Hypo- and hyper-virulence in apricot trees infected by European stone fruit yellows

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Abstract

An apricot orchard, located in an area of north eastern Italy under serious pressure from European stone fruit yellows (ESFY) infection, has been monitored since the year it was planted (1990). During this time, most of the trees displayed symptoms or were shown by PCR analyses to be infected. Two groups of apricot trees were particularly interesting: some trees were asymptomatically infected while others recovered from the symptoms but not from the pathogen. In order to isolate those strains of the phytoplasma characterised by varying virulence, each of the two groups was used as mother plants and propagated. The new trees were used to constitute experimental orchards, where they were observed for the presence of symptoms and in part were tested by PCR, starting in 2003. The results obtained confirmed the presence of strains of the pathogen characterised by varying virulence. The strains originally present in infected apricot trees which recovered from the symptoms of ESFY were seen to be hypovirulent; none of the propagated infected trees ever showed symptoms of the disease. Surprisingly, the strains present in asymptomatic apricot mother plants were hypervirulent and the propagated trees always displayed severe symptoms. In the propagated trees, the transmission of the pathogen was higher in the hypervirulent strains than in the hypovirulent ones. A graft transmission trial carried out in the greenhouse using some of the identified hypo- and hypervirulent strains, confirmed the results obtained in open field. Real time PCR analyses showed that in the trees infected by hypovirulent strains the colonisation of the pathogen was lower than in those infected by the hypervirulent strains. It is possible to affirm that the hypovirulent strains were present in those mother plants which had originally recovered. The research will continue with the aim of verifying the possibility of cross protection among the identified hypo- and hypervirulent strains.

Keywords: 'Candidatus *Phytoplasma prunorum*', real-time PCR, *Prunus*

Introduction

European stone fruit yellows (ESFY), a phytoplasma disease caused by 'Candidatus *Phytoplasma prunorum*' ('Ca. *P. prunorum*') (Seemüller and Schneider, 2004) and spread by *Cacopsylla pruni* (Carraro et al., 1998) is present in several European countries and was previously reported in the Friuli Venezia Giulia region (North East Italy) on several cultivated and spontaneous *Prunus* species (Carraro et al., 2002). Among the cultivated *Prunus* species, *Prunus salicina* and *Prunus armeniaca* are the most frequently affected by ESFY in this region and ESFY frequently became the most relevant phytosanitary problem for these crops, thus reducing the orchard's productive lifespan in economically sustainable terms. Typical ESFY symptoms are: breaking dormancy in winter, yellowing leaves and leaf roll in summer, decline and dieback. Recovery from symptoms was reported but not as commonly as is described in other crops like grapevines and apple-trees (Musetti et al., 2005).

Evidence of variability in virulence between 'Ca. *P. prunorum*' isolates have been reported (Cornaggia et al., 1995; Kison and Seemüller, 2001) and new tools have recently been developed with the aim of obtaining molecular markers for the characterization of the strains according their virulence (Danet et al., 2008; Martini et al., this issue).

The aim of this work was to identify 'Ca. *P. prunorum*' hypovirulent strains and investigate the stability of the hypovirulence in apricot trees in field conditions, and with artificial infection. The final goal of this research was to investigate the feasibility of applying cross protection based on mild strains to reduce losses in commercial orchards.

Material and methods

Field trials: An apricot orchard, cv. 'Reale d'Imola' grafted onto myrobalan rootstocks, which was established in 1990 in an area with a high risk of ESFY infection near Cividale del Friuli (Udine, Italy), was monitored at least twice a year for the expression of ESFY symptoms and samples were collected for molecular analyses in order to confirm the presence of 'Ca. *P. prunorum*'. Two groups of apricot trees were particularly interesting: some trees were asymptomatically infected while others recovered from the symptoms but not from the pathogen. In order to isolate those strains of the phytoplasma characterised by varying virulence, each of the two groups was used as mother plants and propagated. Buds were collected in August 2001 from apricot trees and chip-grafted onto virus-free myrobalan rootstocks. Part of the propagation material underwent heat treatment and buds thus treated were grafted onto virus-free myrobalans.

A total of more than 500 new trees were used to constitute experimental orchards, where they were visually inspected twice a year for the presence of symptoms and in part were tested by PCR, starting in 2003. Healthy controls (virus-free certified 'Reale d'Imola' trees) were planted in the same orchards in order to understand natural ESFY diffusion. Plants were divided into four classes according to symptom severity: class 0 = no symptoms; class 1 = faint symptoms only during the vegetative season, but without a reduction in growth; class 2 = dormancy break, yellowing, leaf roll, no growth reduction, class 3 = dormancy break, yellowing, leaf roll, growth reduction and severe decline.

Greenhouse and screenhouse trials: In July 2006, based on the behaviour of the plants in experimental orchards, two isolates from consistently asymptomatic plants but which gave heavy symptoms in the progeny (hyper-virulent), and two isolates from recovered plants that gave asymptomatic progeny (hypo-virulent) were inoculated by grafting onto sixty seedlings of peach, apricot and myrobalan. As inoculum, 3 apricot buds for plant were used and seedlings were grown in a greenhouse and monitored for graft survival and symptom expression. Leaf samples for molecular analyses were collected in June and September 2007.

The above mentioned ESFY isolates were also used as a source of inoculum for an experimental trial in an insect-proof screenhouse. In August 2007, four groups of ten cv 'Reale d'Imola' apricot trees, each two years old, were grafted with six buds from infected trees. During March 2008 bud survival was recorded; trees were inspected for symptom expression and molecular analyses were carried out in 2008 and 2009.

Molecular analyses: Total DNA from 0.7g apricot midribs was extracted by a slightly modified CTAB method as described by Doyle and Doyle(1990). A nested PCR protocol with P1/P7 and f01/r01 primers was applied in order to detect the presence of 'Ca *P. prunorum*'. Amplified products were visualized by agarose gel electrophoresis and then stained with ethidium bromide. A PCR/RFLP protocol, based on *ace*F genes was adopted to investigate genetic variability of 'Ca. *P. prunorum*' (Danet et al., 2008; Martini et al., this issue). Quantitative detection of phytoplasma, based on a real-time PCR protocol, was performed on some samples following the protocol described by Martini et al., (2008). The plants selected for quantitative experiments were 7 apricots with no ESFY symptoms and 7 apricots with severe ESFY symtpoms.

'Ca. *P. prunorum*' was quantified by SYBR® Green I real-time PCR as the number of 'Ca. *P. prunorum*' genome units (GU)/ng of plant DNA (Marzachì and Bosco, 2005). Ribosomal protein (rp) gene *rpl22* was used as the target for amplification of 'Ca. *P. prunorum*' with primer pair rpLNS2f/rpLNS2r2; whereas the 18S rDNA was chosen as the target for the amplification of plant DNA (Christensen et al., 2004). Standard curves, PCR reactions and cycling conditions were performed as previously described (Martini et al., 2007). The data were analysed using one-directional ANOVA and Student's t-test.

Results

Field trials: After 10 years of observations apricot trees from the original orchard were divided into classes according symptom severity; among them, two groups were considered for further investigation: trees which had never displayed symptoms and trees which recovered. All the trees belonging to the two groups tested positive in PCR.

In the established experimental orchards, according to the adopted clustering method for symptom intensity, the mean score for trees from recovered buds was 0.02. Surprisingly, the strains present in symptom-free apricot mother plants were hypervirulent and the infected trees propagated always showed severe symptoms; over the 5-years period the mean score for symptom intensity was 1.09. The score for ESFY symptoms on control trees naturally infected during the same period was 1.10. Mean graft transmissibility of 'Ca *P. prunorum*' strains from recovered trees to progeny was 10 % while from the symptom-free trees the figure was 84 %. Five years after planting, the natural rate of infection on healthy controls was 73 %; 56 % of the progeny from recovered trees were infected, while the progeny from the symptom-free trees were 100% infected.

PCR analyses on propagated trees at planting time showed the success of heat treatment in the eradication of 'Ca. *P. prunorum*' from infected material. Comparison between heat-treated and untreated material 5 years after planting showed that 77 % of trees obtained from recovered and heat treated material were PCR positive; while 56% of trees obtained from recovered un treated buds were infected.

Greenhouse and screenhouse trials: A graft transmission trial carried out in the greenhouse using some of the identified hypo- and hypervirulent strains, confirmed the results obtained in the open field. The mean transmissibility was 61 % for virulent and 35 % for hypovirulent strains. Among the PCR positive plants grafted with virulent strains 73 % of the trees showed symptoms (100 % of apricot and peach trees, 0 % of myrobalans). The effects of virulent strains on susceptible host apricot and peach trees were stunting, yellowing and dieback. Among the trees which tested positive

for inoculation with hypovirulent strains, only 8% displayed symptoms (17 % apricot, 0 % peach and myrobalans). Symptoms of hypo-virulent strains on apricot were faint leaf-roll and yellowing.

Results from apricot trees artificial infected in a screenhouse highlight the presence of strains with varying degrees of virulence (Figure 1). The first symptoms induced by virulent strains inoculated in August 2007 were dormancy break in February 2008 and severe leaf roll and decline in May-June of the same year.

Fig. 1 Comparison between the effects of strains of 'Ca. *P. prunorum*' in artificial infections on apricot trees in a screenhouse. In the middle a group of trees inoculated with a hyper-virulent strain; on the left and right trees inoculated with a hypo-virulent strain.

Molecular analyses: For quantification of 'Ca. *P. prunorum*' infection level in the tested plant, 18S rDNA served to normalize the data. Slopes of standard curves for quantification of '*Ca.* P. prunorum' DNA (diluted in 20 ng/µl of total DNA from healthy apricot) and quantification of apricot plant DNA indicated PCR efficiencies close to 100 %. Infection levels in symptomatic apricots ranged from 74.53 to $1.25*10^4$ GU of 'Ca. *P. prunorum*'/ng of plant DNA (Figure 2); whereas the infection level in asymptomatic apricots ranged from 32.52 to $1.23x10^3$ GU of 'Ca. *P. prunorum*'/ng of plant DNA (Figure 2). The average infection rate of 'Ca. *P. prunorum*' in symptomatic and asymptomatic apricots was respectively $5.42x10^3$ and $4.66x10^2$ GU of 'Ca. *P. prunorum*'/ng of plant DNA. These results indicated that between the two groups of plants the infection level of '*Ca.* P. prunorum' was higher in the symptomatic plants and more than 10 times different. The ANOVA test demonstrated that the observed divergence was statistically significant for α=0.01.

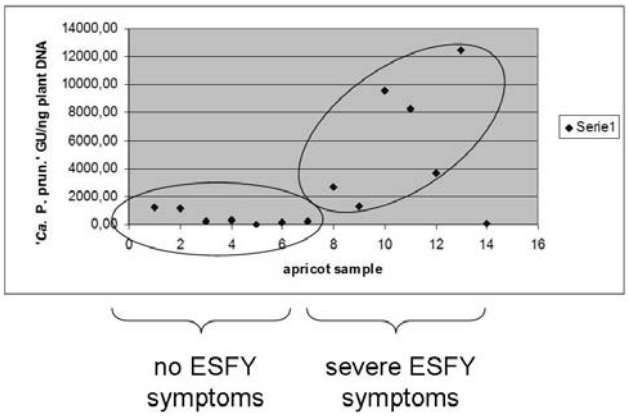

Fig. 2 Quantification of 'Ca. *P. prunorum*' GU/ng plant DNA using SYBR Green I real-time PCR assays in two groups of apricot plants showing respectively no symptoms and severe ESFY symptoms.

Discussion

Previous reports of the presence of 'Ca. *P. prunorum*' strains with varying virulence on apricot and peach trees was confirmed in this study. In particular, by means of long-term monitoring of an apricot orchard in an area with high natural pressure of ESFY infection, it was possible to identify apricot trees that never displayed symptoms but tested positive by PCR analyses. This research focused on two groups of apricot trees: one that never showed symptoms but tested positive in PCR analyses and another group of trees which recovered from the symptoms but not from the pathogen. By artificial infection we demonstrated that in the first case the characteristic of asymptomaticity was plant-related: in fact, progeny obtained from the symptom-free trees themselves displayed symptoms, and artificial infection of several *Prunus* spp. confirm the virulence of this strains, which are also highly transmissible by grafting. By contrast, strains isolated from recovered trees never induced symptoms in the progeny, even in greenhouse test trees, and are only weakly transmissible by grafting. The heat-treated material seemed to be characterized by higher susceptibility to ESFY infection than the un treated material in the first years after planting, (data not shown) but the percentage of infected plants in the groups of apricot trees become similar after 5 years from planting.

A Real-time PCR quantification method was applied so as to better understand the correlations between phytoplasma concentration and symptom intensity. First results seem to correlate low phytoplasma concentration and an absence of symptoms for apricot trees infected with mild strains; strains that induce severe symptoms, on the other hand, seem to be able to achieve high concentrations in the host. The high percentage of symptoms on plants used as negative controls reflects the high disease pressure in the test area, and probably, the greater fitness of virulent strains in these conditions.

Further investigation is needed to clarify the stability of hypovirulence on trees and the interaction between hypo- and hypervirulent strains in mixed infections in order to utilize mild strains for ESFY management.

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