN THE TISSUES OF APRICOT TREES (*PRUNUS ARMENIACA* L.) DURING THE YEAR.

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Link to this article: https://doi.org/10.11118/actaun.2024.012
Received: 23. 9. 2024, Accepted: 28. 11. 2024

Abstract

This study focused on monitoring '*Ca.* P. prunorum' detection rate and titer over the course of the year in one-year-old (January–May) or annual shoots (June–December) of apricot trees. As an experimental material, 3 apricot selections (H990/95, H990/177 and H980/74) each represented by 3 trees of the same age grown in the same orchard were selected and analysed monthly. The real-time PCR assay was used for absolute quantification of '*Ca.* P. prunorum'. The results showed, that phytoplasma was detectable in high rates (above 90 %) from August until December in annual shoots and from January to March and in May in one-year-old shoots. Lower detection rates were observed in April and in June and July, showing, that these months are not reliable for phytoplasma detection. Phytoplasma titer remained the same from January to May in one-year-old shoots. In annual shoots the lowest titers were observed in June, while in the rest of the year, the titers were significantly higher and not differing from the titers in one-year-old shoots collected from January to May. '*Ca.* P. prunorum' quantification also showed, that H990/95 selection had significantly lower phytoplasma titer than the H990/177 and H980/74 selections. This was also supported by the detection rates and symptoms, where H990/95 selection was showing lower values of these parameters than the rest of the selections.

Keywords: ESFY, real-time PCR, phytoplasma quantification, stone fruit species

INTRODUCTION

'*Candidatus* Phytoplasma prunorum', the causal agent of European stone fruit yellows (ESFY) is one of the most detrimental pathogens of stone fruit trees (genus *Prunus*) in Europe (Seemüller and Schneider, 2004). It is an unculturable gram-positive cell-wall deficient bacteria harbouring phloem tissues (IRPCM, 2004). One of the most affected species is apricot (*Prunus armeniaca* L.), where susceptible cultivars may become unproductive 8 to 10 years after planting (Marcone *et al.*, 2010). The most common ESFY symptoms are leafroll, leaf chlorosis or reddening, early bud break, irregular fruit ripening or fruit drop and overall decline of infected trees.

Detection efficiency of '*Ca.* P. prunorum' during the year is not the same (Jarausch *et al.*, 1999; Nečas *et al.*, 2008), which suggests uneven phytoplasma distribution and concentration in plant tissues throughout the year. '*Ca.* P. prunorum' colonizes the root system during the whole year, while its colonisation density decreases in the winter months, where in the highest parts of the tree its presence could be undetectable (Jarausch *et al.*, 1999). Recolonisation of aerial parts of the tree then occurs in the spring with systemic colonization from July to late fall (Marcone *et al.*, 2010).

In previous studies focusing on all-year-long '*Ca.* P. prunorum' monitoring, the detection was based

on PCR or DAPI staining techniques (Jarausch *et al.*, 1999; Nečas *et al.*, 2008). Hence, the concentration of phytoplasma was not assessed. Also, in these studies, the samples were taken either from leaves, roots or unspecified part of branches, where phytoplasma concentration can vary even within one shoot at the same sampling time (Kiss and Nečas, 2022). Currently, commonly used real-time PCR enables absolute or relative quantification of analysed organisms. This method enables also phytoplasma quantification in plant tissues (Nikolić *et al.*, 2010; Christensen *et al.*, 2004) and helped to distinguish *Prunus* genotypes with higher susceptibility to phytoplasmas and differences between phytoplasmal strains (Kiss *et al.*, 2024).

In this work the real-time PCR method was applied to monitor '*Ca.* P. prunorum' titer in plant tissues during the year in one-year-old or annual shoots of three different apricot selections in order to increase the knowledge on '*Ca.* P. prunorum' colonization patterns throughout the year. Phenological phases and meteorological data were also acquired.

MATERIALS AND METHODS

Locality, Plant Material and Sampling

The experiment was conducted in 2022 on three apricot selections (H980/74; H990/177 and H990/95) each represented by three naturally '*Ca.* P. prunorum' infected trees (in total 9 trees). Trees were 10 years old, grown in open vase training system with 5 m × 3 m spacing in the experimental orchard of the Department of Fruit Science at Faculty of Horticulture in Lednice, Mendel University in the Czech Republic. The orchard was planted in loess with a chernozem as top layer without additional irrigation or fertilisation. The mean annual (1961–2009) temperature of the area is 9.5 °C, precipitation 491 mm and duration of sunlight 1775 h (http://www.amet.cz/). The meteorological and phenological data for year 2022 are shown in Fig. 1.

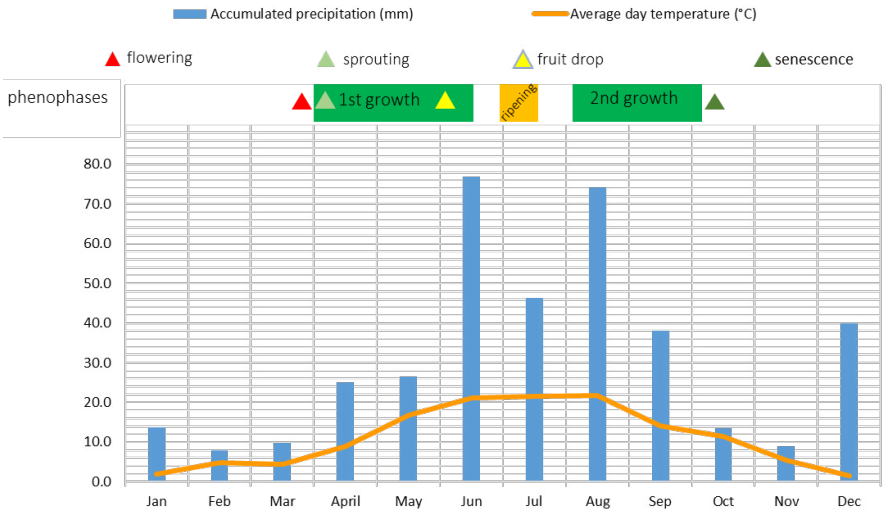
Prior sampling, the tree canopy of each tree was divided into 3 parts (based on main branches). From each part of the tree one one-year-old shoot (shoot grown in the previous year) was collected in the beginning of each month from January to May and an annual shoot (shoot grown in the year of sampling) from June to December. Shoots were collected from the height between 1.5 to 3 m, placed in zip-lock plastic bags and transferred to laboratory for processing. Every month, 27 samples were collected from 9 trees.

DNA Extraction

For total DNA extraction the modified protocol by Maixner *et al.* (1995) described in Kiss *et al.* (2024) was used. From each shoot, approximately 0.3 g of phloem from the basal 3 cm of the shoot was scratched using sterile scalpel and weighed accurately to 1 mg on precise scale Kern EG 620 3NM (KERN, DE). After extraction, the DNA pellets were resuspended in 100 µl of nuclease-free sterile water (Ambion, USA) and stored at -20 °C until use.

Absolute Quantification of '*Ca.* P. Prunorum' by Real-time PCR

For absolute quantification of '*Ca.* P. prunorum', the real-time PCR protocol by Christensen *et al.* (2004) was used. One reaction of a 20 µl volume consisted of 0.3 µM of forward primer, 0.9 µM of reverse primer, 0.2 µM of TaqMan probe, 1 × Luna Universal Probe qPCR Master Mix (New England Biolabs, USA), 2 µl of DNA and nuclease free water (Ambion, USA). Thermal protocol consisted of polymerase activation for 1 min at 95 °C, followed by 40 cycles with 15 s at 95 °C, 60 s at 60 °C and plate read on FAM channel. All samples were tested in duplicates.



1: *Meteorological data and observed phenophases of apricot in 2022*

To set up the standard curve, a plasmid with a cloned PCR product was prepared by Generi Biotech (CZ). The plasmid solution was diluted in phytoplasma negative apricot DNA in 10-fold serial dilution to obtain 10^7 to 10^1 copies.μl^{-1}.

The CFX 96 (Biorad, USA) real-time PCR System and CFX Maestro software (Biorad, USA) was used for fluorescence acquisition, determination of Ct values and baseline and threshold settings. The CFX Maestro software (Biorad, USA) was used for computation of the absolute quantification. The quantity was expressed as number of '*Ca.* P. prunorum' cells per gram of plant phloem (cells.g^{-1}).

Statistical Analysis

Data acquired from absolute quantification were analysed using parametric tests. First, the effect of apricot selection on phytoplasma titer was analysed by analysis of variance (ANOVA) with repeated measures ($p < 0.05$). The effect of sampling time (month of analysis) as well as the interaction of sampling time and apricot selection was analysed by ANOVA ($p < 0.05$). When a significant effect was observed, the differences between variants were further analysed by post-hoc analysis with Tukey's test ($p < 0.05$). All statistical analyses were performed using Statistica 14 software (Tibco, USA).

RESULTS

'*Ca.* P. Prunorum' Detection

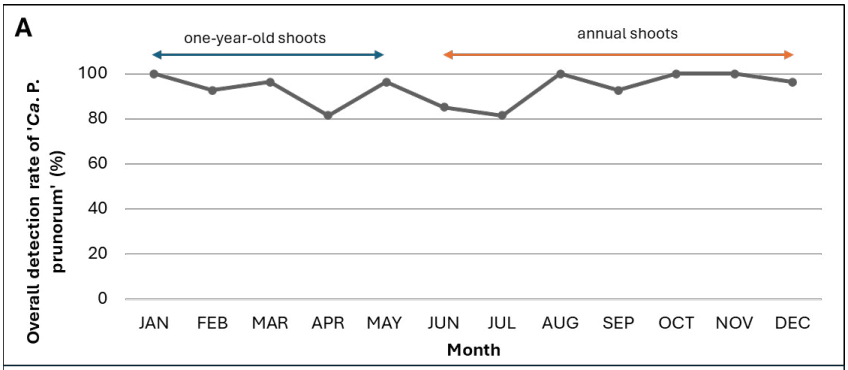
Overall detection rate of '*Ca.* P. prunorum' in tested shoots ranged between 80 and 100 % (Fig. 2A). In one-year-old shoots, the detection rate was decreasing from January (100 %) to April, where the detection rate was the lowest (81.5 %). In May, the detection rate increased to that in March (96.3 %). In annual shoots, the detection rate was, on contrary, increasing from June, when same as in July, the detection rates were the lowest (85 and 81.5 %, respectively). In next months until the end of the year the detection rate was at least 92.3 % with 100 % rates in August, October and November. Interestingly, the detection rate of annual shoots in the end of the year (December) reached similar values of the one-year-old shoots from the beginning of the year (January), showing that the colonisation pattern was reaching the same rate in both winter months.

The detection rates in individual apricot selections showed, that each selection behaved differently (Fig. 2B). Notably, the detection rate of H990/95 selection was more variable (between 56 % and 100 %) than that of the H980/74 and H990/177 (between 78 % and 100 % and between 89 % and 100 %, respectively). Overall, January, August, October and November were the months, where phytoplasma was 100 % detectable regardless the apricot selections.

'*Ca.* P. Prunorum' Titer

Sampling time (month) had a significant effect on '*Ca.* P. prunorum' titer ($p < 0.01$), however this effect was influenced by apricot selection ($p < 0.01$). Therefore, the sampling time was evaluated for each selection separately (Fig. 3).

Despite that '*Ca.* P. prunorum' titer in each apricot selection was slightly decreasing from January to May in one-year-old shoots, no significant difference

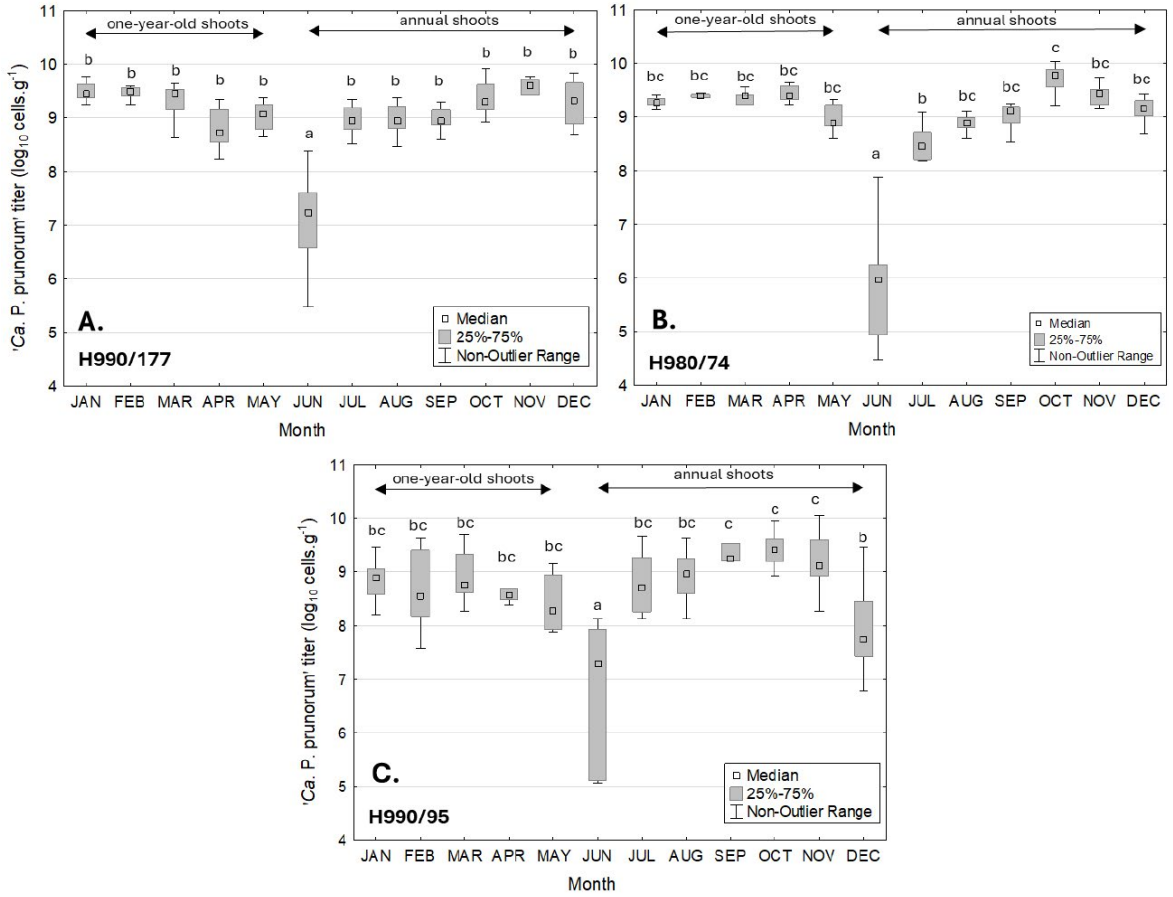


B

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
H 980/74	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	7 (78)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)
H 990/177	9 (100)	8 (89)	9 (100)	8 (89)	8 (89)	8 (89)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	8 (89)
H 990/95	9 (100)	8 (89)	8 (89)	5 (56)	9 (100)	6 (67)	6 (67)	9 (100)	7 (78)	9 (100)	9 (100)	9 (100)

2: *A. Overall detection rate of 'Ca. P. prunorum' during the year in one-year-old and annual shoots. Detection rate was calculated as a percentage of positive detections from all samples tested (n = 27).*

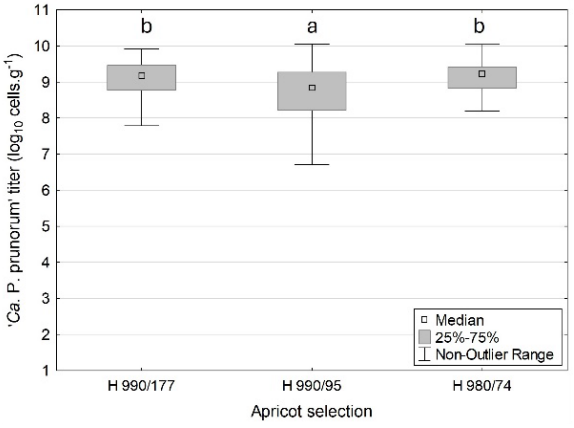
B. Detection rates of 'Ca. P. prunorum' during the year for each apricot selection (H980/74, H990/177 and H990/95) separately. The number in each cell is the number of positive samples and the number in brackets is the percentage of positive samples out of all tested samples of the selection (n = 9).



3: *'Ca. P. prunorum' titer in each tested apricot selection separately (A. H990/177; B. H980/74; C. H990/95). Letters above bars show statistical groups (p < 0.05) based on ANOVA and subsequent post hoc Tukey's test.*

was observed between these months (Fig. 3). In annual shoots, the *'Ca.* P. prunorum' titer in each apricot selection was the lowest in June. In the rest of the months (July–December) the phytoplasma titer in annual shoots varied depending on the apricot genotype. While in H990/177 the *'Ca.* P. prunorum' titer did not change in the rest of the months (July–December) (Fig. 3A), in H980/74 the titer was increasing through July to October, when it reached the highest concentration (Fig. 3B) and in H990/95 the titer was increasing until September–November (the highest titer), but then significantly decreased in December (Fig. 3C). Interestingly, in all apricot selections, except the titers in June, the titers in one-year-old shoots were not different from those in annual shoots.

Finally, significant differences ($p < 0,05$) were observed between the phytoplasma titers in tested apricot selections (Fig. 4). Overall, in tested tissues of H990/95 genotype, the phytoplasma titer (on average 6.85×10^8 cells.g^{-1}) was significantly lower than in H980/74 and H990/177 selections (on average 1.71×10^9 and 1.54×10^9 cells.g^{-1}, respectively). The difference can be seen also in Fig. 3, where in most of the year the phytoplasma titer in H990/95 was under 10^9 cells.g^{-1} while in other two apricot selections it was above 10^9 cells.g^{-1} and also on symptomatology (evaluated in September), where the H990/95 trees were showing weak leafroll and the trees of the other two selections weak leafroll combined with weak leaf chlorosis.



4: *Overall 'Ca. P. prunorum' titer in tested apricot selection throughout the testing year. Letters above bars show statistical groups (p < 0.05) based on ANOVA with repeated measures and subsequent post hoc Tukey's test.*

DISCUSSION

The knowledge of phytoplasma seasonal colonisation patterns in plant hosts is essential for designing protective measures against phytoplasmas but also for determination of the best sampling time periods. '*Ca.* P. prunorum' as well as the other two 16SrX fruit tree phytoplasmas ('*Candidatus* Phytoplasma mali' and '*Candidatus* Phytoplasma pyri') are detectable in root systems of their host all year long (Schaper and Seemüller, 1984; Kaviani *et al.*, 2024; Jarausch *et al.*, 1999). The conventional detection is often performed from leaf or branch samples as sampling roots is not easy. However, the '*Ca.* P. prunorum' detection rate in aerial parts varies during the year. Literature shows, that '*Ca.* P. prunorum' overwinters in lower colonisation densities in aerial parts and the recolonisation of these and newly grown parts happens in the spring with total plant colonisation from July to late fall (Marcone *et al.*, 2010). The results of our study are very similar, where '*Ca.* P. prunorum' was detectable in higher than 90 % rate from August to December in annual shoots and from January to March in one-year-old shoots, showing additionally, that the '*Ca.* P. prunorum' colonised aerial parts in high rates also during winter. In contrary, Nečas *et al.* (2008) observed the lowest detection rate (50 %) during the winter months (November–February). No satisfactory explanation is currently available on different '*Ca.* P. prunorum' colonisation behaviour during the winter. Apart from the influence of host or pathogen genotype, the possible theory could be the winter temperature, where mild (warmer) winters could be beneficial for higher '*Ca.* P. prunorum' overwintering rates in aerial parts compared to those in cold winters. Unfortunately, in previous works, no information about mean temperatures during the winter is available. In presented research, in Lednice region, the winter in the beginning and end of 2022 was very mild, with average month temperatures above 0 °C (Fig. 1) with few occasional days when the mean day temperature was below zero, which could result in favourable overwintering of '*Ca.* P. prunorum' in aerial plant parts.

Similarly, as in Jarausch *et al.* (1999), the detection rates in presented work were the lowest during the spring and early summer (April–July, Fig. 2). Interestingly, in April the same plant parts (one-year-old shoots) were used for detection as in January, February, March and May when the detection rates were higher. The quantification analysis showed, that from January to May the phytoplasma titers in infected samples where the same (Fig. 3). This observation indicates, that the phytoplasma colonisation suddenly changed in April, resulting in some one-year-old shoots with undetectable phytoplasma titers and some with unchanged, high, phytoplasma titers. This observation could indicate a movement of phytoplasmas to newly grown annual shoots that were sprouting in April (Fig. 1), while leaving the plant tissues from which the phytoplasma moved with undetectable titers. Afterwards in May, one-year-old shoots underwent recolonisation resulting in phytoplasma detection rates and titers similar to those before sprouting. In annual, newly, grown shoots the phytoplasma detection rates and titers were low in June. In next months, a phytoplasma movement in newly grown parts as described before (Jarausch *et al.*, 1999; Kiss *et al.*, 2022) was observed, resulting in increased phytoplasma titers from July and detection rates from August until the end of the year.

Overall, the analysis of phytoplasma titer gives a new insight into the behaviour of phytoplasma in plant tissues during the year. Apart from abovementioned observations, the analysis of phytoplasma titer help to identify more and less susceptible apricot selections, where the H990/95 showing only weak ESFY symptoms resulted in significantly lower phytoplasma titer than the other two tested selections (Fig. 4). Lower susceptibility of H990/95 was observed also in detection rates, where in some months (April, June and July) the detection rate dropped below 70 % or even 60 %, while for the other selections the detection rates remained above 70 % all year long, showing that colonisation of less susceptible selections is decreased in the period of sprouting and in the first months of newly grown shoots.

CONCLUSION

In conclusion, presented study showed, that the '*Ca.* P. prunorum' was detectable in high rates (above 90 %) from August until March in one-year-old or annual shoots. Lower rates were observed in April and in June and July, showing, that these months are not reliable for phytoplasma detection. Phytoplasma quantification analysis showed the titers were the same from January to May in one-year-old shoots. In annual shoots the lowest titers were observed in June, while in the rest of the year, the titers were significantly higher and not differing from those in one-year-old shoots collected in January–May. '*Ca.* P. prunorum' quantification also showed, that H990/95 selection had significantly lower phytoplasma titer than the H990/177 and H980/74 selections. This was also supported by the detection rates and symptoms, where H990/95 selection was showing lower values of observed parameters than the rest selections as well.

Acknowledgements

This study was funded by Internal Grant Agency (IGA) of Mendel University under grant agreement number IGA-ZF/2023-SI1-003. This research used the infrastructure acquired by project CZ.02.1.01/0.0/0.0/ 16_017/0002334 Research Infrastructure for Young Scientists, which is co-financed by the Operational Program of Research, Development and Education.

REFERENCES

CHRISTENSEN, N. M., NICOLAISEN, M., HANSEN, M. *et al.* 2004. Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Molecular Plant-Microbe Interactions*. 17(11), 1175–1184.

IRPCM PHYTOPLASMA/SPIROPLASMA WORKING TEAM–PHYTOPLASMA TAXONOMY GROUP. 2004. 'Candidatus Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology*. 54(4), 1243–1255.

JARAUSCH, W., LANSAC, M. and DOSBA, F. 1999. Seasonal colonization pattern of European stone fruit yellows phytoplasmas in different Prunus species detected by specific PCR. *Journal of Phytopathology*. 147(1), 47–54.

KAVIANI, M., GOODWIN, P. H. and HUNTER, D. M. 2024. Pear Rootstock Effects on Seasonal Colonization Patterns of Pear Decline Phytoplasma. *Horticulturae*. 10(2), 129.

KISS, T. and NEČAS, T. 2022. Quantification of 'Candidatus Phytoplasma prunorum' in apricot trees exhibiting uneven European stone fruit yellows symptoms. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*. 70(1), 31–36.

KISS, T., ŠAFÁŘOVÁ, D., NAVRÁTIL, M. and NEČAS, T. 2024. Molecular characterization of 'Candidatus Phytoplasma prunorum' in the Czech Republic and susceptibility of apricot rootstocks to the two most abundant haplotypes. *Microorganisms*. 12(2), 399.

MAIXNER, M., AHRENS, U. and SEEMÜLLER, E. 1995. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology*. 101(3), 241–250.

MARCONE, C., JARAUSCH, B. and JARAUSCH, W. 2010. Candidatus Phytoplasma prunorum, the causal agent Of European stone fruit yellows: An overview. *Journal of Plant Pathology*. 92(1), 19–34.

NEČAS, T., KRŠKA, B., MAŠKOVÁ, V. *et al.* 2008. Influence of the time of sampling on detection of ESFY phytoplasma. *Acta Horticulturae.* 781, 435–441.

NIKOLIĆ, P., MEHLE, N., GRUDEN, K., RAVNIKAR, M. and DERMASTIA, M. 2010. A panel of real-time PCR assays for specific detection of three phytoplasmas from the apple proliferation group. *Molecular and cellular probes*. 24(5), 303–309.

SCHAPER, U. and SEEMÜLLER, E. 1984. Effect of the colonization behaviour of the causal agents of apple proliferation and pear decline on their detectability by fluorescence microscopy. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*. 36(2), 21–25.

SEEMÜLLER, E. and SCHNEIDER, B. 2004. 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma pyri' and Candidatus Phytoplasma prunorum', the casual agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *International Journal of Systematic and Evolutionary Microbiology*. 54(4), 1217–1226.

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