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Double infection of '*Candidatus* Phytoplasma asteris' and "flavescence dorée" phytoplasma in the vector *Euscelidius variegatus*

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Abstract

Competition between chrysanthemum yellows (CY) and "flavescence dorée" (FD70) phytoplasmas was studied. In the experiments the insect vector *Euscelidius variegatus* was allowed to acquire both phytoplasmas by feeding first on CY and then on FD70 infected plants and *viceversa*. Titre of both phytoplasmas was quantified over time in the same insects. Transmission experiments to broad bean, host-plant of both phytoplasmas, were carried out from 14 days after the start of the acquisition until death of leafhoppers. Quantitative Real Time PCR assays demonstrated that FD70 multiplies at a higher rate in *E. variegatus* compared to CY. Both phytoplasmas reached similar titres in single- and double-infected insects. CY titre increases faster when the vector already acquired FD70. In spite of its lower multiplication in the insect body, CY is much more efficiently transmitted than FD70 by double-infected insects.

Key words: Chrysanthemum yellows, "flavescence dorée", quantitative Real Time PCR, mixed phytoplasma infection.

Introduction

Competition among different strains of a pathogen within the same host plant is likely to be important in nature, particularly in areas where more than one pathogen strain occur in the same host plant causing systemic infections. In several host-pathogen combinations, molecular analysis allowed studies of interspecific competition between different strains of viruses and fungi, which may coexist and, therefore, compete in the same host. Multiple phytoplasma infection has been reported for different combinations of pathogen-host plant (Firrao *et al*., 2007). Phytoplasmas are vectored by leafhoppers, planthoppers and psyllids and, in several instances, the same insect may vector different phytoplasma species (Weintraub and Beanland, 2006).

The competition of different phytoplasmas in the same vector may be important in nature and studies are required to clarify its mechanisms. To this purpose we have studied the multiplication and transmission of '*Candidatus* Phytoplasma asteris' (CY strain) and "flavescence dorée" (FD) phytoplasma in *Euscelidius variegatus* Kirschbaum, which is a natural vector of CY (Bosco *et al*., 1997) as well as an experimental vector of FD (Caudwell *et al*., 1972).

Materials and methods

Two phytoplasma strains were used in this study, CY (Conti *et al*., 1988), and FD70 (Caudwell *et al*., 1970). They were maintained by insect transmission in daisy (*Chrysanthemum carinatum* Schousboe) and broad bean (*Vicia faba* L.) plants, respectively.

Healthy colonies of *E. variegatus* (Kirschbaum) were reared on oat (*Avena sativa* L.) under plexiglas and nylon cages in growth chambers at 25 °C, photoperiod L16:D8 and checked by PCR to be phytoplasma-free.

In experiment one, nymphs were initially allowed to feed for a 7-day acquisition access period (AAP) on CY-source daisies and then moved to FD70-source broad bean for a further 7-day AAP. In experiment two, nymphs fed first on FD70-infected broad beans, then on CY-infected daisies. At the end of the two AAPs leafhoppers were transferred onto healthy broad bean every three days for inoculation access periods (IAP) until death (75-87 dpa). Infected *E. variegatus* were sampled 23, 33, 40, 47, 54, 60, 68 days post acquisition (dpa) for quantification of the two phytoplasmas. Inoculated plants were maintained in the greenhouse for symptom evaluation until death. As a control, nymphs were allowed to feed on CY or FD70 only.

Total DNA was extracted from single individuals following a procedure previously described by Marzachì *et al*. (1998). Plant DNA was extracted with the PureLink plant total DNA purification kit (Invitrogen) following the manufacturer's instructions. All plants were sampled for phytoplasmas detection by PCR.

The presence of CY and FD70 in the plants was assayed by conventional nested PCR using the R16F2/R2 followed by R16(I)F1/R1 or R16(V)F1/R1 primers (Lee *et al*., 1993; 1994). Quantification was performed in 96-well plates in BioRad iCycler (BioRad, U.S.), following the procedure described in Marzachì and Bosco (2005), with some modifications. Absolute quantification of insect DNA was achieved as detailed in the original paper. Amplification mix contained appropriate primers, SyberGreen Platinum Quantitative PCR Supermix-UDG (Invitrogen, UK) and templates in a final volume of 25 µl. In each qR-PCR plate agent-free insects and water controls were also included. All the samples were run in triplicate. CY and FD70 DNAs were measured as fg of phytoplasma DNA per ng of insect DNA and then transformed into the number of CY genome units (GU) per

ng of vector DNA as in the original method.

To compare the phytoplasma titres measured in individual leafhoppers on different days after the start of AAP (dpa) one-way ANOVA (followed by the Tukey test for multiple comparisons) was performed. For the analyses, raw data (CY GU/ng of insect DNA) underwent logarithmic transformation, since the standard deviation appeared a function of the mean.

Results

Following single CY acquisition, the phytoplasma titre from 20 dpa until the end of the experiment was measured. CY titre ranged from 7E+03 to 8E+04 GU/ng of insect DNA at any time. Vectors transmitted CY to broad bean plants at any time starting from 25 dpa.

Following single FD70 acquisition, phytoplasma titre ranged from 1.4E+03 at 14 dpa to 2.5E+06 GU/ng of insect DNA at 33 dpa. Vectors transmitted FD70 to broad bean plants at any time starting from 28 dpa.

Following double acquisition of CY and FD70, CY titre was measured from 33 dpa (1E+04 GU/ng of insect DNA) until the end of the experiment (6E+04 GU/ng of insect DNA); FD titre was measured from 14 dpa (4E+03 GU/ng of insect DNA) until the end of the experiment (1E+08 GU/ng of insect DNA).

Vectors transmitted CY only, starting from 17 dpa until the end of the experiment with some discontinuities.

Following double acquisition of FD70 and CY, the former phytoplasma was measured from 14 dpa (3E+04 GU/ng of insect DNA) to the end of the experiment (6E+07 GU/ng of insect DNA); CY was measured from 14 dpa (3.3E+04 GU/ng of insect DNA) to the end of the experiment (6E+04 GU/ng of insect DNA). Vectors transmitted FD70 at 25 and 27 dpa only and CY after one month dpa until the end of the experiment.

Discussion

The experiments indicated that each phytoplasma reached a similar titre following single or double acquisition. FD70 reached higher titres compared to CY at any time following single or double acquisition although the differences were not always significant. FD70 multiplication is faster compared to that of CY, actually FD70 can be quantified earlier after acquisition than CY. Moreover, FD is known to be pathogenic to *E. variegatus* (Bressan *et al*., 2005); this can be explained by the fact that this leafhopper is not a natural vector of FD70 and, therefore, their association is not co-evoluted. When acquired after FD70, CY multiplies faster than following single acquisition, suggesting the existence of an interaction. Transmission pattern of double-infected vectors does not reflect the multiplication pattern. In fact, CY is transmitted much more efficiently than FD70, even when its titre in the insect is below the quantification threshold. This may be due to a more efficient colonization of the salivary glands by CY. CY, when acquired first in double acquisition experiments, showed a shorter latent period compared to the single acquisition experiment. This also suggests an interaction with FD70.

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