Occurrence of European Stone Fruit Yellows (ESFY) in Slovenia: Possibilities for Healthy Mother Plant Cultivation in Insect-Proof Net-Houses

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Keywords: stone fruits, disease control, ESFY, vectors, net-house cultivation, propagating material, bud wood quality

Abstract

Fruit species are affected by severe diseases associated with phytoplasmas. European stone fruit yellows (ESFY) phytoplasma, ('*Candidatus* Phytoplasma prunorum'), causes important disorders and decline of trees in many cultivated *Prunus* species, such as apricot (*P. armeniaca*), Japanese plum (*P. salicina*) and peach (*P. persica*). ESFY phytoplasma is transmitted by the vector *Cacopsylla pruni* and through vegetative propagation. An official survey of the presence of ESFY performed in the last ten years has shown that the pathogen is present in several areas in Slovenia where stone fruits are cultivated. Since the disease is not curable, the production of healthy planting material is regulated as an important preventive measure to control the spread of the disease. Therefore, the maintenance of mother plants in the insect-proof net-house, where the infection pressure from outside is limited, presents a possibility for the production of healthy propagating material. 'Virus free' mother plants of stone fruits were planted in the protected environment of a net-house in spring 2007 with the aim of determining the possibility for production of healthy, technologically properly developed bud wood. Mother trees from the net-house showed good vegetative growth with large amounts of well-developed bud wood for nursery requirements. No vectors were captured inside the net-house during the study period from 2007 to 2009. On control trees outside the net-house one specimen of *C. pruni* was caught on yellow sticky traps in 2009, and a high population of the leafhopper *Asymmetrasca decedens* was found in all three years. The occurrence of *Plum pox virus* (PPV) was confirmed on an outside growing control tree, but no control trees were infected with ESFY in the three years after planting. No vector transmissible diseases were detected on trees inside the net-house.

INTRODUCTION

Production of healthy propagating/planting material presents an important measure to prevent the spread of diseases. Among the pathogens affecting stone fruits, the phytoplasma responsible for European stone fruit yellows (ESFY; '*Candidatus* Phytoplasma prunorum'), which belongs to the apple proliferation group of phytoplasmas (Seemüller and Schneider, 2004), can cause severe symptoms and decline of apricot (*Prunus armeniaca*), Japanese plum (*P. salicina*), and peach (*P. persica*) trees, and can infect several other *Prunus* species (Carraro and Osler, 2003). It is transmitted by the

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vector *Cacopsylla pruni* and through vegetative propagation (Carraro et al., 1998, 2004). The disease is present in several countries in Europe and in the Mediterranean basin (Laimer Da Camara Machado et al., 2001; Carraro and Osler, 2003; Laviňa et al., 2004; Ramel and Gugerli, 2004). In Slovenia, the results of the official survey performed in the last ten years for the presence of ESFY in different *Prunus* spp. revealed a high occurrence of the disease in several areas where stone fruits are cultivated (Mehle et al., 2007; Ambrožič Turk et al., 2008). In such conditions of high infection pressure, maintenance of mother plants in an insect-proof net-house as a protection against vectors should be a proper and most efficient step toward producing healthy propagating material. Besides ESFY phytoplasma, the *Plum pox virus* (PPV), and the bacterium *Xanthomonas arboricola* pv. *pruni* are also quarantine pests which have to be checked for and shown to be absent on propagating/planting material.

The purpose of this work was to maintain healthy mother plants under a protected environment in an insect-proof net-house, and to assess the possibility of production of properly developed bud wood under such modified growing conditions.

MATERIALS AND METHODS

Plant Material, Tree Growth and Budding Experiment

In spring 2007, peaches *Prunus persica* (L.) Batch, apricots (*P. armeniaca* L.) and plums (*P. domestica* L.) of different cultivars, comprising 81 plants, were planted at a spacing of 1.8 m in rows with 3.5 m between rows in an insect-proof net-house of 500 m^2 located at the Fruit Growing Centre of Agriculture and Forestry Service Nova Gorica in Bilje in the southwestern part of Slovenia. Trees were trained to a spindle form. The planting material had virus-free status, derived from a certification scheme. Twelve trees of peach cultivar 'Redhaven' from the net-house were compared with 12 trees of the same cultivar planted outside the net-house as control trees to study the tree growth parameters and the quality of bud wood. Tree height was measured and the amount of buds for budding was counted on the shoots of the 'Redhaven' trees from both environments in the years 2007-2009. To check the quality of bud wood from the net-house trees, and to determine the effect of time of budding, buds from trees growing both inside and outside were grafted at three different times at two-week intervals during the summer 2008 (26 Aug., 9 Sept., and 23 Sept.). The success of budding was evaluated in the following spring. Virus-free 'GF 677' (*P. amygdalus* × *P. persica*) was used as the rootstock in the budding experiment. At each date of budding, buds were taken from different trees (three treatments) and, in addition, a fourth treatment was made in which buds were taken repeatedly from the same trees at each date of budding. In each treatment, three trees were chosen and 10 buds were taken from individual trees. One bud was inserted per rootstock. The success of budding was recorded in the spring 2009 and expressed as a percentage of bud-take. Data were statistically analyzed using the t-test at P=0.05.

Vector Monitoring

During the years 2007-2009, monitoring of vectors of quarantine diseases of *Prunus* plants was carried out, particularly of *C. pruni* (Scopoli), the vector of ESFY phytoplasma. The monitoring was done inside the net-house as well as outside on control trees using yellow sticky traps 24.5×13.5 cm, of the type Terminator (Bioteh, Ljubljana). Four traps were placed on trees at different positions inside the net-house and two traps were placed outside on control trees. Each year the traps were set up from the beginning of March to October. Yellow sticky traps were replaced at two-week intervals and checked in the laboratory under a stereomicroscope for the presence and identity of insects.

Determination of Pathogens in the Laboratory

The presence of quarantine pests ESFY phytoplasma, PPV, and *Xanthomonas arboricola* pv. *pruni* on stone fruits (Council Directive 2000/29/EC) was checked by

laboratory analyses during the years 2007 to 2009 to control the phytosanitary status of trees growing in the net-house and of the control trees growing outside. Samples of roots were taken to check for the presence of ESFY using two molecular approaches: a nested PCR and a real-time PCR. The initial PCR was performed using the universal phytoplasma P1/P7 (Schneider et al., 1995) with slightly modified primers (Hren et al., 2007). The nested PCR reactions that followed were conducted using the AP group specific primers f01/r01 (Lorenz et al., 1995) and a pair of universal phytoplasma primers U3/U5 (Lorenz et al., 1995). A real-time PCR procedure using universal primers UniRNA as described by Hren et al. (2007) was employed to test the fruit tree samples for the presence of phytoplasma (Boben et al., 2007). An eukaryotic 18S rRNA TaqMan assay (Applied Biosystems, USA) was performed alongside the universal testing for the presence of phytoplasmas to evaluate the efficacy of the extraction procedure. For detection of PPV, leaves were tested using the ELISA serological method and, in cases of doubtful results, the molecular PCR method was performed. The presence of *X. arboricola* pv. *pruni* was examined in dormant scion chips according to EPPO diagnostic protocols (EPPO, 2006).

RESULTS AND DISCUSSION

Tree Growth Parameters and the Quality of Bud Wood

In the changed growth conditions inside the net-house, mainly due to reduced light availability, some tree growth parameters and bud wood quality were checked to evaluate the possibility of production of well-developed bud wood for nurseries. The 'Redhaven' trees from net-house were significantly higher than the control 'Redhaven' trees outside the net-house in the studied period from 2007 to 2009 (Table 1). In 2007, the first year after planting, the shoot growth of control trees outside the net-house was suppressed due to the attack of *Cydia molesta* despite the applied insecticide treatments. This can also be seen from the number of buds available for budding, which was considerably reduced in 2007 (Table 1). In the following years, trees from both environments had good vegetative growth, although they were significantly higher inside the net-house. Very good vegetative growth of trees growing inside was also demonstrated through the number of buds available for budding, which was significantly greater compared to the control trees in 2007 and 2009 (Table 1). Buds suitable for budding are considered to be those from the middle part of current-year shoots, since shoot tips as well as the woody basal parts of shoots are unsuitable sources (Webster and Wertheim, 2005).

To compare the quality of bud wood obtained from net-house growing trees with outside growing control trees and to check the effect of time of budding, buds from both environments were grafted to 'GF 677' rootstocks at two-week intervals during the summer 2008, and the bud-take was recorded the following spring. It was seen from the results of the budding experiment that the bud-take was greater (84.4%) if buds were taken from the outside control trees compared to the net-house trees (79.4%), although the difference was not significant. The time of budding (26 Aug., 9 Sept., and 23 Sept.) had no significant influence on the success of bud-take if buds were taken from different trees at a particular date of budding. On the other hand, if buds were taken from the same trees repeatedly at each date of budding, the time of budding had a significant effect on budding success (Table 2).

Entomological Analysis

Entomological analysis of insects, performed in the years from 2007 to 2009 did not confirm either the presence of the vector of ESFY phytoplasma *C. pruni* or the potential vector *Asymmetrasca decedens* (Paoli) inside the net-house. According to the literature, *A. decedens* could have a possible role in the transmission of ESFY phytoplasma in apricots (Pastore et al., 2004). Even inside the net-house no other phytophagous insects were recorded on yellow sticky traps, which confirmed the effectiveness of the net-house in preventing the disease transmission by vectors.

On the control trees outside the net-house, in the studied years, there was only a single *C. pruni* found on yellow sticky traps in the first April decade 2009. On the other hand, many specimens of *A. decedens* were captured on traps outside on control trees during the summer in all three years (data not shown). It can be seen from the results that, in the investigated site, *C. pruni* did not play a significant role in the transmission of ESFY phytoplasma in the studied period, while the abundant presence of the leafhopper *A. decedens* could indicate it is a risk for transmission of the disease.

Plant Health Status

Plant health status of the net-house growing trees and the outside growing control trees was controlled for quarantine pests of stone fruits during the years 2007 to 2009. Before planting in the spring 2007, all trees planted in the net-house and 12 control trees of 'Redhaven' were checked for the presence of ESFY phytoplasma and for PPV. In the following years around 15% of the trees growing in the net-house and all control trees were tested annually for these two diseases. A total of 137 root samples were taken in three years from both environments for ESFY analyses. In case of latent infections when symptoms are not visible, detection is more reliable if root samples are tested for ESFY analyses (Brzin et al., 2005). The results revealed the absence of ESFY phytoplasma in tested samples both from net-house trees and from outside growing control trees in the studied period. Regarding the PPV analyses, 162 leaf samples from both environments were tested from 2007 to 2009. All the samples produced negative results, except for one sample from a control tree growing outside which was PPV positive in 2009, the third year after planting, which may indicate that the disease will spread in the following years on the host plants growing outside.

In winter 2007-2008, *X. arboricola* pv. *pruni* was tested for due to some symptoms expressed as cracks and shoot dieback on overwintering twigs, which was observed mainly on two cultivars inside the net-house. *X. arboricola* pv. *pruni* was not confirmed in sampled tissues. However, a fungal pathogen, *Fusicoccum amygdali*, was found in symptomatic tissues after further laboratory analyses. The affected twigs or branches were cut off, and symptoms were not observed on the trees by further visual inspections.

CONCLUSIONS

From the results obtained it can be seen that mother trees from the net-house had good vegetative growth with large amounts of well-developed bud wood for nursery requirements. No vectors were captured inside the net-house in the study period from 2007 to 2009, while outside, only one specimen of *C. pruni* was detected in 2009 and a high population of *A. decedens* was found on control trees. Regarding the plant health status, the occurrence of PPV was confirmed on an outside growing control tree, but no vector transmissible diseases were detected inside the net-house. These results suggest good potential for *Prunus* mother plant cultivation in protected environments for the production of healthy propagating material.

ACKNOWLEDGEMENT

This research was partly supported by the Ministry of Agriculture, Forestry and Food and by Slovenian Research Agency (project no. V4-0343). The support is gratefully acknowledged.

Literature Cited

Ambrožič Turk, B., Mehle, N., Brzin, J., Škerlavaj, V., Seljak, G. and Ravnikar, M. 2008. High infection pressure of ESFY phytoplasma threatens the cultivation of stone fruit species. Journal of Central European Agriculture 9:795-802.

Boben, J., Mehle, N. and Ravnikar, M. 2007. Optimization of extraction procedure can improve phytoplasma diagnostics. Bulletin of Insectology 60:249-250.

Brzin, J., Petrovič, N., Boben, J., Hren, M., Kogovšek, P., Mehle, N., Žežlina, I., Seljak,

G. and Ravnikar, M. 2005. Fruit tree phytoplasmas. Proc. The 7th Slovenian Conference on Plant Protection. Zreče, Slovenia 8-10 March. p.248-252.
Carraro, L., Osler, R., Loi, N., Ermacora, P. and Refatti, E. 1998. Transmission of European stone fruit yellows phytoplasma by *Cacopsylla pruni*. J. Plant Pathol. 80:233-239.
Carraro, L. and Osler, R. 2003. European stone fruit yellows: a destructive disease in the mediterranean basin. p.113-117. In: A. Myrta, B. Di Terlizzi and V. Savino (eds.), Virus and virus-like diseases of stone fruits, with particular reference to the Mediterranean region, Vol. 45, CIHEAM, Options Mediterraneennes Serie B.
Carraro, L., Ferrini, F., Ermacora, P. and Loi, N. 2004. Transmission of European stone fruit yellows phytoplasma to *Prunus* species by using vector and graft transmission. Acta Hort. 657:449-453.
Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community (OJ L 169/1,10/07/2000).
EPPO. 2006. *Xanthomonas arboricola* pv. *pruni*. Diagnostic protocols for regulated pests. PM 7/64(1). EPPO Bulletin 36:129-133.
Hren, M., Boben, J., Rotter, A., Kralj Novak, P., Gruden, K. and Ravnikar, M. 2007. Real-time PCR detection systems for Flavescence dorée and Bois noir phytoplasmas in grapevine: comparison with conventional PCR detection and application in diagnostics. Plant Pathol. 56:785-796.
Laimer Da Camara Machado, M., Paltrinieri, S., Hanzer, V., Arthofer, W., Strommer, S., Martini, M., Pondrelli, M. and Bertaccini, A. 2001. Presence of European stone fruit yellows (ESFY or 16SrX-B) phytoplasmas in apricots in Austria. Plant Pathology 50:30-135.
Laviňa, A., Sabate, J., Garcia-Chapa, M., Batlle, A. and Torres, E. 2004. Occurence and epidemiology of European stone fruit yellows phytoplasma in Spain. Acta Hort. 657:89-494.
Lorenz, K.H., Schneider, B., Ahrens, U. and Seemüller, E. 1995. Detection of apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology 85:771-776.
Mehle, N., Brzin, J., Boben, J., Hren, M., Frank, J., Petrovič, N., Gruden, K., Dreo, T., Žežlina, I., Seljak, G. and Ravnikar, M. 2007. The results of phytoplasms testing in stone fruits (*Prunus* spp.) in Slovenia (2000-2006). Proc. The 8th Slovenian Conference on Plant Protection. Radenci, Slovenia 6-7 March. p.139-143.
Pastore, M., Raffone, E., Santonastaso, M., Priore, R., Paltrinieri, S., Bertaccini, A. and Simeone, A.M. 2004. Phytoplasma detection in *Empoasca decedens* and *Empoasca* spp. and their possible role as vectors of European stone fruit yellows (16SrX-B) phytoplasma. Acta Hort. 657:507-511.
Ramel, M.E. and Gugerli, P. 2004. Epidemiological survey of European stone fruit yellows phytoplasma in two orchards in western Switzerland. Acta Hort. 657:459-463.
Schneider, B., Seemüller, E., Smart, C.D. and Kirkpatrick, B.C. 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. p.369-380. In: S. Razin and J.G. Tully (eds.), Molecular and diagnostic procedures in mycoplasmology, Vol. 1, Academic Press, San Diego, Calif.
Seemüller, E. and Schneider, B. 2004. '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. International Journal of Systematic & Evolutionary Microbiology 54:1217-1226.
Webster, A.D. and Wertheim, S.J. 2005. Vegetative (asexual) propagation. p.93-106. In: J. Tromp, A.D. Webster and S.J. Wertheim (eds.), Fundamentals of Temperate Zone Tree Fruit Production, Backhuys Publishers, Leiden, The Netherlands.

Tables

Table 1. Tree growth parameters of net-house growing trees in comparison with outside growing control trees of 'Redhaven' in the years 2007 to 2009.

Year	Tree height (cm)		Amount of buds for budding (no./tree)	
	Outside (control) trees	Net-house trees	Outside (control) trees	Net-house trees
2007	134a*	199b	24a	115b
2008	263a	327b	687a	693a
2009	314a	456b	650a	881b

* Means with the same letter in the row are not significantly different using t-test at P=0.05. (Means have to be compared within years but not between years).

Table 2. Budding success at different times of budding in the 'Redhaven' budding experiment.

Time of budding	Budding success (%)	
	Buds taken from different trees	Buds taken repeatedly from the same trees
I. (26 Aug.)	76.7a*	95.0a
II. (9 Sept.)	76.7a	73.3b
III. (23 Sept.)	88.3a	81.7ab

* Means with the same letter in the column are not significantly different using t-test at P=0.05.