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Behaviour and interaction of two '*Candidatus* Phytoplasma mali' strains in artificial infections

Paolo ERMACORA, Marta MARTINI, Luigi CARRARO, Francesca FERRINI, Nazia LOI
Dipartimento di Biologia applicata alla Difesa delle Piante, Università di Udine, Udine, Italy

Abstract

A study was carried out in order to examine colonization pattern of one year old apple trees infected with rpX-A and rpX-D strains of *'Candidatus* Phytoplasma mali'. Plants contemporaneously or successively infected by two strains were obtained by bud grafting and approach grafting. Nested-PCR and RFLP analyses were applied in order to investigate phytoplasma presence and colonization. The attachment of infected buds did not differ between rpX-A and rpX-D-strains. Phytoplasma transmission by bud grafting ranged from 70 to 85%. No differences were observed on symptom expression and latency between the two strains. Based on PCR-RFLP analyses on contemporaneous infections, strain rpX-A appeared faster than strain rpX-D in plant colonization. Mixed infections were present and stable for several months. In superinfections (infections on previously infected plants), the superinoculated strain colonized the previously infected plants several months after the grafting and some mixed infections were present. *Cacopsylla picta* (Foerster) individuals, born on rpX-A + D infected plant, acquired both strains.

Key words: Apple proliferation, *Cacopsylla picta*, mixed infections, nested-PCR.

Introduction

Apple proliferation (AP) is a phytoplasma disease caused by '*Candidatus* Phytoplasma mali'. The presence of strains of the pathogen and their different effects on the host, including virulence, was reported by Kunze (1976) and by Seemüller and Schneider (2007). Molecular tools to investigate '*Ca*. P. mali' biodiversity were developed by Jarausch *et al*. (2000) and, recently, by Martini *et al*. (2007). The same authors showed that, in areas where AP strains are contemporaneously present (north-east Italy), mixed infections are not common; consequently, interactions among strains are not fully known. In the present paper, single and mixed infections of '*Ca*. P. mali' strains, artificially obtained in controlled conditions, were studied with the aim to better understand these interactions.

Materials and methods

In August 2005, a total of 60 one year old apple plants were grafted with AP phytoplasma by chip-budding: 20 of them with 3 rpX-A strain infected buds; 20 with 3 rpX-D strain infected buds; 20 with 3 rpX-A strain and 3 rpX-D strain infected buds (contemporaneous infection). Plants were monitored for symptom expression and leaves were collected for phytoplasma detection three times in 2006 and one time in 2007; roots were also collected during the winter. After symptom appearance, an approach grafting was established among nine rpX-A strain and nine rpX-D strain infected plants.

Total DNA was extracted from samples following a Doyle and Doyle (1990) modified procedure. A nested-PCR protocol, based on ribosomal protein primers rpAP15f2/rp(I)R1A and rpAP15f/rpAP15r was adopted for phytoplasma detection (Martini *et al*., 2007). Amplified products were submitted to RFLP analyses using *Alu*I and *Dra*I endonucleases and polyacrylamide gel electrophoresis was adopted to visualize RFLP patterns. During 2006 about one hundred *Cacopsylla picta* (Foerster) were caught and forced to oviposition on a rpX-A + D infected apple tree. A total of 20 insects of the new generation were collected at different stages and individually analyzed. In 2007 the trial was repeated and 50 *C. picta* were collected and analyzed.

Results

The attachment of grafted buds was 62% for rpX-A strain and 57% for rpX-D strain. In the year after grafting, 17 out of 20 (85%) and 14 out of 20 (70%) plants inoculated respectively with strain rpX-A and strain rpX-D showed typical symptoms; 15 out of 20 (75%) plants with rpX-A+D contemporaneous infection became symptomatic. No differences were observed on the severity of symptoms induced by the two strains or by mixed infections. Nested-PCR and RFLP analyses carried out in May 2006 on contemporaneous infected plants emphasized the presence of strain rpX-A in 9 out of 15 positive plants. Strain rpX-D was prevalent in 2 plants and mixed infections were found in 4 plants. In the following analyses, carried out till May 2007, the prevalence of strain rpX-A and mixed infections decreased. On the contrary, strain rpX-D became present in a higher percentage. Roots of contemporaneous infected plants hosted rpX-A strain in 9 cases, rpX-D strain in 7 cases, and one mixed infection.

The approach graftings were successful in all the nine cases. As shown in table 1, colonization of infected plants by the second strain was slow (four months after grafting no mixed infections were detected); the year after grafting some mixed infections were found, with an higher percentage in the roots. 13 *C. picta* out of 20, born on a mixed infected plant, resulted positive (5 out of 6 adults and 8 out 14 nymphs): 4 were rpX-A strain infected, 8 rpX-D strain infected and one mixed in-

Table 1. Molecular analyses for the presence of '*Ca*. P. mali' strains on apple trees previously infected by a single strain and successively superinfected by a second strain.

Strain previously present	Superinfection with strain	Time of analyses: July 06			October 06			May 07			February 07 (roots)		
		No. of plants infected by strain											
		rpX-A	rpX-D	rpX A+D	rpX A	rpX D	rpX A+D	rpX A	rpX D	rpX A+D	rpX A	rpX D	rpX A+D
rpX-A	rpX-D	9	0	0	8	0	1	2	0	7	1	0	8
rpX-D	rpX-A	0	9	0	0	8	1	0	4	5	0	6	3

fected. During 2007 insects were reared on the same plant used in 2006; in this case, PCR-RFLP analyses evidenced the presence of only rpX-D strain in 50 individuals. This was also confirmed by analyses obtained of the plant source of inoculum that resulted only rpX-D strain infected.

Discussion

The presence of mixed infections of phytoplasmas belonging to different taxonomic clusters were previously reported and considered quite common for several plant species (Lee *et al*., 1994). On the contrary, not much is known about mixed infections caused by strains of the same phytoplasma, such as '*Ca.* P. mali'. The reason is that specific primers are not always available and amplicons from closely related phytoplasmas did not contain frequently different endonucleases sites. Ribosomal proteins primers rpAP15f/rpAP15r are useful for '*Ca*. P. mali' detection and differentiation in at least four subtypes, using appropriate endonucleases. The results obtained in this study show a biological difference between the employed strains. In particular, when the infections are contemporaneous, strain rpX-A seems to be faster than strain rpX-D in host colonization; afterwards strain rpX-D increases its presence in the host plant. Furthermore, in the case of proved mixed infection, the presence of both the strains in leaves of the same plant is not stable during the time.

In plants infected by a single strain and successively superinfected by a second strain, the number of mixed infections is high (11 out of 18 plants) even if the colonization of the second inoculated strain is slow. In these plants rpX-D strain shows easiness to colonize roots previously infected by strain rpX-A.

Finally, the experiment carried out during 2006 using the vector *C. picta* demonstrates that the insect can acquire both strains from a mixed infected plant. At the same time the plant can change its status of mixed infected to singularly infected, as shown from the repetition of the experiment carried out the next year using the same plant: in this case the insect acquires only the subtype detected in the plant by molecular analyses.

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Corresponding author: Paolo ERMACORA (e-mail: paolo.ermacora@uniud.it), Dipartimento di Biologia applicata alla Difesa delle piante, Via delle Scienze 208, 33100 Udine, Italy.