ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume 70 3 Number 1, 2022

QUANTIFICATION OF '*CANDIDATUS* PHYTOPLASMA PRUNORUM' IN APRICOT TREES EXHIBITING UNEVEN EUROPEAN STONE FRUIT YELLOWS SYMPTOMS

Tomáš Kiss[1], Tomáš Nečas[1]

[1] Department of Fruit Science, Faculty of Horticulture in Lednice, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic

Link to this article: https://doi.org/10.11118/actaun.2022.003
Received: 25. 10. 2021, Accepted: 25. 1. 2022

Abstract

This study focused on quantification of '*Candidatus* Phytoplasma prunorum' in apricot trees exhibiting uneven symptoms of European stone fruit yellows. For the research, 17 trees showing early leaf bud break in one part of the crown were selected in winter season, and 13 trees showing leafroll in older part of the shoots and no symptoms in younger part of the same shoots were selected in summer season. Absolute quantification of phytoplasmas was performed by real-time PCR in samples collected from asymptomatic and symptomatic parts. The results showed, that phytoplasma could not be always detected in asymptomatic parts, where 2 out of 17 samples from asymptomatic parts from winter season were negative while in summer season 6 out of 13 samples from asymptomatic parts were negative. Comparison of phytoplasma quantities between symptomatic and asymptomatic plant parts then showed significantly higher quantities in symptomatic parts from samples collected in summer season and no quantity difference in samples collected in winter season.

Keywords: real-time PCR, '*Ca.* P. prunorum', early leaf bud break, leafroll, ESFY, *Prunus armeniaca*

INTRODUCTION

'*Candidatus* Phytoplasma prunorum', the causal agent of European stone fruit yellows disease (ESFY) is one of the most dangerous pathogens affecting fruit trees in the temperate zone in the Europe (Seemuller and Schneider, 2004). It is a cell wall-less grampositive bacteria harbouring phloem tissues of *Prunus* species (IRPCM, 2004) and is naturally vectored from plant to plant by a psyllid *Cacopsyla pruni* (Scopoli) (EPPO, 2020). Together with '*Ca.* P. mali', causal agent of apple proliferation and '*Ca.* P. pyri', causal agent of pear decline, it belongs to the 16SrX group (apple proliferation group). From *Prunus* species the most affected are apricots and Japanese plums, where susceptible cultivars may render unproductive 8 to 10 years after planting (Marcone *et al.*, 2010).

The most typical symptoms of ESFY are leaf yellowing or reddening together with leafroll during the summer, premature fruit drop, prolongation of fruit maturing period or off-season growth and premature leaf bud break, which happens before flowering (Seemuller and Schneider, 2004). Diseased trees, especially apricots, peaches and Japanese plums on susceptible rootstocks may die few years after infection (Carraro and Osler, 2003). Symptoms do not always occur in the whole tree and only a part of the crown (e.g. a branch) might be symptomatic.

Distribution of phytoplasmas within infected plants throughout the year is not even (Jarausch *et al.*, 1999; Nečas and Krška, 2005). '*Ca.* P. prunorum' colonizes the root system during the whole year, while its titer in the phloem tissues decreases during the winter period, where in the highest parts of the tree its presence could be undetectable (Jarausch *et al.*, 1999). Recolonization of aerial parts of the tree then occurs in the spring with systemic colonization from July to late autumn (Marcone *et al.*, 2010).

Little is known about the distribution of '*Ca.* P. prunorum' within symptomatic and asymptomatic parts of the same tree. Especially interesting is the distribution of phytoplasma within a tree during the winter season where some branches exhibit early leaf bud break while the rest of the tree remains dormant, asymptomatic. Also, during the vegetation period it often happens that only a part of the shoot is showing ESFY symptoms. With the real-time PCR and its absolute quantification it is possible to quantify the titer of phytoplasma (Kiss *et al.*, 2016; Nečasová *et al.*, 2016) within symptomatic and asymptomatic parts of the tree to reveal the distribution pattern of this pathogen.

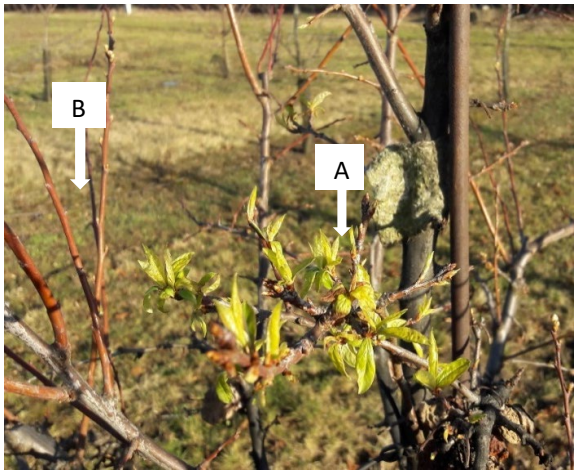
The aim of this study was to use real-time PCR method for absolute quantification of '*Ca.* P. prunorum' within symptomatic and asymptomatic parts of the same tree and to answer the question whether '*Ca.* P. prunorum' is unevenly distributed within these plant parts.

MATERIALS AND METHODS

Plant Material

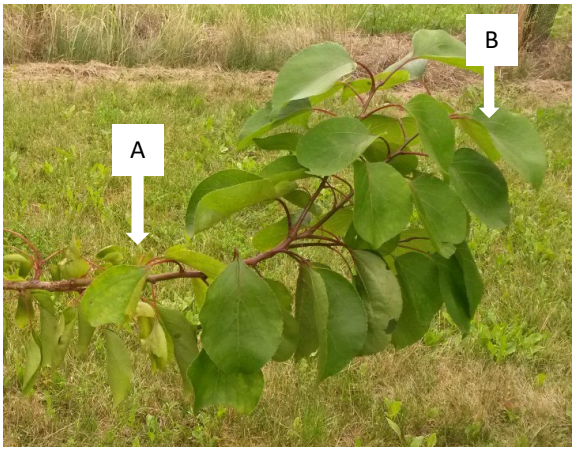
For the research, apricot trees (*Prunus armeniaca*) positively tested for the presence of '*Ca.* P. prunorum' and showing typical ESFY symptoms were used. The trees were grown in the experimental orchard of the Department of Fruit Science at Faculty of Horticulture in Lednice, Mendel University in the Czech Republic. Samples were collected in the winter season (end of February) and in the summer season (end of June).

In the winter season, samples were collected form 17 trees showing early leaf bud break in one part of the crown, while the other part was still dormant and not showing any bud break (Fig. 1). Per each tree, one sample consisting of 3 one-year-old shoots was taken from the part showing early bud break and the second sample was taken from the part not showing any bud break. In total 34 samples were collected from 17 trees.



1: *Part of the crown showing early leaf bud break before flowering (A), while the rest of the crown remains asymptomatic (B)*

In summer season, samples were collected from 13 trees showing leafroll on the first half of the shoot while on the other, younger, half it was asymptomatic (Fig. 2). Per each tree, one sample, consisting of 5 leaves, was taken evenly from the symptomatic part and the second sample was taken from the asymptomatic part of the shoot. In total 26 samples were collected from 13 trees.



2: *One-year-old shoot showing leafroll on the basis of the shoot (A), while the other, younger part of the shoot is asymptomatic (B)*

DNA Extraction

For total DNA extraction the modified protocol by Maixner *et al.* (1995) was used. From the samples collected in summer petioles and midribs, and from winter collected samples phloem was used as an input material. Before extraction, around 0.3 g of plant material was 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 10 µl volume consisted of 0.3 µM of forward primer, 0.9 µM of reverse primer, 0.2 µM of TaqMan probe, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems, USA) and 1 µl of DNA. Thermal protocol consisted of polymerase activation for 10 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 triplicates.

To set up the standard curve, a plasmid with a cloned PCR product was prepared by Generi Biotech (Czech Republic). The plasmid solution was

diluted in 10-fold serial dilution to obtain 10^7 to 10^1 copies.μl^{-1} in phytoplasma negative plant DNA.

The Eco Real-Time PCR System and Software (Illumina, USA) was used for fluorescence acquisition, determination of Ct values and for computation of the absolute quantity of '*Ca.* P. prunorum' that was expressed as number of phytoplasmal cells per gram of plant tissue (leaf stalks and midribs from summer sampling and phloem from winter sampling).

Statistical Analysis

Data acquired from absolute quantification were analysed by Statistica software (Tibco, USA). Initial statistical analyses did not show normal distribution of data, thus non-parametric Wilcoxon test with paired samples was used for evaluation.

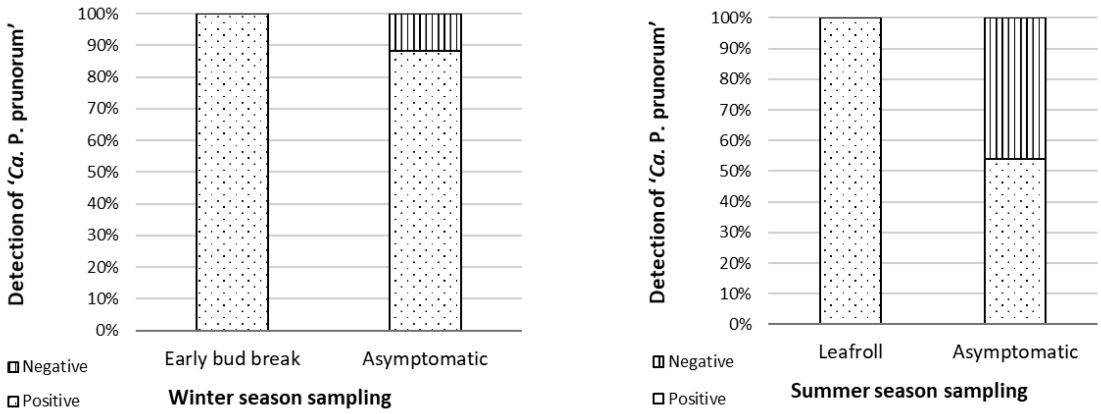
RESULTS

'*Ca.* P. Prunorum' Detection

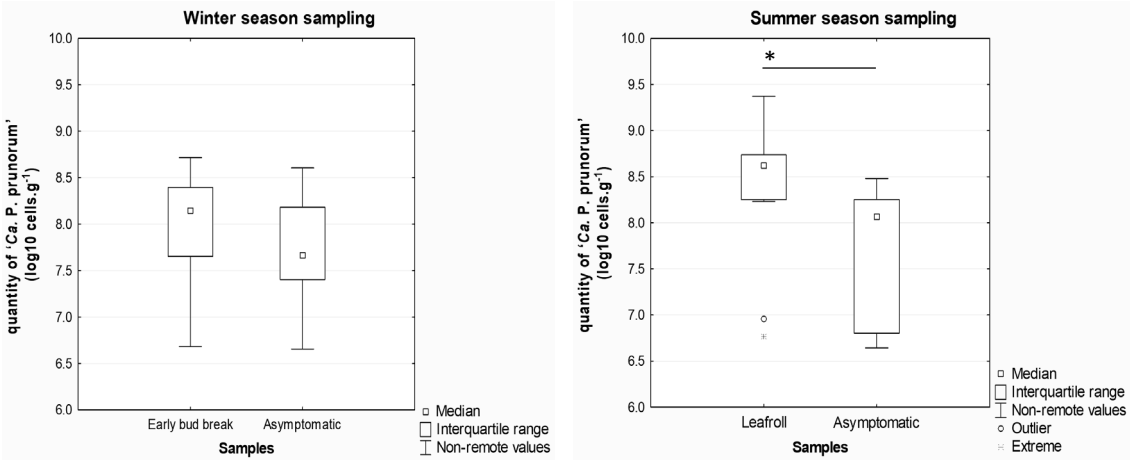
Detection of phytoplasmas deriving from real-time PCR showed, that all samples collected from symptomatic parts, regardless on the season, were positive (Fig. 3). However, not all samples collected from asymptomatic parts were positive. From asymptomatic parts collected in winter season, almost 12% of the samples were negative (2 out of 17 samples) while from the samples collected in summer season 46% were negative (6 out of 13 samples).

Absolute Quantification of '*Ca.* P. Prunorum'

From winter sampling, the quantity of '*Ca.* P. prunorum' in samples collected from shoots showing early bud break ranged between 4.82×10^6 to 5.21×10^8 cells.g^{-1} while in the samples from asymptomatic shoots the quantity ranged between 4.52×10^6 to 4.04×10^8 cells.g^{-1}, showing similar quantities (Tab. I). The '*Ca.* P. prunorum' quantity difference between symptomatic and asymptomatic shoots on a same tree showed that at 5 trees the samples collected from asymptomatic shoots had higher '*Ca.* P. prunorum' quantities than samples from the symptomatic shoots. The rest of the trees had higher '*Ca.* P. prunorum' quantities in symptomatic part, where 7 trees had up to 5 times higher quantities in symptomatic parts and



3: *'Ca. P. prunorum' detection rates from samples collected in summer and winter season*



4: *Average quantities of 'Ca. P. prunorum' in samples from symptomatic (early bud break or leafroll) and asymptomatic parts collected in winter season (left) and summer season (right). Asterisk is showing significant difference between samples of the respected sampling season according to Wilcoxon test with paired samples ($p < 0.05$).*

I: *'Ca. P. prunorum' quantities in collected samples from winter season and differences in quantities between symptomatic and asymptomatic part of the same tree*

Tree	*Prunus armeniaca*	Quantity of '*Ca.* P. prunorum' (cells.g^{-1})		'*Ca.* P. prunorum' quantity difference between sympt. and asympt. part (x times)
		Early bud break	Asymptomatic	
W1	Bergeron	1.04×10^{7}	1.21×10^{8}	0.09
W2	Paviot	4.98×10^{8}	ND	NA
W3	Seker pare 1	2.17×10^{7}	5.67×10^{6}	3.82
W4	Chuan sin	5.21×10^{8}	2.53×10^{7}	20.61
W5	Ivone Liverani	3.10×10^{7}	3.49×10^{8}	0.09
W6	Palumella	4.93×10^{7}	4.53×10^{7}	1.09
W7	Seker pare 2	5.64×10^{7}	3.33×10^{7}	1.69
W8	NJA-2	1.75×10^{8}	4.58×10^{7}	3.82
W9	LE-10816	3.46×10^{8}	1.52×10^{8}	2.28
W10	Velika luka	2.48×10^{8}	1.21×10^{8}	2.06
W11	Fantasme	1.32×10^{8}	8.81×10^{7}	1.50
W12	Cegledi Bibor	4.51×10^{7}	1.93×10^{8}	0.23
W13	Koliza rife	2.07×10^{8}	4.04×10^{8}	0.51
W14	Reale d'Imola	4.82×10^{6}	6.02×10^{6}	0.80
W15	Hybr 165	4.75×10^{8}	4.29×10^{7}	11.07
W16	Hybr 130	2.22×10^{8}	ND	NA
W17	Hybr 20	1.40×10^{8}	4.52×10^{6}	30.85

ND: not detected; NA: not applicable

II: *'Ca. P. prunorum' quantities in collected samples from summer season and differences in quantities between symptomatic and asymptomatic part of the same tree*

Sample	*Prunus armeniaca*	Quantity of '*Ca.* P. prunorum' (cells.g^{-1})		'*Ca.* P. prunorum' quantity difference between sympt. and asympt. part (x times)
		Leafroll	Asymptomatic	
S1	Poyer	1.78×10^{8}	ND	NA
S2	Polonais	2.69×10^{8}	ND	NA
S3	Luizet	4.98×10^{8}	4.39×10^{6}	113.32
S4	Lefrosta	4.22×10^{8}	4.95×10^{7}	8.52
S5	Veselka	4.94×10^{8}	ND	NA
S6	Velikyj	3.21×10^{8}	1.15×10^{8}	2.79
S7	M59	8.98×10^{6}	ND	NA
S8	Favorit	5.79×10^{6}	6.37×10^{6}	0.91
S9	Hungarian c235	1.69×10^{8}	ND	NA
S10	Roxana	2.36×10^{9}	ND	NA
S11	Saldcot	5.44×10^{8}	$1,79 \times 10^{8}$	3.05
S12	Raduga	1.16×10^{9}	3.02×10^{8}	3.86
S13	Sunglo	9.20×10^{8}	1.74×10^{8}	5.29

ND: not detected; NA: not applicable

3 trees had more than 5 times higher quantities in symptomatic parts. Finally, the statistical analysis showed, that there is no significant difference ($p = 0.307$) between quantities of '*Ca.* P. prunorum' between symptomatic and asymptomatic parts of the same tree (Fig. 4).

From summer sampling, the quantity of '*Ca.* P. prunorum' in samples collected from shoots showing leafroll ranged between 5.79×10^6 to 2.36×10^9 cells.g^{-1} while in the samples from asymptomatic shoot parts the quantity ranged between 4.39×10^6 to 3.02×10^8 cells.g^{-1} (Tab. II). The '*Ca.* P. prunorum' quantity difference between symptomatic and asymptomatic part of the shoot on a same tree showed that at 1 tree the sample collected from asymptomatic part had higher '*Ca.* P. prunorum' quantity than from the symptomatic part. The rest of the samples had higher '*Ca.* P. prunorum' quantities in symptomatic part, where 3 trees had up to 5 times higher quantities in symptomatic parts and 3 trees had more than 5 times higher quantities in symptomatic parts. Finally, the statistical analysis showed, that '*Ca.* P. prunorum' quantity in symptomatic parts is significantly higher ($p = 0.028$) than in the asymptomatic parts of the same tree (Fig. 4).

In both sampling seasons, there were trees where '*Ca.* P. prunorum' was undetectable in asymptomatic parts, which resulted in impossibility to compare '*Ca.* P. prunorum' quantities between symptomatic and asymptomatic parts (Tab. I, II). However, despite negatives from asymptomatic part, symptomatic parts did not have the lowest quantities of '*Ca.* P. prunorum'. Except for one tree (S7), where symptomatic part had 8.98×10^6 '*Ca.* P. prunorum' cells.g^{-1}, while the rest of the trees had '*Ca.* P. prunorum' quantities in order of 10^8 cells.g^{-1} in symptomatic parts.

DISCUSSION

As inactivation of sieve tubes does not occur in *Prunus* species, '*Ca.* P. prunorum' might persist in lower titers in aboveground parts during the winter (Jarausch *et al.*, 1999; Nečas *et al.*, 2008). A common ESFY symptom occurring during the winter is an early leaf bud break. This symptom is connected with the activity of the remaining sieve tubes from the late season where phytoplasmas multiply (Jarausch *et al.*, 1999), indicating higher phytoplasma quantities in parts showing leaf bud break. However, according to our results the quantity of 'Ca. P. prunorum' in asymptomatic, still dormant parts and parts showing early bud break was not different, suggesting that there is other mode of symptom development than that deriving from phytoplasma multiplication.

Another case when uneven manifestation of ESFY symptoms can be observed is in the early summer season. Here, the leafroll symptom often occurs in the basis of the shoot, while the youngest part of the shoot does not exhibit any symptoms. Almost no information has been available about the phytoplasma titer in these plant parts. The results of this study showed, that there is a significant decrease in phytoplasma quantity in asymptomatic, youngest parts of the shoots. Moreover, 6 out of 13 samples taken from asymptomatic parts resulted negative in phytoplasma detection. The low phytoplasma quantity in asymptomatic young part in contrast with high phytoplasma quantity in symptomatic older part of the same shoot might be connected with shoot prolongation growth periods of apricots. Here, the first growth period takes place in spring time which is followed by the second growth period in the early summer period. It is well known that the phytoplasma spreads to newly growing plant parts from the older parts, which have higher phytoplasma quantities (Jarausch *et al.*, 1999). Therefore, while the phytoplasma is already multiplicated in high quantities in the symptomatic shoot part grown in the first growth period, lower phytoplasma quantities and no symptoms are observed in newly grown parts from the second growth period. Later, in late summer period the analysed shoots were symptomatic in whole length, suggesting successful colonization of '*Ca.* P. prunorum' in shoot parts from the second growth period.

The results of this paper might help improve the way of sample collection for phytoplasma detection. In all cases it is preferred to collect samples from symptomatic plant parts for successful detection. This might not be that necessary in the winter season when early leaf bud break occurs, but it is crucial in the summer season, when younger parts are asymptomatic. However, when trees are asymptomatic during the summer sample collection, the leaf samples should be preferably collected from the oldest shoot parts and due to phytoplasma spreading this will be true also for collection of shoots for phloem samples. In the case of winter sampling from asymptomatic trees, the best way would be sampling of the root parts (Jarausch *et al.*, 1999).

CONCLUSION

According to the results, the '*Ca.* P. prunorum' quantities were not different between symptomatic and asymptomatic parts of the same tree in winter season, showing, that symptom occurrence in winter season does not correlate with multiplication of '*Ca.* P. prunorum' in symptomatic parts. Thus, the reason of symptom occurrence in winter season is still not clearly known. On the other hand, significant differences were observed between '*Ca.* P. prunorum' quantities in symptomatic

and asymptomatic parts in summer season, where significantly higher '*Ca.* P. prunorum' quantities were observed in symptomatic parts. These findings agree with the colonization behaviour of '*Ca.* P. prunorum' and its transgression into newly growing shoot parts.

Acknowledgements

This research was supported by the project NAZV QK1920058 provided by the Ministry of Agriculture of the Czech Republic. Laboratory equipment was acquired under project CZ.02.1.01/0.0/0.0/16_017/0002334 Research infrastructure for young scientists.

REFERENCES

CARRARO, L. and OSLER, R. 2003. European stone fruit yellows: a destructive disease in the Mediterranean basin. *Options Méditerranéennes*, 45: 113–117.

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.

EPPO. 2020. PM 7/62 (3) 'Candidatus Phytoplasma mali', 'Ca. P. pyri' and 'Ca. P. prunorum'. *OEPP/EPPO Bulletin*, 50(1): 69–85.

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., BLIOT, C. *et al.* 1999. Phytoplasma transmission by in vitro graft inoculation as a basis for a preliminary screening method for resistance in fruit trees. *Plant Pathology*, 48(2): 283–287.

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.

KISS, T., NEČAS, T. and NEČASOVÁ, J. 2016. Comparison of real-time PCR protocols in detection and quantification of fruit tree 16SrX group phytoplasmas. *Genetika*, 48(2): 629–642.

MAIXNER, M., AHRENS, U. and SEEMULLER, 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.

NEČAS, T. and KRŠKA, B. 2005. Detection of Phytoplasma ESFY in apricot trees using phloem and petioles. *Plant protection science,* 41(4): 132–140.

NEČASOVÁ, J., NEČAS, T., EICHMEIER, A. and KRŠKA, B. 2016. Use of real-time PCR for the characterization of variable symptoms in ESFY phytoplasma disease. *Acta Horticulturae,* 1110(1): 57–62.

SEEMULLER, 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.

Contact information

Tomáš Kiss: tomas.kiss@mendelu.cz (corresponding author)
Tomáš Nečas: tomas.necas@mendelu.cz