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Effect of different strains of '*Candidatus* Phytoplasma mali' in susceptible and resistant *Malus* genotypes inoculated by *in vitro* grafting

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Abstract

In vitro culture techniques were employed to study the effect of two different strains of '*Candidatus* Phytoplasma mali' in susceptible cultivars of *Malus domestica* and on apomictic, resistant genotypes deriving from *M. sieboldii.* Inoculation was done by *in vitro* grafting. The effect of the two '*Ca.* P. mali' strains in the different genotypes was analysed by recording the quality of the grafts, the transmission rates, and the survival rates of infected plants 6 and 12 months after grafting. Significant differences of all parameters were observed among the two strains. As further parameters the symptoms of the inoculated plants were recorded and the phytoplasma concentration was determined by quantitative PCR at different time points after grafting. All infected apomictic genotypes had lower phytoplasma concentrations than the susceptible controls for both strains. A highly significant difference between the strains was found for the phytoplasma concentration, especially in the susceptible genotypes. In contrast to the susceptible genotypes, the resistant ones did not show apple proliferation-specific symptoms or growth reduction compared to the healthy control. After transferring infected and healthy *in vitro* plants *ex vitro* these results were confirmed. The *in vitro* system is an interesting new tool to study differences in virulence of phytoplasma strains as well as susceptibility of the host plant.

Key words: micrografting, quantitative real-time PCR, apple proliferation, *Malus sieboldii*.

Introduction

'Candidatus Phytoplasma mali', a yet uncultured, wall-less bacterium of the class *Mollicutes* is responsible of apple proliferation (AP) disease. Apple proliferation is one of the most important phytoplasmoses in Europe that causes considerable economic losses. As there is no cure of the phytoplasma infection, the most promising approach to face AP appears to be the use of resistant plant material.

All currently grown cultivars and rootstocks are susceptible to the disease. Natural resistance to AP was discovered only in wild, apomictic *Malus* species, namely in *Malus sieboldii*. Crossings of these wild *Malus* species with *M. domestica* were carried out in the 1950s and 1970s, but the resulting progeny turned out to be too vigorous for modern apple cultivation. A certain number of genotypes, like selections D2212 (Bisognin *et al.,* 2007b) and H0909 (Seemüller *et al.,* unpublished data), remained resistant to AP disease. They never developed symptoms or recovered within few years. Phytoplasma titres in the roots were significant lower in resistant *M. sieboldii* progenies than in standard stocks.

Considerable variation in genetic variability and in pathogenicity was observed among different isolates of '*Ca*. P. mali' (Seemüller and Schneider, 2007). *In vitro* grafting has been applied previously with success to transmit 'Ca. P. mali' to healthy test plants *in vitro* (Jarausch *et al.,* 1999). Based on these results an *in vitro* resistance screening system for AP resistance has been established (Bisognin *et al.,* 2007a). In the present study this system was further exploited to study the differences in virulence of different strains of '*Ca.* P. mali' in susceptible and resistant *Malus* genotypes.

Materials and methods

Shoot cultures of healthy genotypes of cvs. Golden Delicious, Rubinette, M9 and apomictic resistant genotypes *M. sieboldii* and its hybrids H0909 and D2212 were obtained according to the protocol of Jarausch *et al*. (1996) as described by Bisognin *et al*. (2007a). Infected cultures were derived from cv. Rubinette harbouring '*Ca.* P. mali' subtype AP (denominated "PM4") and from cv. Golden Delicious, infected with a '*Ca*. P. mali' subtype AT2 (named "PM6"). Micrografting was done as previously described (Jarausch *et al.,* 1999; Bisognin *et al.,* 2007a) in several repetitions. The graft contact was maintained for 1.5 months. Parameters of quality of the grafts, transmission rates, mortality 3 months post inoculation (p.i.) and survival rate of infected plants 6 and 12 months p.i. were recorded as described (Bisognin *et al.,* 2007a). Total DNA of plants with successful grafts was extracted according to Doyle and Doyle (1990) and direct PCR was carried out with '*Ca.* P. mali'-specific primers fAT/rAS (Smart *et al.,* 1996). The concentration of phytoplasmas was measured by quantitative PCR based on a TaqMan™ assay (Baric and Dalla Via, 2004) with some modifications (Bisognin *et al.,* 2007a).

Healthy and infected *in vitro* plants were acclimatized *ex vitro* according to standard protocols in spring, at least 12 months p.i..

Results and discussion

An *in vitro* system based on *in vitro* graft-inoculation was employed to study the effect of two '*Ca.* P. mali' strains on different *Malus* genotypes. The quality of the grafts – as specified by Jarausch *et al.* (1999) – was significantly higher with strain PM6 (67-82%) compared to strain PM4 (53-79%). The transmission rates – as determined by '*Ca.* P. mali'-specific PCR – were consequently higher with strain PM6 (45-79%), except for M9. Higher percentages of mortality 3 months p.i. were recorded for grafts inoculated with strain PM6 compared to PM4 but these differences were not statistically significant. Important differences were found regarding the survival of inoculated shoots. Whereas almost all plants of the different genotypes infected with strain PM6 survived at least one year, this was only true for genotypes Golden Delicious, M9 and D2212 infected with strain PM4. All plants of genotypes *M. sieboldii* and H0909 died within the period of 12 months p.i..

As the symptoms were only analysed on well established infected cultures after 12 months no data were available for the genotypes *M. sieboldii* and H0909 infected with strain PM4. The resistant genotype D2212, however, did not show apple proliferation-specific symptoms or growth reduction compared to the healthy control. For this genotype no difference among the strains was observed. On the contrary, the susceptible genotypes Golden Delicious and M9 showed typical apple proliferation symptoms such as proliferation and enlarged stipules with both strains. A slight difference was observed between the strains regarding the growth reduction compared to the healthy control. Whereas strain PM4 induced severe stunting *in vitro* the growth of plants infected with strain PM6 was reduced but generally not stunted.

In vitro shoot samples were periodically subjected to phytoplasma detection and quantification. For data analysis a relative quantification approach was adopted using the mean values of all inoculated plants per genotype. *M. sieboldii* infected with strain PM6 showed the lowest phytoplasma concentration among the apomictic genotypes. Its concentration was therefore used as reference to compare the phytoplasma concentration in all other genotypes. D2212 had comparable low phytoplasma titres when infected with strain PM6 (ratio 1.06) but a higher titre with strain PM4 (ratio 1.91). An inverse relationship was observed for H0909: a higher titre with strain PM6 (ratio 2.15) and a low one with strain PM4 (ratio 1.03). In contrast, strains PM6 and PM4 multiplied to higher concentrations in the susceptible controls Golden Delicious (ratios 2.54 and 4.76, respectively) and M9 (ratios 2.94 and 3.20, respectively). By analysing all data together a highly significant difference was found between both strains. Strain PM4 multiplied to higher concentrations in the *in vitro* plants than strain PM6. This effect was especially pronounced in cv. Golden Delicious.

Infected and healthy *in vitro* plants were transferred *ex vitro* and were maintained under controlled conditions. All resistant genotypes did not show characteristic symptoms in the first two years after acclimatization with either strain. Instead, all plants of susceptible genotypes exhibited typical symptoms like enlarged stipules and witches' brooms. As in the *in vitro* system, low phytoplasma concentrations were found in all resistant genotypes infected with strain PM6 and in D2212 plants infected with strain PM4. Also in *ex vitro* conditions strain PM4 multiplied to higher concentrations than strain PM6. Thus, the resistance trait and the different virulence of the strains could be confirmed under *in vitro* conditions as well as under *ex vitro* conditions.

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